Conformational Mobility in the Active Site of a Heme Peroxidase*

Sandip K. Badyal, M. Gordon Joyce, Katherine H. Sharp, Harriet E. Seward, Martin Mewies, Jaswir Basran, Isabel K. Macdonald, Peter C. E. Moody, and Emma Lloyd Raven

From the Department of Chemistry, Henry Wellcome Building, University of Leicester, University Road, Leicester LE1 7RH, United Kingdom and Department of Biochemistry and Henry Wellcome Laboratories for Structural Biology, Henry Wellcome Building, University of Leicester, Lancaster Road, Leicester LE1 9HN, United Kingdom

Conformational mobility of the distal histidine residue has been implicated for several different heme peroxidase enzymes, but unambiguous structural evidence is not available. In this work, we present mechanistic, spectroscopic, and structural evidence for peroxide- and ligand-induced conformational mobility of the distal histidine residue (His-42) in a site-directed variant of ascorbate peroxidase (W41A). In this variant, His-42 binds “on” to the heme in the oxidized form, duplicating the active site structure of the cytochromes but, in contrast to the cytochromes, is able to swing “off” the iron during catalysis. This conformational flexibility between the on and off forms is fully reversible and is used as a means to overcome the inherently unreactive nature of the on form toward peroxide, so that essentially complete catalytic activity is maintained. Contrary to the widely adopted view of heme enzyme catalysis, these data indicate that strong coordination of the distal histidine to the heme iron does not automatically undermine catalytic activity. The data add a new dimension to our wider appreciation of structure/activity correlations in other heme enzymes.

The heme peroxidase enzymes catalyze H₂O₂-dependent oxidation of a range of substrates through a mechanism that, in all cases, involves formation of an oxidized ferryl intermediate (known as Compound I; see Equation 1) that is subsequently reduced by substrate (Equations 2 and 3) (1, 2). In the majority of cases, reduction of Compound I occurs by two successive single-electron transfer steps, as follows (where P = peroxidase, HS = substrate, S = 1-electron oxidized form of substrate).

$$k_1 \quad P + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (\text{Eq. 1})$$

$$k_2 \quad \text{Compound I} + HS \rightarrow \text{Compound II} + S \quad (\text{Eq. 2})$$

$$k_3 \quad \text{Compound II} + HS \rightarrow P + S + H_2O \quad (\text{Eq. 3})$$

Structural information is available for a number of heme peroxidase enzymes, and in all cases the heme iron is poised in a 5- or 6-coordinate environment with the sixth ligand provided by a weakly coordinated water molecule. This differs from the heme coordination geometry in other noncatalytic heme proteins that do not require binding of an exogenous ligand at the metal site. The classic example is the cytochromes, which typically have a strong endogenous protein ligand at the sixth site (usually His or Met) and no vacant coordination site for iron-catalyzed chemistry to occur. The prevailing view that has emerged, therefore, is that the catalytic enzymes (which include the heme peroxidases but also embraces other, more complex heme enzymes such as heme oxygenase, the cytochrome P450s, and cytochrome c oxidase) usually contain 5-coordinate or weakly 6-coordinate heme groups that allow facile reaction with substrate, whereas the electron transfer proteins, for example the cytochromes, have no vacant site at the metal ion for catalysis to occur.

In line with the above considerations, there are no known examples of a genuine heme peroxidase with bis-histidine ligation, but there are a few examples in the literature of different heme peroxidases, or site-directed variants thereof, in which coordination of the distal histidine residue has been proposed on the basis of spectroscopic studies (3–8). These examples include: the W51A (7) and D235N (6) variants of cytochrome c peroxidase; thermally inactivated manganese peroxidase (4); and manganese peroxidase at alkaline pH (8). Removal of the ligands coordinating to the bound K⁺-site in ascorbate peroxidase also leads to formation of a low-spin species (3). In none of these cases has unambiguous structural information been obtained, however. In this work, we present the first crystallographically defined example of a functional peroxidase enzyme with bis-histidine ligation in the W41A variant of ascorbate peroxidase. This variant duplicates the heme coordination geometry of the cytochromes $b$ in the oxidized form but remains fully competent for formation of the catalytic Compound I and Compound II intermediates, as well as for sub-

*This work was supported by Project Grants 91/B19083 and B11469 and a studentship from the Biotechnology and Biological Sciences Research Council (to S. K. B.) and by a fellowship from The Leverhulme Trust (to S. K. B.) and by a fellowship from The Leverhulme Trust (to S. K. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 2GGN, 2GHc, 2GHD, 2GHE, 2GHH, 2GHK) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

To whom correspondence should be addressed. Tel.: 44-116-229-7047; Fax: 44-116-252-2789; E-mail: emma.raven@le.ac.uk.
strate oxidation, by means of a reaction mechanism in which a conformationally mobile ligand (His-42) binds “on” and then swings “off” the iron during catalysis. This switch between the on and off forms is triggered by reaction with hydrogen peroxide (or other ligands) and, under catalytic conditions, is fully reversible, allowing essentially complete activity to be maintained. These data indicate that strong coordination of the distal histidine residue to the heme iron does not automatically undermine peroxidase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—l-Ascorbic acid (Aldrich), guaiacol (Sigma), and the chemicals used for buffers (Fisher) were of the highest analytical grade (more than 99% pure) and were used without further purification. Hydrogen peroxide solutions were freshly prepared by dilution of a 30% (v/v) solution (BDH Chemicals); exact concentrations were determined using the published absorption coefficient ($\varepsilon_{240} = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$) (9). Aqueous solutions were prepared using water purified through an Elgastat Option 2 water purifier, which itself was fed with deionized water. All pH measurements were made using a Russell pH-electrode attached to a digital pH-meter (Radiometer Copenhagen, model PHM 93).

