The Asp245→Asn Mutant of *Coprinus cinereus* Peroxidase. Characterization by ^{1}H-NMR Spectroscopy and Comparison with the Wild-Type Enzyme†

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ABSTRACT: The resting, fluoride-ligated and cyanide-ligated states of the Asp245→Asn mutant of *Coprinus cinereus* peroxidase (D245N CIP) have been characterized using ^{1}H-NMR spectroscopy in conjunction with parallel studies of the wild-type enzyme. Analysis of the spectra of resting state D245N CIP over the pH range 5−10 has uncovered the existence of three high-spin species in dynamic equilibrium with each other. The predominant species at neutral pH is six-coordinate high-spin (6-c HS), with a distal water molecule as the sixth ligand. This species is in slow exchange on the NMR time scale with a second six-coordinate high-spin species (6-c HS*) and a five-coordinate high-spin species (5-c HS**), toward acidic and alkaline pH values, respectively. The 6-c HS* species appears to be unique and is proposed to differ from the 6-c HS species by protonation of the proximal His residue, whereas the 5-c HS** species lacks the proximal His ligand and is coordinated by a hydroxyl group. In sharp contrast, wild-type CIP is a five-coordinate high-spin (5-c HS) species over the same pH range. The D245N CIP mutant also exhibits a greater affinity for fluoride than wild-type CIP. The ^{1}H-NMR spectrum of cyanide-ligated D245N CIP, assigned using two-dimensional methods, differs significantly from that of the wild-type enzyme. Perturbations to heme and heme-linked proton resonances are rationalised in terms of the loss or significant weakening of the hydrogen bond between His183 Nδ1H and the side-chain of residue 245 when Asp is replaced by Asn. This subtle interaction directly affects the heme pocket structure of CIP both proximal and distal to the heme plane.

The plant peroxidase superfamily is divided into three distinct structural classes, with class II containing enzymes of fungal origin (Welinder, 1992). In addition to the well-known lignin and manganese-dependent peroxidases, which occur as multiple isoenzymes, this class also includes a single heme peroxidase secreted by the ink-cap fungus, *Coprinus cinereus* (Morita et al., 1988). An efficient expression system in *Aspergillus oryzae*, leading to the availability of recombinant enzyme and a large number of site-directed mutants, has made *C. cinereus* peroxidase (CIP)[1] something of a paradigm for exploring structure:function relationships within heme peroxidases (Welinder & Andersen, 1993).

All enzymes included in the plant peroxidase superfamily have as a common feature a number of invariant amino acids, one of which is an Asp residue neighboring the proximal His residue coordinated to the heme iron atom. Crystal structure data from representatives of each of the three classes has confirmed that the side chain of this Asp residue is involved in a key hydrogen bond to the Nδ1H atom of proximal His (Wang et al., 1990; Kunishima et al., 1994; Schuller et al., 1996). The function of this residue has been investigated most fully in yeast cytochrome *c* peroxidase (CCP), using both wild-type enzyme and the CCP mutants, D235A, D235E, and D235N. Three main roles have been suggested on the basis of X-ray (Wang et al., 1990), resonance Raman (Smulevich et al., 1988; Spiro et al., 1990), NMR (Satterlee et al., 1990; Ferrer et al., 1994), EPR (Goodin & McRee, 1993), and enzyme kinetics (Vitello et al., 1992), namely, that Asp235 of CCP acts to keep the sixth coordination state of the heme iron vacant, indirectly stabilises the higher oxidation states of Fe known as compounds I and II, and engages in additional hydrogen bonded interactions with Trp191 in order to optimize the orientation of this key side-chain with respect to the heme and proximal His175.

In this study, the role of the analogous Asp245 residue of *C. cinereus* peroxidase has been investigated using proton NMR spectroscopy, as no data comparable with that for CCP is available at present for any class II fungal peroxidase. The NMR spectra of resting, fluoride- and cyanide-ligated states of the D245N CIP mutant are compared with those obtained for wild-type CIP and contrasted with corresponding data for the D235N CCP mutant (Satterlee et al., 1990). Wild-type CIP from both commercial and recombinant sources has been characterized previously by NMR (Lukat et al., 1989; Veitch et al., 1994) and a variety of other spectroscopic

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[1] Abbreviations: ARP, *Arthromyces ramosus* peroxidase; CCP, cytochrome *c* peroxidase; CIP, *Coprinus cinereus* peroxidase; D235A CCP, the Asp235→Ala mutant of CCP; D235E CCP, the Asp235→Glu mutant of CCP; D235N CCP, the Asp235→Asn mutant of CCP; D245N CIP, the Asp245→Asn mutant of CIP; HRP C, horseradish peroxidase isoenzyme C; 5-c HS, five-coordinate high-spin; 6-c LS; six-coordinate low-spin; 6-c HS, six-coordinate high-spin.

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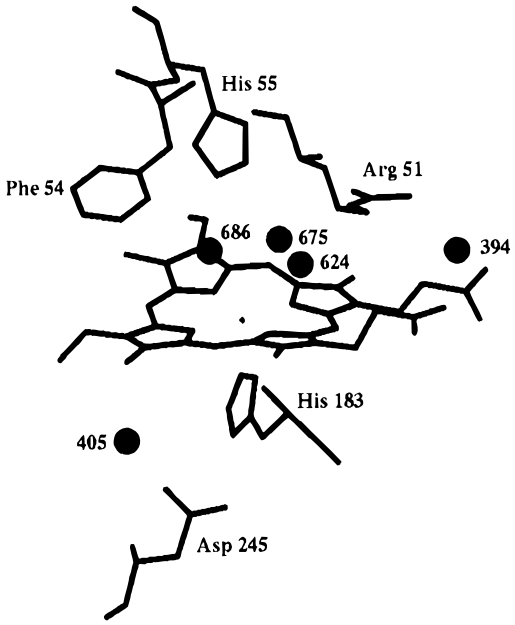

FIGURE 1: Schematic illustration of the heme binding pocket of *C. cinereus* peroxidase, after Kunishima et al. (1996). Heme iron is coordinated in the proximal position by His183. A number of significant structural water molecules are also shown, of which Wat686, at a distance of 3.9 Å, is nearest to heme iron. This water molecule is hydrogen-bonded to the Nϵ2 atom of distal His55.

techniques (Andersen et al., 1991; Smulevich et al., 1994), and X-ray data to 2.6 Å resolution is available for recombinant CIP (Petersen et al., 1994). Additional studies published under the species names *Coprinus macrorhizus* (DePillis & Ortiz de Montellano, 1989; Dugad & Goff, 1992) and *Arthromyces ramosus* (Farhangrazi et al., 1994) must also be considered, as the corresponding heme peroxidases are identical to CIP except in their degree of glycosylation (Kjalke et al., 1992). *A. ramosus* has been proposed to be a new species (Shinmen et al., 1986), but is not yet described in the taxonomic literature, while *C. macrorhizus* is a synonym of *C. cinereus* and not a separate taxon. The crystal structure of *A. ramosus* peroxidase (ARP) has been solved to 1.8 Å resolution, and data for cyanide- and triiodide-bound forms are also available, representing a valuable source of three-dimensional structural information which will be referred to as a standard for the present work (Kunishima et al., 1994, 1996; Fukuyama et al., 1995). An outline structure of the heme binding region of this enzyme is given in Figure 1 (Kunishima et al., 1996). Note that amino acids are numbered throughout according to the published sequence of CIP in order to maintain consistency with other studies (Baunsgaard et al., 1993).

