Expression, Purification, Characterization, and NMR Studies of Highly Deuterated Recombinant Cytochrome *c* Peroxidase†

Marina I. Savenkova,‡ James D. Satterlee,*,‡ James E. Erman,§ William F. Siems,|| and Gregory L. Helms⊥

Department of Chemistry, Laboratory for Biotechnology and BioAnalysis, and NMR Spectroscopy Center, Washington State University, Pullman, Washington 99164-4630, and Department of Chemistry and Biochemistry, Northern Illinois University, De Kalb, Illinois 60115

Received May 30, 2001; Revised Manuscript Received August 1, 2001

ABSTRACT: Two forms of extensively deuterated *S. cerevisiae* cytochrome *c* peroxidase (CcP; EC 1.11.1.5) have been overexpressed in *E. coli* by growth in highly deuterated medium. One of these ferriheme enzyme forms (recDCcP) was produced using >97% deuterated growth medium and was determined to be approximately 84% deuterated. The second form [recD(*H*is)CcP] was grown in the same highly deuterated medium that had been supplemented with excess histidine (at natural hydrogen isotope abundance) and was also approximately 84% deuterated. This resulted in direct histidine incorporation without isotope scrambling. Both of these enzymes along with the corresponding recombinant native CcP (recNATCcP), which was expressed in a standard medium with normal hydrogen isotope abundance, consisted of 294 amino acid polypeptide chains having the identical sequence to the yeast-isolated enzyme, without any N-terminal modifications. Comparative characterizations of all three enzymes have been carried out for the resting-state, high-spin forms and in the cyanide-ligated, low-spin forms. The primary physical methods employed were electrophoresis, UV−visible spectroscopy, hydrogen peroxide reaction kinetics, mass spectrometry, and ^{1}H NMR spectroscopy. The results indicate that high-level deuteration does not significantly alter CcP's reactivity or spectroscopy. As an example of potential NMR uses, recDCcPCN and recD(*H*is)CcPCN have been used to achieve complete, unambiguous, stereospecific ^{1}H resonance assignments for the heme hyperfine-shifted protons, which also allows the heme side chain conformations to be assessed. Assigning these important active-site protons has been an elusive goal since the first NMR spectra on this enzyme were reported 18 years ago, due to a combination of the enzyme's comparatively large size, paramagnetism, and limited thermal stability.

Cytochrome *c* peroxidase (EC 1.11.1.5; CcP)[1] from *Saccharomyces cerevisiae* is a ∼34 kDa naturally paramagnetic heme enzyme (*1*−*5*) that has been well characterized by a variety of techniques including X-ray crystallography (*6*−*15*), kinetics (*16*−*31*), protein engineering in combination with physical methods (*9*, *11*, *13*−*15*, *18*, *22*, *27*, *28*, *32*−*39*), NMR (*40*−*48*), and modeling (*49*). It occurs naturally in the yeast mitochondrial intermembrane space where it probably functions as a cytotoxic protective agent by catalyzing cytochrome *c*-dependent hydrogen peroxide decomposition (*4*, *5*), and it may also be involved in oxidative stress signaling (*50*). The CcP-catalyzed decomposition of hydrogen peroxide involves oxidation of ferrocytochrome *c* in a process that has been extensively studied in vitro and has been found to involve intermolecular electron transfer within discrete complexes of CcP and cytochrome *c* (*3*, *4*, *28*−*31*, *49*, *51*−*54*). Perhaps the most intriguing aspects of these complexes are that CcP binds two cytochrome *c* molecules at low ionic strength (*29*−*31*), and the fact that

† J.D.S. acknowledges support for this research from the National Institutes of Health (GM 47645). J.E.E. acknowledges support from the National Science Foundation (MCB-9513047). The WSU NMR Center equipment is supported by NIH Grants RR0631401 and RR12948, NSF Grants CHE-9115282 and DBI-9604689, and the Murdoch Charitable Trust.

* Correspondence should be addressed to this author at the Department of Chemistry, Washington State University, Pullman, WA 99164-4630. Phone: 509-335-8620; Fax: 509-335-8867; Email: hemeteam@wsu.edu.

‡ Department of Chemistry, Washington State University.

§ Department of Chemistry and Biochemistry, Northern Illinois University.

|| Laboratory for Biotechnology and BioAnalysis, Washington State University.

⊥ NMR Spectroscopy Center, Washington State University.