**Mutagenesis and Protein Purification**—Site-directed mutagenesis on recombinant soybean cytosolic APX (rsAPX) was performed according to the QuickChange® protocol (Stratagene Ltd., Cambridge, UK). Two oligonucleotides encoding the desired mutation were synthesized and purified (Invitrogen). For W41A, the primers were: 5’-GCTCCGTTTGGCAGGCACACTCTGTGGAACC-3’ (forward primer) and 3’-GGTCCAGAGGTCGGTGCCAACGGAGC-5’ (reverse primer). DNA sequencing of the entire coding region using an Applied Biosystems 3730 DNA analyzer was used to confirm the desired mutation and the absence of spurious mutations.

Bacterial fermentation of cells and purification of rsAPX and W41A was carried out according to published procedures (10, 11). Enzyme purity was assessed by examination of the soret/280 value of the enzyme. Values for $K_\text{m}$ were determined by a fit of the data to the Michaelis-Menten equation using a nonlinear regression analysis program (KaleidaGraph, version 3.09, Synergy Software). All reported values are the mean of three independent assays.

**Transient-state Kinetics**—Transient-state measurements (sodium phosphate, pH 7.0, $\mu = 0.1$ m, [enzyme] = 25 nm, 25 °C) were performed according to published protocols (14). Oxidation of ascorbate was monitored at 290 nm ($\varepsilon_{290} = 2.8 \text{ M}^{-1} \text{ cm}^{-1}$) (15) and initial rates were multiplied by a factor of 2 to account for the fast disproportionation ($k \approx 10^6 \text{ M}^{-1} \text{ s}^{-1}$) of the monodehydroascorbate radical to ascorbate and dehydroascorbate (16). For oxidation of guaiacol, stock solutions (100 mm) were prepared in sodium phosphate buffer containing 30% ethanol (17), and oxidation to tetraguaiacol was monitored at 470 nm ($\varepsilon_{470} = 22.6 \text{ M}^{-1} \text{ cm}^{-1}$) (17). Values for $k_\text{cat}$ were calculated by dividing the maximum rate of activity ($\mu$M$^{-1}$ s$^{-1}$) by the micromolar concentration of the enzyme. Values for $K_\text{m}$ were determined using Data Explorer software (Applied Biosystems). The EPR spectra were accumulated in the range of 5000 to 35,000 Da with a protein mass standard kit (Sequazyme, Applied Biosystems). Spectra were accumulated in the same mass range using an average of at least 250 laser shots. The spectra were analyzed using Data Explorer software (Applied Biosystems). The MALDI-TOF mass spectrum of W41A gives a mass of 28,209.36 ± 0.05% Da (calculated mass for W41A = 28,203.74 Da), indicating that no posttranslational modification has occurred.

**Steady-state Kinetics**—Steady-state measurements (sodium phosphate, pH 7.0, $\mu = 0.1$ m, [enzyme] = 25 nm, 25 °C) were carried out according to published protocols (14). Oxidation of ascorbate was monitored at 290 nm ($\varepsilon_{290} = 2.8 \text{ M}^{-1} \text{ cm}^{-1}$) (15) and initial rates were multiplied by a factor of 2 to account for the fast disproportionation ($k \approx 10^6 \text{ M}^{-1} \text{ s}^{-1}$) of the monodehydroascorbate radical to ascorbate and dehydroascorbate (16). For oxidation of guaiacol, stock solutions (100 mm) were prepared in sodium phosphate buffer containing 30% ethanol (17), and oxidation to tetraguaiacol was monitored at 470 nm ($\varepsilon_{470} = 22.6 \text{ M}^{-1} \text{ cm}^{-1}$) (17). Values for $k_\text{cat}$ were calculated by dividing the maximum rate of activity ($\mu$M$^{-1}$ s$^{-1}$) by the micromolar concentration of the enzyme. Values for $K_\text{m}$ were determined using Data Explorer software (Applied Biosystems). The EPR spectra were accumulated in the range of 5000 to 35,000 Da with a protein mass standard kit (Sequazyme, Applied Biosystems). Spectra were accumulated in the same mass range using an average of at least 250 laser shots. The spectra were analyzed using Data Explorer software (Applied Biosystems). The MALDI-TOF mass spectrum of W41A gives a mass of 28,209.36 ± 0.05% Da (calculated mass for W41A = 28,203.74 Da), indicating that no posttranslational modification has occurred.