EXPERIMENTAL PROCEDURES

Recombinant CIP and D245N CIP were obtained by heterologous expression in *Aspergillus oryzae* and purified as described previously (Veitch et al., 1994). Enzyme samples, stored as ammonium sulfate suspensions and kept at 4 °C, were spun down in a microcentrifuge prior to NMR experiments and the supernatant discarded. The pellets were redissolved first in a solution of 2 mM calcium chloride, before concentration at 4 °C in Amicon Centricon 10 microconcentrators and several cycles of solvent exchange. Initial solution conditions for the titrations of resting state samples were either 10 mM KH_2PO_4, D_2O, pH 7.8, or 10 mM KH_2PO_4, 20 mM H_3BO_3, D_2O, pH 7.8. Fluoride-ligated enzyme samples were prepared in 10 mM KH_2PO_4, 50 mM NaF, D_2O, at pH 5.6, and cyanide-ligated samples were prepared in 10 mM KH_2PO_4, 15 mM KCN, D_2O, at pH 7.0. pH adjustments were made by addition of small aliquots of 10- or 20-fold diluted DCl or NaOD. All pH measurements were made using a Radiometer pH26 meter and are uncorrected for the small deuterium isotope effect. Specific readings in D_2O solutions are denoted by pH* in the text. Enzyme concentrations were determined spectrophotometrically using an absorption coefficient of 109 cm^{-1} mM^{-1} at 405 nm (Andersen et al., 1991) and were typically between 0.5 and 1.0 mM.

All ^{1}H-NMR experiments were carried out on either Varian or Bruker 500 MHz instruments. The general procedures for acquisition and processing of both one- and two-dimensional experiments have been described in detail elsewhere (Veitch et al., 1994). One-dimensional spectra of resting state enzymes were obtained using a spectral width of 100 kHz, 8K to 16K transients, and a recycle time of 5 s^{-1}. The 90° pulse width was 6.2−6.6 μs, although a flip angle of 45° was preferred. Spectral processing introduced a line-broadening factor of 30 Hz (40 Hz was used for the spectra of the fluoride-ligated enzymes). Base line correction was necessary for all one-dimensional spectra. A mixing time of 30 ms was used in NOESY experiments, while in two-dimensional TOCSY experiments, the isotropic mixing time was 18.6 ms. All experiments were undertaken at 30 °C unless otherwise stated. Chemical shift measurements are referenced to 1,4-dioxan as an internal standard with a resonance at 3.74 ppm relative to 2,2-dimethyl-2-silapentane-5-sulfonate.

RESULTS

Resting State Enzymes: Dependence of Spin- and Coordination-State on pH

A previous comparison of the ^{1}H-NMR spectra of resting state wild-type *C. cinereus* peroxidases from both commercial and recombinant sources found them to be identical with respect to hyperfine-shifted resonances (Veitch et al., 1994). The chemical shift and line width parameters of these resonances, recorded at neutral pH, were typical of those of a 5-c HS species, the identity of which has been confirmed by other spectroscopic measurements including resonance Raman (Smulevich et al., 1994). In Figure 2, a comparison of the behavior of the hyperfine-shifted resonances of resting state D245N and wild-type CIP between neutral and acidic pH is given. It can be seen from examination of Figure 2B, that the downfield region of the spectrum of wild-type CIP at 30 °C and neutral pH comprises four heme methyl resonances at typical chemical shift values between 50 and 90 ppm, namely, 85.3, 72.0, and 64.5 (overlap of two heme methyls) ppm, and a set of single proton resonances between 30 and 50 ppm usually associated with vinyl C3^{1}H and C8^{1}H, propionate C13^{2}H$_2$ and C17^{2}H$_2$, and proximal histidine CβH$_2$ protons. This distribution of hyperfine-shifted resonances is similar to that found in the analogous spectrum of resting state horseradish peroxidase isoenzyme C (HRP C), a predominantly 5-c HS species for which specific proton resonance assignments are available (La Mar et al., 1980; De Ropp & La Mar, 1991). A very broad single proton resonance, known to occur in the spectra

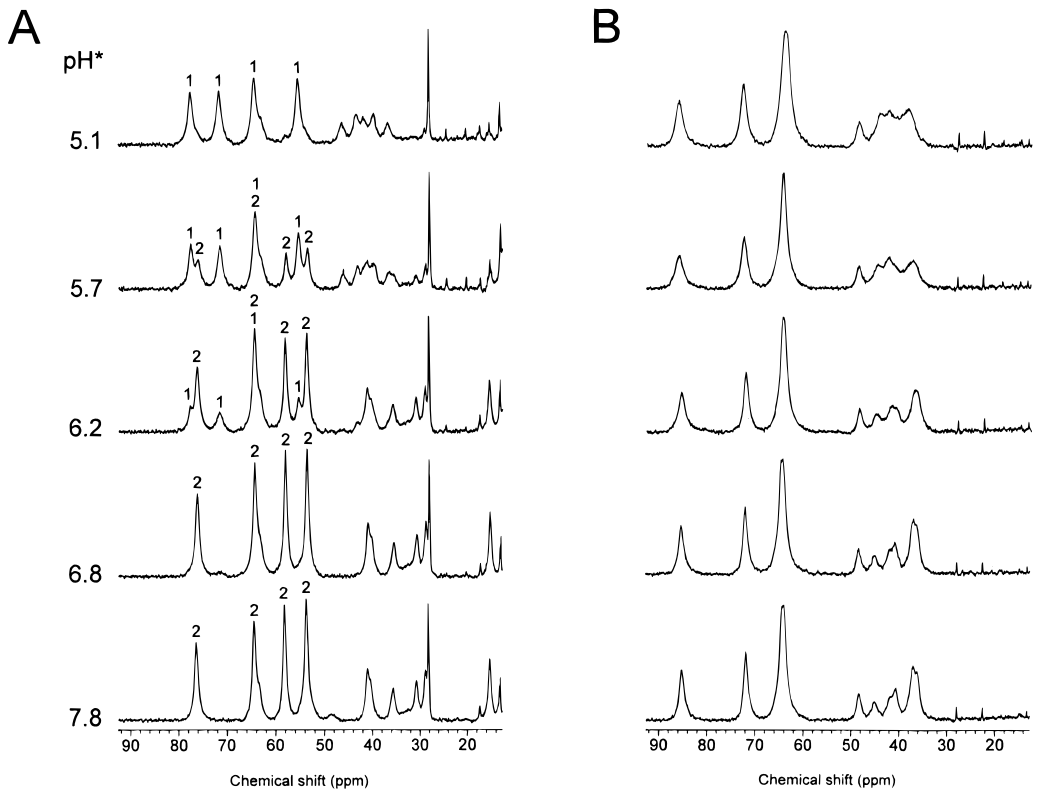

FIGURE 2: One-dimensional ^{1}H-NMR spectra of (A) resting state D245N CIP and (B) resting state wild-type CIP, showing the pH dependence of hyperfine-shifted resonances from neutral to acidic conditions. Heme methyl resonances are distinguished by labels "1" and "2" corresponding to the HS1 and HS2 species, respectively. The spectra were recorded at 500 MHz with solution conditions of 10 mM potassium phosphate, D_2O, 30 °C.

of some resting state heme peroxidases between 90 and 100 ppm when recorded in deuterium oxide solutions, is absent from the corresponding spectra of *C. cinereus* peroxidases. This exchangeable resonance has been ascribed to proximal His Nδ1H, and its presence or absence is presumed to be linked to the degree of solvent accessibility in this region (La Mar et al., 1980; Banci et al., 1992; De Ropp & La Mar, 1993).

The chemical shift values of the heme methyl resonances of resting state wild-type CIP are largely unaffected by the transition from neutral to acid pH. A number of relatively small shifts and intensity changes occur for the resonances in the 30−50 ppm region as the pH is lowered although overall the appearance of the spectrum is not changed markedly. In contrast, the corresponding spectra of the D245N CIP mutant shown in Figure 2A are remarkably different.

At neutral pH, the four heme methyl resonances of resting state D245N CIP are well resolved at 76.5, 64.6, 58.3, and 53.8 ppm. The mean value of these methyl shifts is, at 63.3 ppm, significantly lower than that for wild-type CIP at 71.6 ppm. As the pH is lowered, a second set of heme resonances appears with altered chemical shift values of 77.6, 71.7, 64.5, and 55.5 ppm (mean of 67.3 ppm). The original set of heme resonances observed at neutral pH decrease in intensity as the pH is lowered while at the same time, the new set increase in intensity. There are no accompanying chemical shift changes to either set during the course of the titration. The spectrum acquired at pH* 5.7 affords a particularly elegant example of this phenomenon, as all eight heme methyl resonances can be observed simultaneously. This indicates that two high-spin species are present which are in slow exchange with respect to the NMR time scale. These are denoted as HS1 and HS2, for acidic and neutral pH values respectively. The pK_a value for this transition appears to be close to 6.0. The spectral region between 30 and 50 ppm is also of interest and differs between HS1 and HS2 species. At pH* values below 4.5 and 5.1 (for wild-type and mutant enzymes, respectively), sample precipitation becomes a problem. This may be due both to the proximity to the pI value, reported to be 3.5 for the wild-type enzyme (Morita et al., 1988), and to the relatively high concentrations of enzymes used in the experiments.