**Conformational Mobility in a Heme Peroxidase**

Conformational Mobility in a Heme Peroxidase (rsAPX) was calibrated in the range of 5000 to 35,000 Da with a protein mass standard kit (Sequazyme, Applied Biosystems). Spectra were accumulated in the same mass range using an average of at least 250 laser shots. The spectra were analyzed using Data Explorer software (Applied Biosystems). The MALDI-TOF mass spectrum of W41A gives a mass of 28,209.36 ± 0.05% Da (calculated mass for W41A = 28,203.74 Da), indicating that no posttranslational modification has occurred.

2 The abbreviations used are: APX, ascorbate peroxidase; rsAPX, recombinant soybean cytosolic ascorbate peroxidase; sh, shoulder; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight.
Conformational Mobility in a Heme Peroxidase

solving a few crystals of potassium cyanide in 10 ml of mother liquor (0.1 M Hepes, pH 8.3, and 2.25 M lithium sulfate). Crystals of the NO-bound heme complexes were obtained by soaking crystals in sodium dithionite in the mother liquor followed by the addition of crystals of potassium nitrite, which reacts with excess dithionite to produce nitric oxide (19). For \( \text{H}_2\text{O}_2 \) soaks of W41A, crystals were soaked in 0.1 M \( \text{H}_2\text{O}_2 \), left for 5 min, and then frozen in liquid nitrogen. The heme coordination geometry in cytochrome c peroxidase is known to be sensitive to the presence of noncoordinating anions (e.g. phosphate, nitrate; see for example, Refs. 6, 7, and 20–23). These anions were not used in the crystallization experiments reported here. Glycerol also was not used in the crystallization studies.

Diffraction data for W41A and the NO- and cyanide-bound forms were collected at beamline ID23-EH1 using an ADSC Quantum-315 detector, whereas data for the W41A-\( \text{H}_2\text{O}_2 \) soak was collected at beamline ID14-EH4 using an ADSC Quantum-4 detector all at the ESRF, Grenoble. Data for the rsAPX-NO and rsAPX-CN complexes were collected at DESY (Hamburg) at beamline X-11 using a MAR 165 mm CCD detector. All synchrotron data were collected at 100 K. Data collected at the ESRF was indexed and scaled using MOSFLM (24) and SCALA (25), and data collected at DESY was indexed and scaled using the HKL programs DENZO and SCALPACK (26). Data collection and processing statistics are shown in Table 1; 5% of the data were flagged for the calculation of \( R_{\text{free}} \) and excluded from subsequent refinement. The structures were refined from a model derived from the 1.45-Å rsAPX-ascorbate complex (Protein Data Bank accession code 1OAF) by the removal of bound ligand and water molecules. Several cycles of refinement using REFMAC5 (27) from the CCP4 suite (25) and manual rebuilding of the protein model using COOT (28) followed by the addition of water molecules were carried out until the \( R_{\text{free}} \) and \( R_{\text{factor}} \) values converged. In total, six crystal structures and diffraction data have been deposited with the following identifiers: Protein Data Bank codes 2GGN (ferric W41A), 2GHC (W41A-NO complex), 2GHD (W41A-CN complex), 2GHE (W41A-\( \text{H}_2\text{O}_2 \) soak), 2GHH (rsAPX-NO complex), and 2GHK (rsAPX-CN complex). The final refinement statistics of all structures are presented in Table 1.

RESULTS AND DISCUSSION

Heme Coordination Geometry—The electronic spectrum of the ferric derivative of W41A is shown in Fig. 1. The spectrum (\( \lambda_{\text{max}}/\text{nm} (\epsilon/\text{mM} \cdot \text{cm}^{-1}) = 405 (125), 525, 564, 630 \) differs from that of the wild type enzyme (\( \lambda_{\text{max}}/\text{nm} (\epsilon/\text{mM} \cdot \text{cm}^{-1}) = 407 (107), 525, \sim 630 (11); \) Fig. 1) and shows a peak in the visible region (564) that is consistent with the presence of low-spin heme. (The corresponding W41A variant in pea cytosolic APX was also examined (data not shown); in this variant similar wavelength maxima are observed but the Soret band is shifted to 412 nm.) The high-spin peak at \( \sim 630 \) nm in the wild type enzyme is still visible in the variant (Fig. 1). No evidence for the formation of (low-spin) hydroxide-bound heme was observed at alkaline pH (data not shown); hence, the low-spin species was tentatively assigned as arising from coordination of an (internal) protein ligand.

Reaction of ferric W41A with various noncatalytic ligands is informative because it allows us to assess whether the proposed protein ligand is reversibly or irreversibly bound to the metal. Addition of potassium cyanide to the ferric derivative of W41A leads to a spectrum in which complete formation of low-spin heme is now observed (\( \lambda_{\text{max}}/\text{nm} = 418, 540, 561 \) nm; Fig. 1). This suggests that addition of a strong exogenous ligand leads to displacement of the existing (internal) ligand. As shown below, the crystal structure of the cyanide-bound derivative of W41A confirms this observation. Additionally, we have observed that NO binds to the reduced heme (\( \lambda_{\text{max}} = 417, 541, 574 \) nm); the crystal structure of the NO-bound derivative also confirms that His-42 is displaced (see below).

EPR spectroscopy provides further evidence in support of a low-spin heme species (Fig. 2). The EPR spectrum of ferric W41A is dominated by a rhombic low-spin species with observed g-values of 3.22 and 2.05 (the third feature is too broad to be observed). A second, minor component with g-values of
5.67 and 1.99 is consistent with an axial, high-spin species. The low-spin species is the majority species at 10 K and is consistent with bis-histidine ligation; the g-values are consistent with an orientation in which the imidazole planes are not parallel to each other. Neither species resembles those observed in the EPR spectrum of the wild type enzyme, which has a rhombic high-spin species (g = 6.04, 5.27, and 1.98) and a small amount of rhombic low-spin heme (g = 2.69, 2.21, and 1.79) (13, 29). A minor low-spin rhombic species (g = 2.95 as a shoulder on the g = 3.22 feature) and the positive lobe of g feature (g = 2.30) in W41A are likely to arise from a histidine/histidine-ligated heme in which the imidazole planes are parallel to each other (note that there is also evidence for multiple conformations of the proximal histidine in the crystal structure of the cyanide-bound form of W41A; see above).

X-ray Crystallography of W41A and Its Cyanide- and NO-bound Derivatives—The crystal structure of the cyanide-bound derivative of W41A (Fig. 3 and Table 1) confirms the observations made in solution. In this structure (Fig. 3B), which closely maps onto that for the cyanide-bound form of rsAPX (Fig. 3A), the iron is ligated by the cyanide ligand and the nitrogen of the bound ligand is hydrogen-bonded (2.8 Å) to N8 of His-42 (2.5 Å in rsAPX). For the W41A-CN complex, the electron density observed for the proximal His-163 residue is consistent with two orientations of this side chain (Fig. 3B). One orientation has His-163 hydrogen-bonded to Asp-208 (3.3 Å) as for rsAPX (3.1 Å); the other orientation has His-163 hydrogen-bonded (2.8 Å) to the backbone carbonyl of Ser-160 (3.8 Å in rsAPX). In the rsAPX-CN structure a single conformation of His-163 is observed; however, the data are to lower resolution (2.0 Å compared with 1.4 Å), and a second orientation of the

![Figure 3. A and B, structures of the cyanide-bound complexes of rsAPX (A) and W41A (B) showing coordination of the diatomic ligand to the heme. For W41A, there are two orientations of His-163 observed. C and D, structures of the NO-bound complexes of rsAPX (C) and W41A (D) in the region of the active site. The loss of the indole of Trp-41 in W41A allows the NO molecule to adopt two orientations. Hydrogen bonds are shown as black dotted lines. Water molecules are shown as red spheres. This figure was created using PyMOL (40).](image-url)

| TABLE 1: Data collection and refinement statistics |
|--------------------------------------------------|
| **Statistics** | **W41A** | **W41A-CN** | **rsAPX-CN** | **W41A-NO** | **rsAPX-NO** | **W41A-H2O2** |
| Resolution (Å) | 28.88-1.35 | 36.81-1.4 | 58.200 | 45.88-1.25 | 18.86-2.01 | 28.99-1.75 |
| Outer shell | (1.42-1.35) | (1.436-1.4) | (2.07-2.0) | (1.32-1.25) | (2.064-2.01) | (1.84-1.75) |
| Total observations | 599,082 | 248,232 | 58,820 | 299,270 | 59,132 | 360,725 |
| Unique observations | 56,193 | 52,940 | 15,798 | 69,773 | 16,455 | 26,381 |
| I/|I2 | 17.8 (4.0) | 13.3 (3.1) | 15.9 (2.98) | 12.8 (3.9) | 15.8 (3.12) | 30.6 (11.6) |
| Rmerge | 0.097 | 0.088 | 0.052 | 0.107 | 0.051 | 0.059 |
| Completeness | 99.4 (99.9) | 92.8 (96.6) | 95.22 (95.7) | 98.6 (99.9) | 97.32 (95.9) | 99.8 (100) |
| Multiplicity | 10.7 | 4.7 | 3.7 | 4.3 | 3.6 | 13.7 |
| Refinement |
| Rfactor | 0.190 | 0.206 | 0.187 | 0.189 | 0.183 | 0.179 |
| Rfree | 0.205 | 0.229 | 0.235 | 0.207 | 0.258 | 0.220 |
| r.m.s.d. angle (°) | 1.086 | 1.110 | 1.290 | 1.049 | 1.457 | 1.188 |
| r.m.s.d. bonds (Å) | 0.007 | 0.008 | 0.012 | 0.006 | 0.015 | 0.011 |
| PDB accession code | 2GGN | 2GHD | 2GHK | 2GHC | 2GHH | 2GHE |
proximal histidine may not be observed because of the large effect of the heme electron density.

The crystal structure of the NO-bound derivative of W41A also confirms that His-42 is displaced (Fig. 3D). In this case, and in contrast to rsAPX-NO (Fig. 3C), removal of the hydrogen bond to Trp-41 means that the NO ligand now adopts two conformations.