The titration experiment was extended to cover the neutral to alkaline pH range by use of a buffer solution containing 10 mM potassium phosphate and 20 mM boric acid in D_2O. Inspection of Figure 3B confirms that the spectrum recorded at pH* 7.8 for wild-type CIP in this buffer is identical to that obtained at the same pH* in 10 mM potassium phosphate in D_2O, as shown in Figure 2B. The corresponding spectra of D245N CIP recorded at pH* 7.8 in these buffers differ only with respect to the intensity of a single resonance at 15.4 ppm, according to Figures 2A and 3A. In contrast, the differences between the pH dependence of resting state wild-type and D245N CIP over this range are dramatic. The effect of increasing pH on the spectrum of the wild-type enzyme is minimal, as is evident from Figure 3B. In contrast, a new high-spin species is observed for resting state D245N CIP at alkaline pH values (Figure 3A). This species has heme methyl resonances with chemical shift values of 67.9, 54.6, 52.5, and 48.1 ppm, with a mean value of 55.8 ppm. As the pH is increased toward alkaline values the set of heme methyl resonances corresponding to HS2 decrease in intensity while those representing the alkaline form, HS3, increase in intensity, with a pK_a value for the transition of approximately 8.8. There are no accompanying chemical shift changes,

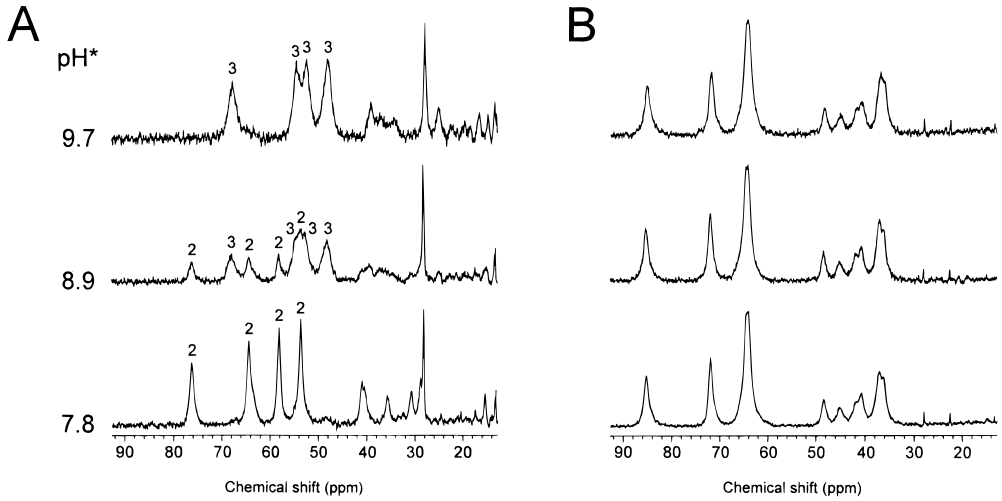

FIGURE 3: One-dimensional ^{1}H-NMR spectra of (A) resting state D245N CIP and (B) resting state wild-type CIP, showing the pH dependence of hyperfine-shifted resonances from neutral to alkaline conditions. Heme methyl resonances are distinguished by labels "2" and "3" corresponding to the HS2 and HS3 species, respectively. The spectra were recorded at 500 MHz with solution conditions of 10 mM potassium phosphate, 20 mM boric acid, D_2O, 30 °C.

which confirms that the species HS2 and HS3 are also in slow exchange on the NMR time scale. At pH* values greater than 10.5 a marked decrease in the intensity of the hyperfine-shifted resonances was noted for both wild-type and D245N CIP. These higher pH forms were not further investigated, although it appears that the associated transitions are reversible, as judged by analysis of NMR spectra recorded after lowering the pH of the samples.

In addition to the characteristic HS hyperfine-shifted resonances in the spectra of resting state D245N CIP, a number of resonances of narrower line width occur in the spectral region between 30 and 15 ppm. Two near-coincident heme methyl resonances close to 28.0 ppm are particularly notable. These are typical of 6-c LS species such as the cyanide-ligated form of heme peroxidases. The chemical shift values and intensities of this set of resonances in resting state D245N CIP are unaffected by pH, as is evident from Figures 2A and 3A. Similar resonances are seen in the corresponding spectra of wild-type CIP (Figures 2B and 3B), although these are of greatly reduced intensity with respect to the HS hyperfine-shifted resonances. The chemical shift values of these resonances are also unaffected by pH changes.

Comparison between Fluoride-Ligated D245N and Wild-Type CIP

Fluoride binds directly to the heme iron atom of peroxidases in the undissociated acid form, as is also the case for azide and cyanide (Dunford & Stillman, 1976). The fluoride-bound form of HRP C, for example, is a 6-c HS species, with magnetic susceptibility data characteristic of an axially symmetric high-spin electronic state (Theorell, 1942). A crystal structure for the related fluoride-bound complex of CCP has been determined and refined to 1.85 Å (Edwards & Poulos, 1990). The distal arginine residue Arg48 is displaced by 2.0 Å in order to optimize a hydrogen bond between this side chain and bound fluoride. A smaller adjustment to the distal histidine residue His52 also occurs, together with some reorganization to the hydrogen-bonded network of four water molecules in the distal pocket. The fluoride ligand is thus a useful probe of spin- and coordination state changes in the heme-linked region.

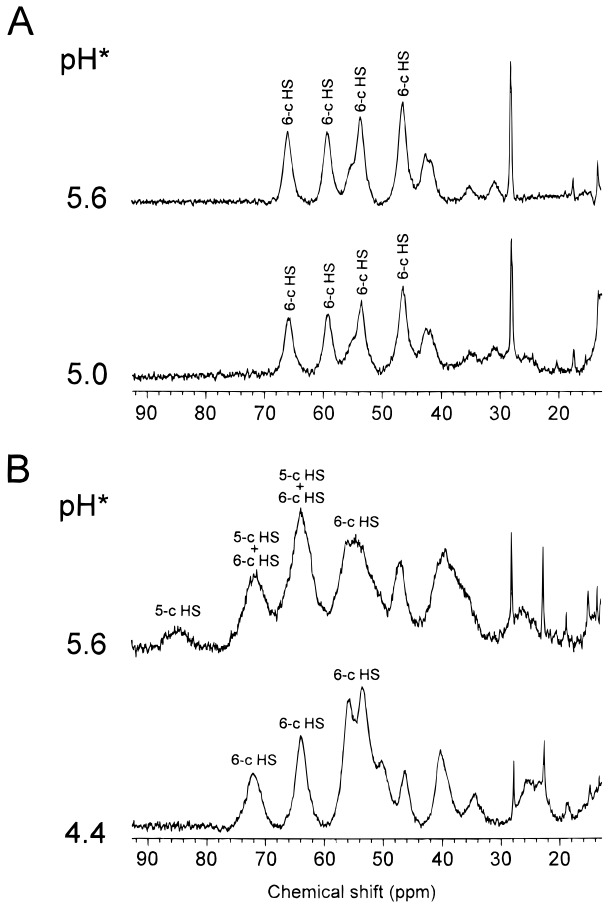

FIGURE 4: One-dimensional ^{1}H-NMR spectra of (A) fluoride-ligated D245N CIP and (B) fluoride-ligated wild-type CIP. The spectra were recorded at 500 MHz with solution conditions of 10 mM potassium phosphate, 50 mM sodium fluoride, D_2O, 30 °C. Heme methyl resonances are labeled "5-c HS" and "6-c HS", corresponding to resting and fluoride-ligated states, respectively. Chemical shift values may be consulted in Table 2.