To clarify the nature of the low-spin heme in ferric W41A, diffraction data to 1.75 Å were obtained (Fig. 4 and Table 1). W41A is very similar in its overall structure to the wild type protein; the root-mean-square deviation between Cα positions (residues 2–249) for this structure and ferric rsAPX is 0.260 Å (determined using LSQKAB (30)). The structure of W41A shows that space previously occupied by the Trp side chain is now filled by two water molecules (labeled 1 and 2 in Fig. 4A). Although the overall structure and most of the active site structure is similar to rsAPX, there are local changes in protein conformation around His-42 (Fig. 4B). Hence, the main chain of His-42 moves toward the heme in W41A such that N° of His-42 is now within bonding distance (2.3 Å, compared with 5.5 Å in rsAPX) of the iron. We refer to this as the "on" form. A further water molecule (labeled 3 in Fig. 4A) is located in the region that was previously occupied by His-42.

The Fe-N°(His-163) distance is essentially identical in both W41A and rsAPX (2.1 and 2.0 Å, respectively). This new histidine ligand replaces a water molecule that is bonded to the iron in the wild type protein (2.1 Å). This movement of the main chain of His-42 toward the heme in the on form and the subsequent alteration in heme geometry are the consequences of the removal of the bulky Trp-41 residue, which allows the His-42 side chain to ligate to the iron.

Examination of the electron density for W41A around His-42 indicates that there is positive \( F_o - F_c \) density above His-42 and also close to the main chain of His-42 (carbonyl oxygen; Fig. 4). This density overlays with the orientation of His-42 in rsAPX and is consistent with the presence of a minority (presumed high-spin) heme species in which His-42 is not ligated. There are two explanations of this observation. First, the spectroscopic data presented above are also consistent with some high-spin heme in the ferric form; in this case, the residual electron density would arise from a mixed population of high- and low-spin iron in the crystal. Second, although we used ferric enzyme in our experiments, we recognize that partial reduction of heme is possible during data collection (31). The electronic spectrum of ferrous W41A shows maxima (\( \lambda_{\text{max}}/\text{nm} = 428, 556, 581 \)) that are similar to those for rsAPX (\( \lambda_{\text{max}}/\text{nm} = 431, 555, 585 \)) and are not consistent with bis-histidine ligation, indicating that dissociation of His-42 probably occurs on reduction. In this case, the residual electron density would arise from partial reduction of the heme during data collection. We were not able to obtain crystallographic data for the ferrous W41A derivative. Whatever the origin of the residual density, the important feature is that the data clearly indicate that His-42 is flexible and can adopt more than one conformation.

Reactivity toward \( \text{H}_2\text{O}_2 \)—The data presented above indicate that, although the heme is largely 6-coordinate and low-spin in ferric W41A (with two strong histidine ligands), His-42 is not irreversibly bound to the heme and may dissociate under certain conditions. We refer to this dissociated form as the "off" form. If this is the case, then reaction with \( \text{H}_2\text{O}_2 \) and turnover of substrate may still be possible. Because there is, to our knowledge, no unambiguous example of a crystallographically defined bis-histidine-ligated peroxidase in the literature, it was of critical interest to establish whether strong axial ligation,
Conformational Mobility in a Heme Peroxidase

FIGURE 5. A, plot of observed rate constant, $k_{1,obs}$ versus [H$_2$O$_2$] for the reaction of W41A with hydrogen peroxide (sodium phosphate buffer, pH 7.0, 0.10 m, 25.0 °C). The solid line is a fit of the data to Equation 3. B, spectra of reaction intermediates, obtained using photodiode array, from reaction of ferric W41A with H$_2$O$_2$. Solid line, ferric W41A; dashed line, Compound I; dotted line, Compound II (pH 7.0, 5.0 °C, [W41A] = 3 μM, [H$_2$O$_2$] = 3 μM).

Analogous to that observed for example in the cytochromes $b$, would preclude reaction with H$_2$O$_2$.

Rate constants for Compound I formation in W41A were determined under pseudo-first-order conditions ([H$_2$O$_2$] = 10−125 μM). The W41A variant was shown to be competent for formation of Compound I under these conditions, albeit at a lower rate, as evidenced by a decrease in absorbance at 405 nm. These changes in absorbance duplicate those observed for rsAPX (11). Observed rate constants for this process, $k_{1,obs}$, showed a clearly nonlinear dependence on the concentration of hydrogen peroxide (Fig. 5A). This is in contrast to the data for rsAPX, in which a linear dependence on [H$_2$O$_2$] is observed in the experimentally accessible concentration range ($k_1 = (3.3 ± 0.1) \times 10^2$ M$^{-1}$ s$^{-1}$) (11). The nonlinear dependence is consistent with a mechanism that requires a conformational change of the protein, proposed to be conversion between the on and off forms, prior to reaction with H$_2$O$_2$, as shown in Equations 4 and 5 (CI = Compound I).

$$W41A_{on} \xrightarrow{k_1} W41A_{off} \quad \text{(Eq. 4)}$$

$$W41A_{off} + H_2O_2 \rightarrow CI + H_2O \quad \text{(Eq. 5)}$$

In the presence of excess H$_2$O$_2$, the observed rate constant, $k_{1,obs}$, can be expressed as follows (Equation 6).

$$k_{1,obs} = \frac{k_1 k_2 [H_2O_2]}{k_1 + k_2 [H_2O_2]} \quad \text{(Eq. 6)}$$

A fit of these data for W41A to Equation 6 (Fig. 5A) yields values for the limiting first-order rate constant, $k_1$, of 2370 s$^{-1}$ and the composite second order rate constant, $k_1 k_2/k_{-1}$, of $6.6 \times 10^6$ M$^{-1}$ s$^{-1}$.