A comparison between the ^{1}H-NMR spectra of fluoride-bound forms of wild-type and D245N CIP is given in Figure 4, with data obtained at several different pH* values. At pH* 5.6 the spectrum of fluoride-ligated wild-type CIP consists of two sets of partially overlapping resonances due solely to high-spin species. One set is characteristic of the

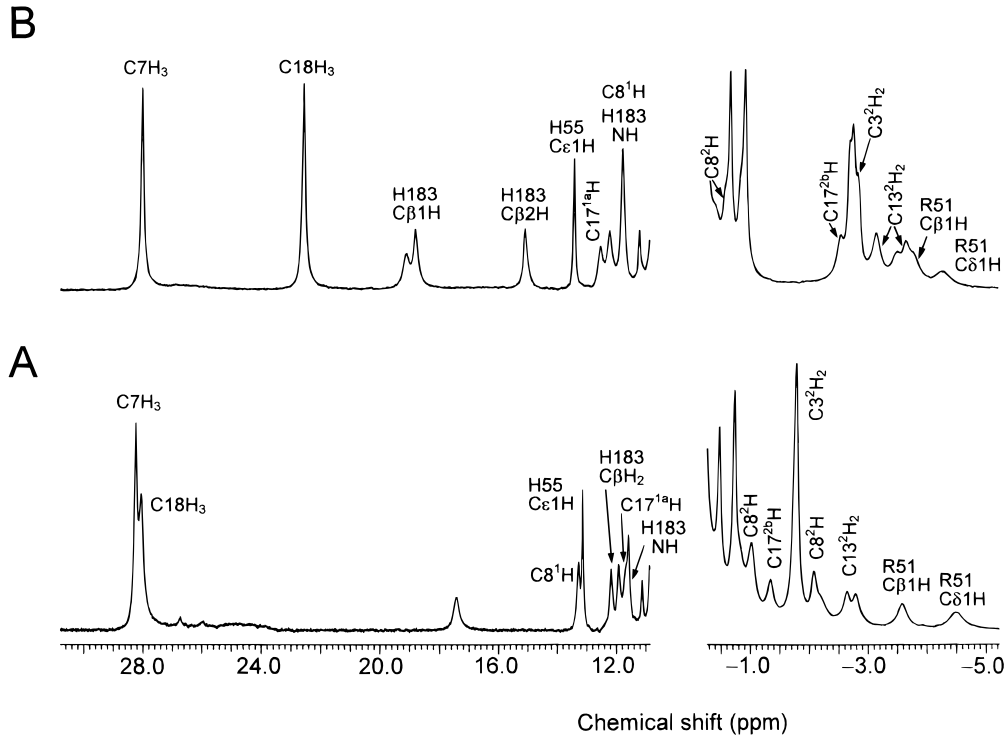

FIGURE 5: Comparison between the hyperfine-shifted regions of the one-dimensional ^{1}H-NMR spectra of (A) cyanide-ligated D245N CIP and (B) cyanide-ligated wild-type CIP. A number of resonance assignments for heme and heme-linked protons are given for reference. The resonance of the vinyl protons, $C3^2H_2$, in spectrum A overlaps with that of an unassigned methyl group. Spectra were recorded at 500 MHz with solution conditions of 10 mM potassium phosphate, 15 mM potassium cyanide, D_2O, at pH 7.0 and 30 °C.

5-c HS species of resting state CIP, while the second arises from the 6-c HS fluoride-ligated species. The heme methyl resonances of this species are upfield of their 5-c HS counterparts, with chemical shift values of 72.1, 64.0, 55.9, and 53.5 ppm and a mean value of 61.4 ppm. At pH* 4.4, the resonances due to the 5-c HS species are essentially absent and the spectrum is dominated by the resonances of the fluoride-bound 6-c HS species. The data further indicate that the binding of the protonated fluoride ligand to wild-type CIP is characterized, in terms of dynamics, by the slow exchange regime. Analogous data for the fluoride complex of D245N CIP are presented in Figure 4A. In contrast to the wild-type enzyme, D245N CIP forms a fully-bound fluoride complex at pH* 5.6 under near-identical solution conditions and enzyme concentrations. No spectral changes were noted at lower pH* values, as is evident from Figure 4A where the single set of hyperfine-shifted resonances corresponding to the 6-c HS form is unchanged. The four heme methyl resonances occur at somewhat different chemical shift values to those of fluoride-ligated wild-type CIP, namely, 66.0, 59.3, 53.7, and 46.4 ppm, with a mean value of 56.4 ppm. Notice that the resonances due to the 6-c LS form in the resting state enzymes are still present in the spectra of the fluoride-ligated enzymes.

Comparison between Cyanide-Ligated D245N and Wild-Type CIP

Analysis of the spectra acquired for resting and fluoride-ligated states of wild-type and D245N CIP highlights a number of significant differences between these enzymes. Extending the study to the 6-c LS cyanide-ligated state offers the additional advantages of improved spectral resolution of hyperfine-shifted resonances and the ability to make specific proton resonance assignments. The one-dimensional ^{1}H-NMR spectra of cyanide-ligated wild-type and D245N *Coprinus* peroxidases are given in Figure 5. A small number of assignments have been reported previously for cyanide-ligated wild-type CIP (Dugad & Goff, 1992; Veitch et al., 1994). This set is extended here for purposes of comparison with the spectrum of cyanide-ligated D245N CIP, and assignment procedures for both enzymes are discussed further below. It is of interest to note that the spectrum of cyanide-ligated wild-type CIP reported here in Figure 5B does not include any of the additional hyperfine-shifted resonances due to a minor form of the enzyme found in previous preparations of both commercial and recombinant origin (Lukat et al., 1989; Dugad & Goff, 1992; Veitch et al., 1994).

The downfield region of the spectrum of cyanide-ligated D245N CIP is remarkable for the near-coincidence of heme $C7H_3$ and $C18H_3$ resonances at 28.24 and 28.07 ppm, respectively. This represents an unusually large chemical shift perturbation of +5.51 ppm for $C18H_3$ with respect to the value for cyanide-ligated wild-type CIP as reported in Table 1. The two heme methyl resonances can be distinguished unambiguously by exploiting their differential temperature dependence, as illustrated in Figure 6. At higher temperatures it can be seen that the line width of the $C18H_3$ resonance is also slightly greater than that of the $C7H_3$ resonance. In addition to these observations, the chemical shift perturbations to the proximal histidine CβH$_2$ resonances are notable. Their chemical shift values in cyanide-ligated D245N CIP are 12.17 and 11.92 ppm, representing upfield shifts of −6.45 and −3.04 ppm, respectively, from cyanide-ligated wild-type CIP values. Other resonances in the same spectral region are less strongly affected as a result of the Asp to Asn substitution as can be judged from Table 1. Upfield regions of the spectra shown in Figure 5 also differ considerably in appearance despite containing resonances

Table 1: Comparison between ^{1}H-NMR Resonance Assignments for Cyanide-Ligated States of the D245N Mutant of *C. cinereus* Peroxidase and Wild-Type Enzyme[a]

proton[b]		chemical shift in ppm: D245N CIP	wild-type CIP	difference
$C3^1H$	2-H_α	7.20	8.54	−1.34
$C3^2H$	2-H_β	−1.77	−2.74	+0.97
$C3^2H$	2-H_β	−1.77	−2.88	+1.11
$C7H_3$	3-CH_3	28.24	28.02	+0.22
$C8^1H$	4-H_α	13.27	11.75	+1.52
$C8^2H$	4-H_β	−1.03	0.20	−1.23
$C8^2H$	4-H_β	−2.08	−0.67	−1.41
$C13^{2a}H$	6-H_β	−2.64	−3.17	+0.53
$C13^{2b}H$	6-$H_{\beta'}$	−2.79	−3.66	+0.87
$C17^{1a}H$	7-H_α	11.68	12.52	−0.84
$C17^{1b}H$	7-$H_{\alpha'}$	9.08	7.94	+1.14
$C17^{2a}H$	7-H_β	1.40	0.50	+0.90
$C17^{2b}H$	7-$H_{\beta'}$	−1.35	−2.53	+1.18
$C18H_3$	8-CH_3	28.07	22.56	+5.51
C20H	δ-meso	7.58	8.65	−1.07
Arg51 Cδ1H		−4.49	−4.28	−0.21
Arg51 Cβ1H		−3.58	−3.77	+0.19
His55 Cϵ1H		13.14	13.36	−0.22
His183 NH		11.59	11.76	−0.17
His183 CαH		10.37	10.65	−0.28
His183 Cβ1H		12.17	18.62	−6.45
His183 Cβ2H		11.92	14.96	−3.04
His183 Cδ2H		24.6	26.8	−2.2
His183 Cϵ1H		−23.1	−21.8	−1.3

[a] Chemical shift data obtained at 30 °C with solution conditions of 10 mM potassium phosphate, 15 mM potassium cyanide, D_2O, at pH 7.0. [b] The older Fischer nomenclature for heme protons is also given for reference.

from essentially the same set of protons. The complex group of overlapping resonances between −4.50 and −2.50 ppm is characteristic of cyanide-ligated wild-type CIP, whereas corresponding resonances for cyanide-ligated D245N CIP are slightly more dispersed. Nevertheless, the interpretation of the spectrum of the latter is also complicated by overlap of resonances. This can be resolved to some extent by careful analysis of temperature dependence as shown in Figure 6, together with recourse to two-dimensional experimental data. A final point of comparison is with the proximal His183 Cϵ1H resonance which appears in the −20.0 to −25.0 ppm spectral region. This shows an upfield shift of −1.3 ppm to −23.1 ppm as a result of the substitution.

Proton Resonance Assignments in Cyanide-Ligated D245N and Wild-Type CIP: Heme Group and Proximal His183 Side Chain

The assignment of a number of proton resonances in the spectrum of cyanide-ligated D245N CIP was achieved in the first instance by comparison of patterns of NOE connectivities in two-dimensional NOESY experiments with those obtained for cyanide-ligated wild-type CIP. This enables the two near-coincident heme methyl resonances at 28.07 and 28.24 ppm to be distinguished and assigned as shown in Figure 7. The analysis recognizes the proximity of the $C18H_3$ group to the C17 propionate side chain and the $C7H_3$ group to the C8 vinyl side chain. This leads to the assignments of the $C17^1H_2$ and $C17^2H_2$ protons and the $C8^1H$ and $C8^2H_2$ protons, respectively. Scalar-coupled cross-peaks were also detected for this vinyl side chain in a corresponding

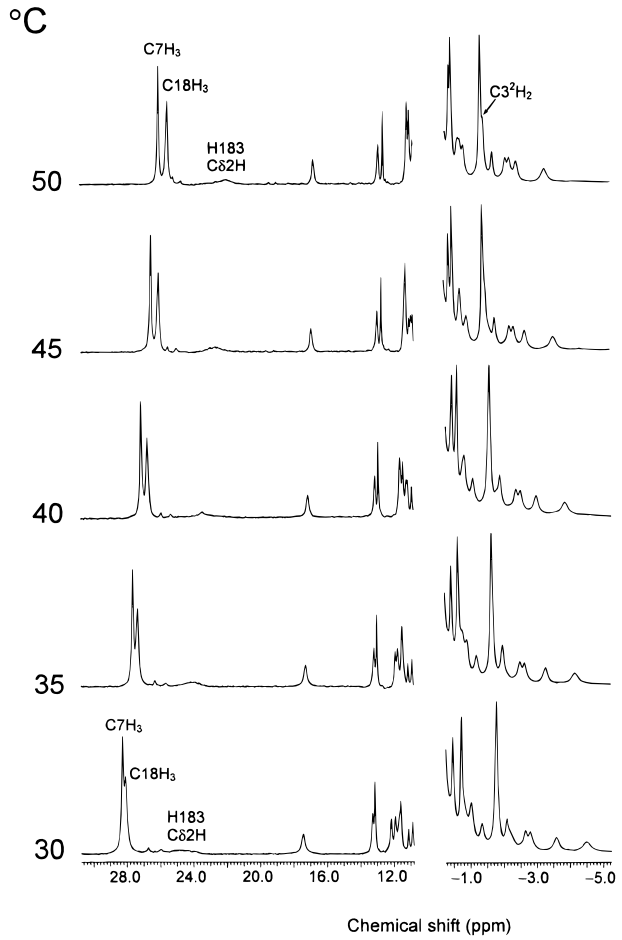

FIGURE 6: Temperature dependence of the hyperfine-shifted regions from the one-dimensional ^{1}H-NMR spectrum of cyanide-ligated D245N CIP. Spectra were recorded at 500 MHz with solution conditions of 10 mM potassium phosphate, 15 mM potassium cyanide, D_2O, at pH 7.0.

TOCSY experiment (not shown). The $C7H_3$ resonance in the NOESY spectrum of cyanide-ligated wild-type CIP also exhibits NOE connectivities to a second vinyl side-chain, although these are of weaker intensity than to the C8 vinyl side-chain. Re-examination of this spectrum and corresponding TOCSY data indicate that one of the original assignments for the $C3^2H_2$ protons should be corrected from −0.88 to −2.88 ppm (Veitch et al., 1994). The $C3^2H_2$ proton resonances are almost coincident at −2.74 and −2.88 ppm and occur within the complex envelope of hyperfine-shifted resonances illustrated in Figure 5. A similar problem is encountered with cyanide-ligated D245N CIP where only one TOCSY cross-peak representing the C3 vinyl side chain can be detected, indicating that the $C3^2H_2$ protons are coincident at −1.77 ppm. These resonances also overlap with an additional methyl group resonance which has the same chemical shift value as $C3^2H_2$ at 30 °C. This methyl resonance can be clearly distinguished from the $C3^2H_2$ resonances at higher temperatures, as seen in Figure 6.

The resonances of protons associated with His183 in the spectra of cyanide-ligated D245N CIP were also located by comparing patterns of NOE connectivities with the corresponding spectra of cyanide-ligated wild-type CIP. Assignments of His183 NH, CαH, and CβH_2 protons were confirmed by TOCSY data. Two much broader His183 ring resonances, at −23.1 and 24.6 ppm, are assigned to Cϵ1H and Cδ2H, respectively.

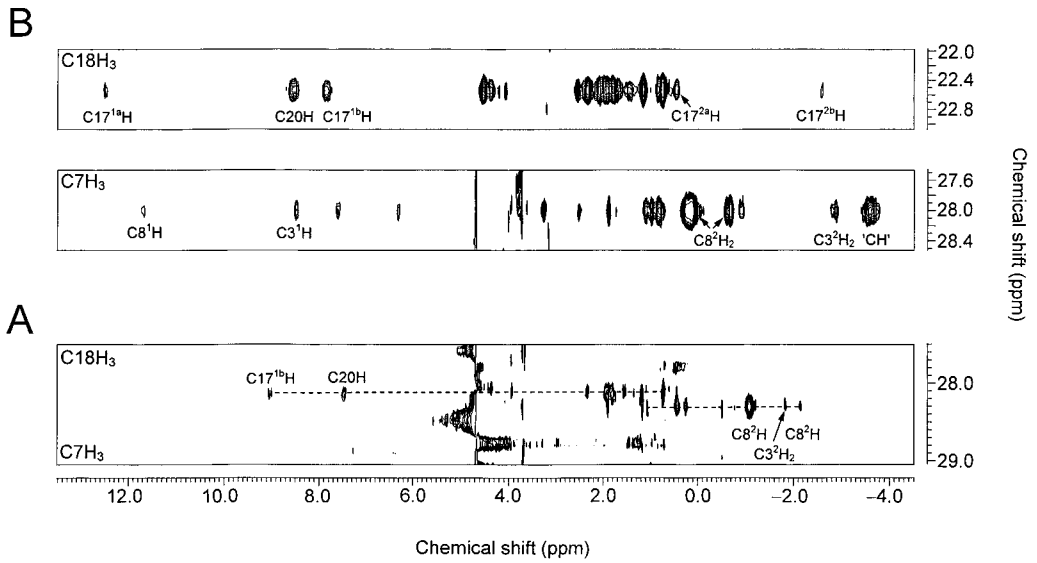

FIGURE 7: Characteristic NOE connectivities for heme methyl resonances $C7H_3$ and $C18H_3$, as extracted from the two-dimensional NOESY spectra of (A) cyanide-ligated D245N CIP and (B) cyanide-ligated wild-type CIP. Spectra were recorded at 500 MHz with solution conditions of 10 mM potassium phosphate, 15 mM potassium cyanide, D_2O, at pH 7.0 and 30 °C. Mixing times of 30 ms were used in both experiments.