In separate experiments, the reaction of W41A with H$_2$O$_2$ was studied using photodiode array detection (Fig. 5B). Data collected over a period of 500 ms were best-fitted to a two-step model (A $\rightarrow$ B $\rightarrow$ C, as shown previously for rsAPX (11)), where A is ferric W41A, B is Compound I, and C is Compound II. Spectra for the Compound I ($\lambda_{max}/nm = 410, 530, 569^{sh}$, 640) and Compound II ($\lambda_{max}/nm = 414, 530, 560^{th}$) intermediates are in agreement with those observed for rsAPX ($\lambda_{max}/nm = 409, 530, 569^{sh}$, and 655 for Compound I and $\lambda_{max}/nm = 417, 529$, and 560; this work and Ref. 11). Formation of Compound I (i.e. A $\rightarrow$ B) for W41A occurs with an observed rate constant ($k_{1,obs}$) of 26 ± 0.2 s$^{-1}$, which is ∼10-fold slower than for rsAPX ($k_{1,obs} = 218 ± 2.6$ s$^{-1}$). Formation of Compound II (i.e. B $\rightarrow$ C) occurs with similar rate constants for both W41A and rsAPX ($k_{2,obs} = 3.8 ± 0.05$ and $2.7 ± 0.1$ s$^{-1}$, respectively). These data clearly indicate that reaction of ferric W41A with H$_2$O$_2$ leads to the formation of genuine Compound I and Compound II intermediates, as observed for rsAPX, and suggest that conformational rearrangement of the protein is involved during catalysis.

Reactivity toward Substrates—The W41A variant was also shown to be competent for oxidation of two types of substrate. In addition to its physiological substrate (ascorbate), APX is known to oxidize other aromatic substrates that are typical of the class II and III peroxidases (32).
Conformational Mobility in a Heme Peroxidase

FIGURE 7. Overlay of the structures of ferric W41A (green) and ferric W41A after reaction with H$_2$O$_2$ (yellow). Water molecules in the two structures are shown in green and yellow, respectively. The orientation of His-42 after reaction with H$_2$O$_2$ (yellow) overlays with that of rsAPX (see Fig. 4B). This figure was created using PyMOL (40).

FIGURE 8. Electronic spectra of ferric W41A prior to the addition of H$_2$O$_2$ (solid line) and ferric W41A immediately after reaction with 1 equivalent of H$_2$O$_2$ (dashed line) and after reaction with 1 equivalent of H$_2$O$_2$ followed by the addition of 1 equivalent of ascorbate (dashed-dotted line). The visible region has been multiplied by a factor of 5 (sodium phosphate, pH 7.0, $\mu$ = 0.1 M, 25.0 °C).

two types of substrate differs: ascorbate binds close to the heme 6-propionate (at the so-called $\gamma$-heme edge (18)), and aromatic substrates are thought to bind close to the $\delta$-heme edge (33, 34).

For ascorbate oxidation by W41A, steady state data obeyed the Michaelis-Menten equation. The kinetic data presented above allow three major conclusions to be drawn. (a) Removal of Trp-41 leads to increased conformational mobility around the active site. This makes His-42 more mobile such that it now coordinates to the heme in the ferric derivative of W41A (the on form). (b) The inherent flexibility of His-42 means that it may dissociate from the heme under certain conditions (the off form), for example in the presence of a strong exogenous ligand (e.g. cyanide, NO). (c) This switch between the on and off forms is also used during catalysis through a mechanism that involves dissociation of His-42 on reaction with H$_2$O$_2$ and subsequent oxidation of substrate through the normal catalytic route.

We sought more explicit information on the conformational mobility of His-42 during catalysis. Hence, we conducted an experiment in which crystals of ferric W41A were soaked in H$_2$O$_2$ (100 mM) for 5 min and then frozen immediately in liquid nitrogen. The overall structure of H$_2$O$_2$-soaked W41A, including most of the active site region, is very similar to both ferric W41A (root-mean-square deviation between C$_\alpha$ positions is 2.1 Å) and to ferric rsAPX (0.10 Å), but in comparison with the ferric W41A structure there are local changes in conformation around His-42 (Fig. 7). Hence, His-42 now swings off the iron to occupy a position that is identical to that observed in the ferric rsAPX structure. Clear electron density is observed directly above the iron in a region that for ferric rsAPX (18), as well as for other peroxidases, has been assigned as a water molecule (Fig. 7). The distance between the iron and this distal water molecule (Fig. 7). The distance between the iron and this distal water molecule is very similar (2.2 Å) to that observed in ferric W41A structure; local changes in conformation around His-42 during catalysis. Hence, we conducted an experiment in which crystals of ferric W41A were soaked in H$_2$O$_2$ (100 mM) for 5 min and then frozen immediately in liquid nitrogen. The overall structure of H$_2$O$_2$-soaked W41A, including most of the active site region, is very similar to both ferric W41A (root-mean-square deviation between C$_\alpha$ positions is 2.1 Å) and to ferric rsAPX (0.10 Å), but in comparison with the ferric W41A structure there are local changes in conformation around His-42 (Fig. 7). Hence, His-42 now swings off the iron to occupy a position that is identical to that observed in the ferric rsAPX structure. Clear electron density is observed directly above the iron in a region that for ferric rsAPX (18), as well as for other peroxidases, has been assigned as a water molecule; we have also assigned this density as arising from a water molecule (Fig. 7). The distance between the iron and this distal water molecule is very similar (2.2 Å) to that observed in ferric rsAPX (2.1 Å).