Proton Resonance Assignments in Cyanide-Ligated D245N and Wild-Type CIP: Distal Heme Pocket His55 and Arg51 Side Chains

Assignment of the His55 Cϵ1H resonance in cyanide-ligated D245N CIP follows readily from comparison of the spectra presented in Figures 5 and 6. At 13.14 ppm, this resonance is only slightly upfield-shifted from that for cyanide-ligated wild-type CIP at 13.36 ppm. The side-chain resonances of distal Arg have proved relatively difficult to locate in the spectra of cyanide-ligated class II fungal peroxidases studied to date. This is in contrast to peroxidases of classes I and III, such as cytochrome *c* peroxidase and HRP C, respectively, where the distal Arg Cδ1H and Cβ1H resonances are usually well resolved in the upfield-shifted region of the spectrum of the cyanide-ligated state (Satterlee & Erman, 1991; Thanabal et al., 1987). A recent approach adopted with cyanide-ligated lignin peroxidase from *Phanerochaete chrysosporium* involves the determination of the magnetic susceptibility tensor and calculation of contact and pseudocontact terms to assist with proton resonance assignments (Banci et al., 1995), a procedure used previously for a number of *b*- and *c*-type cytochromes (Veitch et al., 1990; Gao et al., 1991). The assignments achieved by this method for the distal Arg side chain in cyanide-ligated lignin peroxidase confirm the suggestion that the upfield shifts are smaller overall than those for the analogous distal Arg side-chain protons of class I and class III peroxidases (De Ropp et al., 1991). An additional problem is experienced with the assignment of the Arg51 side-chain resonances of cyanide-ligated wild-type CIP, which derives principally from the difficulties inherent in interpreting the particularly complex set of upfield-shifted resonances. The assignment process was, however, aided by comparisons with the spectrum of cyanide-ligated D245N CIP, which has a different pattern of hyperfine-shifted resonances in this upfield region (Figure 5). In both cases, the two most upfield-shifted resonances can be assigned to protons of the Arg51 side chain on the basis of three supporting lines of evidence.

(1) The resonances at −4.28 and −3.77 ppm in the spectrum of cyanide-ligated wild-type CIP exhibit essentially the same pattern of NOE connectivities in NOESY spectra as the resonances at −4.49 and −3.58 ppm in the corresponding spectra of cyanide-ligated D245N CIP.

(2) These resonances, and the NOE connectivities associated with them in NOESY spectra, are absent from the corresponding spectra of the cyanide-ligated Arg51→Leu CIP mutant (N. C. Veitch, Y. Gao, & K. G. Welinder, unpublished experiments).

(3) Analysis of the NOESY spectrum of cyanide-ligated wild-type CIP as shown in Figure 8 allows the assignment of a pair of scalar-coupled single proton resonances at −3.17 and −3.66 ppm to the propionate side chain, $C13^2H_2$. This follows from the observation that the $C13^{2a}H$ proton resonance at −3.17 ppm shows a NOE connectivity to $C17^{2b}H$ at −2.54 ppm and, furthermore, that the $C13^2H_2$ and $C17^{2b}H$ resonances all show NOE connectivities to a resonance at 5.67 ppm assigned to the heme meso-proton C15H. In addition, a weak NOE connectivity can be detected between this latter resonance and $C17^{1a}H$ at 12.52 ppm, confirming the assignment of C15H. The $C13^{2a}H$ resonance exhibits a weak NOE connectivity to the Arg51 side-chain proton at −4.28 ppm. According to crystal structure data for CIP, Arg51 CδH_2 represents the closest heme pocket side-chain contact to $C13^2H_2$ (Kunishima et al., 1994). The resonance at −4.28 ppm is therefore assigned to Arg51 Cδ1H and that at −3.77 ppm to Arg51 Cβ1H, on the basis of the strong NOE connectivity between them. This represents a similar distribution of distal Arg side-chain resonances with regard to chemical shift parameters to those found in the spectra of cyanide-ligated cytochrome *c* peroxidase, horseradish peroxidase isoenzyme C, and cationic peanut peroxidase (Satterlee & Erman, 1991; Thanabal et al., 1987; Barber et al., 1995). The corresponding Arg51 resonances were located in the spectrum of cyanide-ligated D245N CIP by a similar procedure.

An additional point of interest in the spectra of both cyanide-ligated enzymes is the presence of a single proton resonance at 19.12 ppm (wild-type CIP) and 17.44 ppm (D245N CIP). The resonance is highly temperature dependent, as is clear from Figure 6, indicating that the proton in question is either from the heme group itself or from an

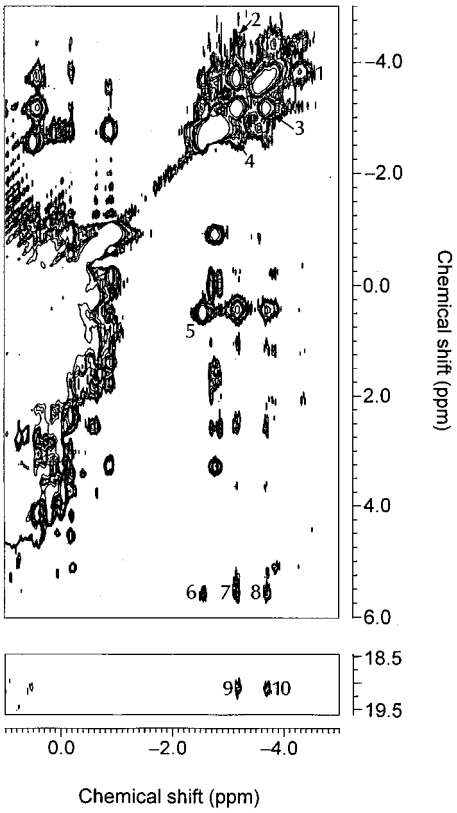

FIGURE 8: Upfield-shifted region from the two-dimensional NOESY spectrum of cyanide-ligated wild-type CIP. The spectrum was recorded at 500 MHz with solution conditions of 10 mM potassium phosphate, 15 mM potassium cyanide, D_2O, at pH 7.0 and 30 °C. A mixing time of 30 ms was used. Cross-peak assignments are as follows: (1) Arg51 Cδ1H:Arg51 Cβ1H, (2) Arg51 Cδ1H:C13^{2a}H, (3) C13^{2b}H:C13^{2a}H, (4) C13^{2a}H:C17^{2b}H, (5) C17^{2b}H:C17^{2a}H, (6) C17^{2b}H:C15H, (7) C13^{2a}H:C15H, (8) C13^{2b}H:C15H, (9) C13^{2a}H:C13^{1a}H, and (10) C13^{2b}H:C13^{1a}H. The assignment for C13^{1a}H is tentative.

amino acid side chain subject to a considerable pseudocontact (dipolar) shift. Cross-peaks in NOESY spectra to the C13^{2}H$_2$ protons have been detected in both cases (Figure 8). The corresponding resonance is also present in the spectrum of the cyanide-ligated Arg51→Leu CIP mutant, indicating that it does not originate from a slowly exchanging Arg51 side-chain NH proton. One solution is that this resonance represents one of the heme propionate C13^{1}H$_2$ protons. It has not yet been possible to detect scalar correlations from this resonance in order to substantiate this proposal, although it cannot be discounted that this is due solely to line width considerations. In general, it is one of the heme propionate C17^{1}H$_2$ protons that is subject to a substantial downfield shift, thereby placing its proton resonance in the spectral region between 15.00 and 26.00 ppm for a number of cyanide-ligated class I and III peroxidases (Satterlee & Erman, 1991; Thanabal et al., 1987; Barber et al., 1995). The downfield shift for C17^{1}H$_2$ is less in the case of cyanide-ligated class II fungal peroxidases, typical chemical shift values being between 10.00 and 13.00 ppm, which may reflect an altered distribution of electron density across the heme group (De Ropp et al., 1991; Banci et al., 1992). It is conceivable therefore that one of the C13^{1}H$_2$ protons could be considerably downfield-shifted in the case of cyanide-ligated *Coprinus* peroxidases. However, further comparative data are required to substantiate this tentative assignment.