Examination of Catalytic Intermediates—The heme geometry observed in the structure of H$_2$O$_2$-soaked W41A, in which His-42 swings off the heme, is different from the majority of low-spin species observed spectroscopically at both room temperature (electronic spectra) or cryogenic temperature (EPR spectra) or from that observed in the crystal structure of the ferric derivative (see above). For this reason we carried out solution experiments, on a timescale identical to that of the crystallography experiments, to authenticate the nature of the heme.

$^3$ For rsAPX, data for oxidation of ascorbate did not obey Michaelis kinetics, and these data were fitted to the Hill equation instead to extract values for $k_{cat}$ and $K_m$ (11).
species observed. Hence, ferric W41A was reacted with H₂O₂ (varied from 1–100 equivalents) and the spectrum collected immediately. This spectrum (Fig. 8), shows clear evidence for formation of a Compound II intermediate, as observed for W41A in the transient kinetic experiments (Fig. 5B). This Compound II species persisted for >15 min for W41A; subsequent reduction by ascorbate gave a spectrum that was essentially identical to the original ferric low-spin heme species (i.e. predominantly bis-histidine ligated) (Fig. 8). This is interpreted as evidence for reversibility between the on and off forms and is consistent with the steady state data presented above. No evidence for the presence of majority high-spin heme was observed under any conditions; high-spin heme is easily identifiable because ferric (water-bound) rsAPX has a clear spectroscopic signature (see above) that is not observed for W41A.

These data provide clear evidence for reversible switching between the on and off forms and are in agreement with the mechanism proposed above and with the crystallographic data. In the light of these data, and because no evidence for a predominantly high-spin (water-bound) heme was observed spectroscopically, the crystallographic data presented in Fig. 7 are most sensibly rationalized as arising from a Compound II-derived structure. The iron-oxygen bond, at 2.2 Å, is longer than those previously reported for other Compound II intermediates (reported as 1.87 Å (35), 1.92 Å (36), 1.82 Å (37), 1.8 Å (31)), which may reflect the fact that partial conversion back toward the on form, as observed spectroscopically, has occurred.

Conclusions—Collectively, the spectroscopic, mechanistic, and crystallographic data presented here illustrate that removal of Trp-41 in ascorbate peroxidase leads to increased internal mobility of His-42 and clearly indicate that this residue can ligate to the heme in the oxidized derivative. The major findings are summarized in Scheme 1. Contrary to expectation, strong coordination of His-42 to the iron to form a bis-histidine-ligated heme does not preclude reaction with H₂O₂ because His-42 is conformationally mobile and can reversibly interconvert between on and off forms to allow reaction with peroxide to occur normally. In this sense, W41A is able to act as a bona fide peroxidase, because substrate binding at both the γ-heme edge (for ascorbate) and the δ-heme edge (for aromatic substrates) is still possible. More generally, any alteration in peroxidase structure that allows increased mobility of His-42, either through removal of the hydrogen bonding interaction between His-42 and Asn-71 or by removal of steric bulk in the heme cavity (near Trp-41), should encourage conversion to cytochrome b-type geometry. Introduction of bulk on the distal side (e.g. by replacement of Asn-71 with a more bulky residue) may have the same effect by pushing His-42 toward the iron.

These results provide a new perspective on the widely held view of heme enzyme catalysis in which strong axial ligation, as exemplified most famously in the cytochromes, is presumed to inhibit reaction with ligands at the heme iron. We note that reversible coordination of the distal histidine is also possible in some hemoglobins (38) and some heme-based sensors (39), indicating that a similar mechanism for heme-catalyzed biological activity might be used more widely in other, more complex heme enzymes.

Acknowledgments—We thank Kuldeep Singh for technical assistance. We are grateful to Professor Andrew Thomson and Dr. Myles Cheesman for the use of EPR facilities and to Dr. Clive Metcalfe for collection of preliminary mass spectrometry data. Dr. Sharad Mistry, Dr. Andrew Bottrill, and Shaibabanu Ashra are also acknowledged for assistance with MALDI data collection. We also thank Dr. David Leys and Dr. Alexandre R. Gingras for synchrotron data collection and Nicholas Putz for assistance with graphics.