DISCUSSION

Identification of the High-Spin Species of Resting State D245N CIP

The D245N mutant of *C. cinereus* peroxidase is remarkable for the diversity of spin- and coordination-state species displayed in solution, hitherto without comparison in other heme peroxidase systems. Characterization of the pH dependence of the resting state enzyme using NMR techniques has uncovered an apparently unique interdependence of species quite distinct from the behavior of the wild-type enzyme. A summary of relevant NMR data is presented in Table 2 as an overall guide to the species observed, with particular reference to the chemical shift values of the four heme methyl groups. The major difference between resting state D245N and wild-type CIP over the pH range studied is the presence in the former of three distinct high-spin species. According to the NMR data, these high-spin species are interrelated by the following pH-dependent equilibria:

$$\text{HS1} \rightleftharpoons \text{HS2} \rightleftharpoons \text{HS3}$$

The HS2 species, which predominates at neutral pH, is in slow exchange with respect to both HS1 and HS3 as the solution conditions move toward acid and alkaline, respectively.

It is useful to compare the mean heme methyl chemical shift values for resting state CIP and fluoride-ligated CIP, representing the transition from 5-c HS to 6-c HS (with the proximal His Nϵ2 atom retained as the fifth ligand in both cases), with those for resting state CIP and the HS2 species of D245N CIP. The distribution of the chemical shifts of the contact-shifted heme methyl groups is directly linked to the electronic state of the heme Fe atom, which in turn is affected by a number of factors including unpaired electron spin density, the geometry of the heme and the nature of the axial ligands. The 5-c HS species which characterizes resting state CIP comprises an axially symmetric electronic state subject to a significant rhombic distortion, whereas the electronic state of the fluoride-ligated form is wholly axially symmetric, as in the analogous case of resting state and fluoride-ligated HRP C, for which *g*-values and magnetic susceptibility data are available (Theorell, 1942). It can be predicted therefore that this transition from a 5-c HS to a 6-c HS state, where the fifth ligand is unaltered, is accompanied by an overall decrease in the magnitude of the heme methyl chemical shifts. This is also supported by data for resting state and fluoride-ligated HRP C included in Table 2 (Veitch et al., 1996). On this basis, the HS2 species of resting state D245N is predicted from NMR data to be 6-c HS, with a water molecule providing the sixth ligand. The assignment of this 6-c HS species to HS2 of D245N CIP is strongly supported by complementary resonance Raman and electronic absorption data (Smulevich et al., 1996).

Assignment of ligand type to the HS1 and HS3 species from NMR data alone is less straightforward in view of the fact that spectra of related species have not been reported in the literature. According to resonance Raman and electronic absoption data, resting state D245N CIP is predominantly 6-c HS over the pH range from 3.8 to 8.8 (Smulevich et al., 1996). However, the NMR results confirm that two species are present in this range, indicating that HS1 is an additional 6-c HS species, designated here as 6-c HS*, which is only

Table 2: Chemical Shift Data for Heme Methyl Resonances in High-Spin States of Heme Peroxidases[a]

peroxidase	species	ligands	heme methyl chemical shift values at 30 °C	mean heme methyl chemical shift
resting state CIP	5-c HS	His183 Nϵ2	85.3, 72.0, 64.5, 64.5	71.6
fluoride-ligated CIP	6-c HS	His183 Nϵ2, (H)F	72.1, 64.0, 55.9, 53.5	61.4
resting state D245N CIP, pH* 5.1	6-c HS*	His183 Nϵ2H$^+$, H_2O	77.6, 71.7, 64.5, 55.5	67.3
resting state D245N CIP, pH* 7.8	6-c HS	His183 Nϵ2, H_2O	76.5, 64.6, 58.3, 53.8	63.3
resting state D245N CIP, pH* 9.7	5-c HS**	OH^-	67.9, 54.6, 52.5, 48.1	55.8
fluoride-ligated D245N CIP	6-c HS	His183 Nϵ2, (H)F	66.0, 59.3, 53.7, 46.4	56.4
resting state HRP C	5-c HS	His170 Nϵ2	79.6, 73.2, 69.2, 53.1	68.8
fluoride-ligated HRP C[b]	6-c HS	His170 Nϵ2, (H)F	63.6, 59.5, 55.2, 52.6	57.7

[a] Data were obtained with solution conditions as given in the Experimental Procedures. [b] Spectrum published in Veitch et al. (1996).

observed by NMR. The mean heme methyl chemical shift of HS1 is intermediate between that of 5-c HS resting state CIP and the 6-c HS HS2 species. Furthermore, HS1 exhibits an altered pattern of hyperfine-shifted resonances in the 30−50 ppm region of the NMR spectrum where the resonances of His183 CβH$_2$ occur, compared with that of HS2 (Figure 2A). It is likely, therefore, that it is the proximal His183 ligand which is altered in this new species, while the distal ligand remains unchanged. Resonance Raman spectra also indicate that while the D245N CIP mutant is predominantly six-coordinate high-spin at pH 3.8, a small proportion of the enzyme is in the 5-c HS* form. This latter species, which predominates at pH 3.8 in wild-type CIP, has been characterized as containing a proximal His to heme iron bond which is either significantly weakened or absent (Smulevich et al., 1996). We propose that the 6-c HS* species, HS1, is an intermediate state in which the proximal His residue is protonated. This new species precedes the formation of the 5-c HS* species in D245N CIP at pH 3.8. The two species, 6-c HS and 6-c HS*, cannot be distinguished in resonance Raman spectra as their core marker bands will overlap. However optical spectra for resting state D245N CIP at pH 7.0 and 5.4 show a 3 nm red-shift of the Soret band from 407 to 410 nm, respectively (Smulevich et al., 1996), which may be related to the formation of the 6-c HS* species.

The HS3 species can be identified as five-coordinate high-spin as its appearance at higher pH values in the NMR spectra of Figure 3A exactly matches comparable data obtained using resonance Raman spectroscopy (Smulevich et al., 1996). These authors also note that the alkaline transition for the mutant is much lower than that for wild-type CIP. The species in question has been designated as 5-c HS**, where the proximal His183 Nϵ2 coordinate bond to Fe is absent and the fifth ligand is hydroxyl, hydrogen bonded to distal Arg51 NϵH and His55 Nϵ2 (Smulevich et al., 1996). The proposed assignments for the HS1 and HS3 species find additional support from the dynamic behavior of these species noted from the corresponding NMR spectra, as slow exchange regimes are typically associated with conformational change or bond-breaking or formation events.

The NMR data for resting state wild-type CIP indicate that only the 5-c HS species is observable over the pH range from 5 to 10. The 5-c HS* species, detected by resonance Raman spectroscopy for resting state wild-type CIP at a pH value of 3.8, appears to be inaccessible with the solution conditions used for the NMR experiments (Smulevich et al., 1996). Subtle spectral changes noted between 30.0 and 50.0 ppm in the pH titration of resting state wild-type CIP may, however, reflect the onset of the gradual transition to the 5-c HS* state. A 6-c LS form with a hydroxyl ligand coordinated to the heme iron atom has also been detected at pH 12.0 by optical and resonance Raman spectroscopy (Smulevich et al., 1996). The loss of intensity of the hyperfine-shifted resonances in the NMR spectrum of wild-type CIP at pH* values above 10.5 is likely to be related to the transition between the 5-c HS species and this low-spin species. Aspects of the unfolding and refolding of wild-type CIP at high pH values and under a variety of experimental conditions have been described fully in a recent report (Tams & Welinder, 1996).

NMR Studies of the Cytochrome c Peroxidase Mutants D235N and D235A: Comparison with Data for D245N CIP

Two NMR studies of proximal Asp mutants of the class I cytochrome *c* peroxidase have been reported (note that Asp235 of CCP is the equivalent residue to Asp245 of CIP) (Satterlee et al., 1990; Ferrer et al., 1994). Resting state D235N CCP, the mutant directly comparable with D245N CIP, does not exhibit the unique range of high-spin species described for the latter, although only the neutral to acid pH range has been studied. At pH* 6.8 and 22 °C, a mixture of 6-c LS and 6-c HS species is present, with the 6-c LS species the major component. This was described as a hydroxide-bound species on account of the high degree of similarity between its NMR spectrum and that of hydroxide-bound metmyoglobin. A spin-state equilibrium was postulated in which the population of the 6-c HS species increases at higher temperatures. The proportion of 6-c HS is also increased by lowering the pH* to 5.4. This species was classified as a water-bound form (Satterlee et al., 1990). Although a 6-c LS form is present in the resting state spectra of D245N CIP, there is no evidence to suggest that it is in equilibrium with any of the high-spin species. The pH dependent properties of a second CCP mutant, D235A, have also been discussed in a more recent investigation (Ferrer et al., 1994). Three species were described in the pH range from 4.5 to 8.2 based on optical and NMR spectroscopy, while at higher pH values, denaturation was observed. At low pH values, 6-c HS and 6-c LS species are present with the latter species becoming dominant toward neutral pH. The additional sixth ligands were proposed to be water and hydroxide respectively, so that in this respect the distribution of species is similar to that of the D235N CCP mutant. In addition, these two species were described as being in a state of slow exchange with respect to the NMR time scale. This does not appear to be in agreement with the spectra presented however, which show chemical shift changes for the LS species as the pH is increased from 4.5 to 6.7. A second LS species, present between pH 6.7 and 8.2, was proposed

to originate from bishistidyl coordination. This second LS species in D235A CCP has no known counterpart in either D235N CCP or D245N CIP.

Fluoride-Ligated Forms of D245N CIP and Wild-Type CIP

The spectra presented for the fluoride-ligated forms of the enzymes demonstrate convincingly the greater affinity of D245N CIP for this ligand compared with wild-type CIP. Under near-identical solution conditions, wild-type CIP only forms a fully-bound fluoride complex at a lower pH value (where the effective concentration of the undissociated ligand is higher) than that required for formation of the analogous complex with D245N CIP. This observation is entirely in agreement with results presented for fluoride binding to D235N CCP (Satterlee et al., 1990), where it was shown that bound fluoride can be removed readily by dialysis of fluoride-ligated wild-type CCP but not of fluoride-ligated D235N CCP. It can be confirmed from these parallel studies that the presence of the Asp side chain reduces significantly the possibility of a sixth ligand binding to the heme iron atom, both in CCP and CIP. This finds additional support from the crystal structure of the D235N CCP(MI) mutant which has been solved to 2.2 Å resolution (Wang et al., 1990). One important result in terms of the discussion here, is that distal Wat595 is displaced toward the plane of the porphyrin by 0.6 Å, while the heme iron atom is displaced 0.2 Å toward the distal side of the heme plane, compared to the wild-type structure. This places the water molecule within the primary coordination sphere of the iron atom and favors a 6-c HS state. This is in contrast to the wild-type enzyme where Wat595, at a distance of 2.7 Å from heme iron, is effectively excluded from coordinate bond formation to give a 6-c state. In wild-type CIP at pH 4.5, the equivalent distal pocket water molecule, Wat686, is 3.9 Å from the heme iron atom (Figure 1), which once again precludes the formation of a 6-c water-bound state (Kunishima et al., 1996).

Six-Coordinate Low-Spin Species in Resting and Fluoride-Ligated States of D245N CIP

The presence of a 6-c LS species in all spectra recorded for resting and fluoride-ligated states of D245N CIP indicates that it is not a true enzyme form but rather one which is artifactual. Smaller amounts of the same species have also been seen in analogous spectra of wild-type CIP. This additional low-spin species originates from an adventitious donor ligand binding at the sixth coordination site, with a ligand field strength greater than that of F- or O-donor ligands. *C. cinereus* peroxidase isolated from culture broths of the fungus was in fact found to contain a small proportion of the cyanide-ligated 6-c LS form, which was only removed on treatment with *para*-chloromercuribenzoate (Morita et al., 1988). The chemical shift values of the two heme methyl resonances of the 6-c LS form in D245N CIP also appear identical to those for $C7H_3$ and $C18H_3$ of the cyanide-ligated form of the enzyme. In addition, it has been demonstrated recently that samples of *A. ramosus* peroxidase in ammonium sulfate solutions contain a low-spin species which becomes more abundant as the pH is increased from 6.0 to 9.0 (Kunishima et al., 1996). As expected, NH_3 was identified as the effective sixth ligand. Note that the D245N CIP mutant contains a greater proportion of the 6-c LS species than wild-type CIP, which underlines its propensity for forming six-coordinate states.

Analysis of NMR Spectra of Cyanide-Ligated D245N CIP

The proton resonance assignments obtained for the mutant enzyme provide critical data on the differences in heme pocket structure between this and the wild-type enzyme. As expected, chemical shift values for His183 protons are considerably altered, reflecting a combination of local structural change due to the Asp to Asn substitution and electronic factors. The large upfield shifts experienced by the His183 CβH$_2$ protons in comparison with those of the wild-type indicate a transition from imidazolate to neutral imidazole character of the His ring (La Mar et al., 1982). This supports the conclusion reached from resonance Raman data that the hydrogen bond between His183 Nδ1H and Asp245 is either absent or weakened significantly when the Asp residue is replaced by Asn, as in D245N CIP (Smulevich et al., 1996). Similar results have been reported following NMR studies of the cyanide-ligated forms of cytochrome *c* peroxidase mutants D235A and D235N (Satterlee et al., 1990; Ferrer et al., 1994). Chemical shift perturbations for His175 CβH$_2$ of D235A CCP (-7.3 and -3.5 ppm) match those of His183 of D245N CIP (-6.5 and -3.0 ppm) more closely than those for His175 CβH$_2$ of D235N CCP (-0.1 and -1.0 ppm). However, the His175 Cϵ1H resonance of both CCP mutants was strongly downfield shifted compared with the more modest upfield shift experienced by the analogous His183 Cϵ1H resonance of D245N CIP. This indicates that the relative shifts of the proximal His Cϵ1H proton are not as reliable an indicator of the degree of imidazolate character of this side chain, as has been maintained in previous studies (Banci et al., 1991). These differing patterns of chemical shift perturbations reflect subtle variations in heme pocket structure at this proximal site between class I (CCP) and II (CIP) peroxidases.

One important consequence of the loss or significant weakening of the His183 to Asp245 hydrogen bond is the effect on the electronic structure of the heme group, mediated by the proximal His residue. The spin density across the heme group is altered considerably in cyanide-ligated D245N CIP, as reflected in the significant perturbations to resonances of heme protons such as $C18H_3$, which are more remote from the site of mutation. This phenomenon is commonly observed in the spectra of cyanide-ligated derivatives of heme peroxidase mutants (Satterlee et al., 1990; Veitch et al., 1992).

CONCLUSIONS

(1) Resting state D245N CIP comprises three pH-dependent high-spin species in the pH range 5−10, two of which are unique (6-c HS* and 5-c HS**) and in slow exchange with a third 6-c HS species. Proton NMR spectra of these species are presented for the first time and should aid future identification of similar species in peroxidase mutants. Wild-type CIP appears as a 5-c HS species over the same pH range.

(2) The D245N CIP mutant binds fluoride more readily than wild-type CIP, a further indication of the ease with which it binds a sixth ligand. This supports the general view that this Asp residue, which is invariant among peroxidases

of the plant peroxidase superfamily, assists in the maintenance of a free sixth coordination site at the heme iron atom.

(3) The critical hydrogen bond between His183 and Asp245 is absent or weakened significantly in the D245N CIP mutant, as judged from analysis of the NMR spectra of the cyanide-ligated form of the enzyme and related data. This has a profound effect on the chemical shifts of resonances of heme and heme-linked protons compared with those recorded for cyanide-ligated wild-type CIP.

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