REFERENCES
1. Everse, J., Everse, K. E., and Grishham, M. B. (1991) Peroxidases in Chemistry and Biology, Vol. 1 and 2, CRC Press, Boca Raton, FL
2. Dunford, H. B. (1999) Heme Peroxidases, John Wiley, Chichester, United Kingdom
3. Cheek, J., Mandelman, D., Poulos, T. L., and Dawson, J. H. (1999) *J. Biol. Inorg. Chem.* 4, 64–72
4. Sutherland, G. R. J., Zapanta, S., Tien, M., and Aust, S. D. (1997) *Biochemistry* 36, 3654–3662
5. Smulevich, G., Miller, M. A., Kraut, J., and Sprio, T. G. (1991) *Biochemistry* 30, 9546
6. Vitello, L. B., Erman, J. E., Miller, M. A., Mauro, J. M., and Kraut, J. (1992)
Conformational Mobility in a Heme Peroxidase

Biochemistry 31, 11524–11535

7. Turano, P., Ferrer, J. C., Cheesman, M. R., Thomson, A. J., Banci, L., Bertini, I., and Mauk, A. G. (1995) Biochemistry 34, 13895–13905

8. Youngs, H. L., Moenne-Loccoz, P., Loehr, T. M., and Gold, M. H. (2000) Biochemistry 39, 9994–10000

9. Nelson, D. P., and Kiesow, L. A. (1972) Anal. Biochem. 49, 474–478

10. Metcalfe, C. L., Ott, M., Patel, N., Singh, K., Mistry, S. C., Goff, H. M., and Raven, E. L. (2004) J. Am. Chem. Soc. 126, 16242–16248

11. Lad, L., Mewies, M., and Raven, E. L. (2002) Biochemistry 41, 13774–13781

12. Antonini, M., and Brunori, E. (1971) Hemoglobin and Myoglobin and Their Reactions with Ligands, North Holland Publishers, Amsterdam

13. Jones, D. K., Dalton, D. A., Rosell, F. I., and Lloyd Raven, E. (1998) Arch. Biochem. Biophys. 360, 173–178

14. Mittler, R., and Zilinskas, B. A. (1991) Plant Physiol. 97, 962–968

15. Asada, K. (1984) Methods Enzymol. 105, 422–427

16. Kelly, G. J., and Lattko, E. (1979) Naturewissenschaften 66, 617–618

17. Santimone, M. (1975) Can. J. Biochem. 53, 649–657

18. Sharp, K. H., Mewies, M., Moody, P. C. E., and Raven, E. L. (2003) Nat. Struct. Biol. 10, 303–307

19. Leys, D., Backer, K., Meyer, T. E., Hagen, W. R., Cusanovich, M. A., and Van Beeumen, J. J. (2000) J. Biol. Chem. 275, 16050–16056

20. Ferrer, J. C., Turano, P., Banci, L., Bertini, I., Morris, I. K., Smith, K. M., Smith, M., and Mauk, A. G. (1994) Biochemistry 33, 7819–7829

21. Vitello, L. B., Huang, M., and Erman, J. E. (1990) Biochemistry 29, 4283–4288

22. Vitello, L. B., Erman, J. E., Miller, M. A., Wang, J., and Kraut, J. (1993) Biochemistry 32, 9807–9818

23. Erman, J. E., Vitello, L. B., Miller, M. A., Shaw, A., Brown, K. A., and Kraut, J. (1993) Biochemistry 32, 9798–9806

24. Leslie, A. G. W. (1992) Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography, No. 26

25. Collaborative Computational Project (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763

26. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 227, 366–396

27. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 240–255

28. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 2126–2132

29. Gadsby, P. M., and Thomson, A. J. (1990) J. Am. Chem. Soc. 112, 5003–5011

30. Kabsch, W. (1976) Acta Crystallogr. Sect. A 32, 922–923

31. Berglund, G. I., Carlsson, G. H., Smith, A. T., Szoke, H., Henriksen, A., and Hajdu, J. (2002) Nature 417, 463–468

32. Raven, E. L. (2003) Nat. Prod. Rep. 20, 367–381

33. Sharp, K. H., Moody, P. C. E., Brown, K. A., and Raven, E. L. (2004) Biochemistry 43, 8644–8651

34. Bursey, E. H., and Poulos, T. L. (2000) Biochemistry 39, 7374–7379

35. Bonagura, C. A., Bhasker, B., Shimizu, H., Li, H., Sundaramoorthy, M., McRee, D. E., Goodin, D. B., and Poulos, T. L. (2003) Biochemistry 42, 5600–5608

36. Hersleth, H. P., Dalhus, B., Gorbiz, C. H., and Andersson, K. K. (2002) J. Biol. Inorg. Chem. 7, 299–304

37. Green, M. T., Dawson, J. H., and Gray, H. B. (2004) Science 304, 1653–1656

38. Trent, J. T., III, and Hargrove, M. S. (2002) Trends Plant Sci. 8, 387–393

39. Sasakura, Y., Yoshimura-Suzuki, T., Kurokawa, H., and Shimizu, H. (2006) Acc. Chem. Res. 39, 37–43

40. Delano, W. L. (2002) The PyMOL Molecular Graphics System, Delano Scientific, San Carlos, CA