[1] Abbreviations: D, deuterium, ^{2}H; H, protium, ^{1}H; CcP, generic abbreviation for resting-state cytochrome *c* peroxidase; natWTCcP, resting-state wild-type cytochrome *c* peroxidase isolated from yeast at natural proton isotope abundance; recNATCcP, recombinant form of resting-state cytochrome *c* peroxidase at natural proton isotope abundance with the exact 294 amino acid sequence of the yeast-isolated wild-type enzyme; recDCcP, recombinant form of resting-state cytochrome *c* peroxidase with the exact 294 amino acid sequence of the yeast-isolated wild-type enzyme incorporating high-level replacement of H by D in all amino acids, essentially perdeuterated CcP; recD(*H*is)CcP, recombinant form of resting-state cytochrome *c* peroxidase with the exact 294 amino acid sequence of the yeast-isolated wild-type enzyme specifically incorporating natural proton (hydrogen) isotope abundance histidine into otherwise high-level replacement of H by D; CcPCN, generic abbreviation for low-spin, cyanide-ligated cytochrome *c* peroxidase, a general notation throughout, where the suffix "CN" attached to any CcP abbreviation indicates formation of the low-spin cyanide-ligated form; HRPCN, cyanide-ligated horseradish peroxidase; Kan, kanamycin; Amp, ampicillin; δ-ALA, δ-aminolevulinic acid; 1D, one-dimensional; 2D, two-dimensional; LBB-I, -II, Washington State University Laboratory for Biotechnology and Bioanalysis, units I and II, respectively; WET, water suppression enhanced through T_1 effects.

10.1021/bi0111000 CCC: $20.00
Published on Web 09/13/2001

the electron transfers between heme groups on the oxidized and reduced proteins are over relatively long distances (*49*, *51*−*53*).

Two types of recombinant CcP genes are currently being used for expression in *E. coli* by various laboratories. These are the nuclear gene sequence (*4*, *5*, *55*) and the sequence of the yeast-isolated enzyme (*4*, *5*, *56*). These two primary sequences differ at two positions, 53 and 152 (*57* and references cited therein). Within these two primary sequence categories, the recombinant CcP genes that are currently in use differ in the number and type of non-native amino acids found at the enzyme's amino terminus (*57* and references cited therein). Here we have used the recombinant gene that produces the exact 294 amino acid protein sequence (*4*, *5*, *56*) of the yeast-isolated enzyme, natWTCcP[1] (with T53 and D152), without any N-terminal sequence changes from that of the wild-type enzyme. We have achieved high-level expression of two extensively deuterated forms of cytochrome *c* peroxidase. One of these (recDCcP) was produced in *E. coli* using 97−99.9% D medium. The other deuterated cytochrome *c* peroxidase form is one in which excess histidine, at natural proton isotope abundance, was added to the same highly deuterated growth medium in order to determine whether His would be incorporated into the highly deuterated recombinant enzyme without degradation and concomitant isotope scrambling.

This work had several other objectives. One of our goals was to determine if any perdeuterated forms of yeast cytochrome *c* peroxidase could be produced in *E. coli*. We also sought to determine whether, if successfully expressed and isolated, these enzymes would be stable for the periods of time required for characterization studies; and whether their chemical properties varied from those of the corresponding wild-type (natWTCcP) and recombinant native (recNATCcP) enzymes that contained natural proton isotope abundance.

A prime motivation for making these two deuterated enzymes was to assess their potential uses for NMR studies. The first test, reported here, was to determine whether a complete set of unambiguous heme proton resonance assignments could be obtained using the low-spin, CN-ligated forms of the two deuterated enzymes [recDCcPCN and recD(*H*is)CcPCN]. The holoenzymes were accordingly assembled from each of the two individual highly deuterated apoenzymes by combination with exogenous heme that possessed natural proton isotope abundance.

Making complete heme proton assignments for CcPCN has been an elusive goal since the first attempt in 1983 (*40*). As detailed under Results and Discussion, the heme-centered paramagnetism, the enzyme's size (molecular mass ∼34 kDa), and its limited thermal stability have been the primary complicating factors in this effort. However, achieving this goal is profoundly important because the heme is the enzyme's active site. To-date only about half of the heme protons have been correctly assigned, and this fact has so far compromised all NMR attempts to unambiguously study the active site solution chemistry of this enzyme. For example, several currently incorrect heme proton assignments have lead to incorrect assignments of heme pocket amino acid protons (*42*−*44*) and questionable conclusions about active site chemistry. It seems clear that comprehensive NMR studies of CcPCN solution-state chemistry cannot successfully proceed until all hyperfine-shifted proton resonances, owing to the heme and heme-pocket amino acids, are unambiguously assigned. The first step in this effort is to achieve unambiguous and complete heme proton assignments, which will subsequently lead to hyperfine resonance assignments of active site amino acids. These will provide the necessary basis for expanding solution studies of active site dynamics. We demonstrate here a straightforward chemical technique that overcomes existing hurdles and allows complete, unambiguous, stereospecific heme proton hyperfine resonance assignments by using highly deuterated enzyme. Such an approach is potentially applicable to other cofactor-containing holoproteins that can be assembled from the corresponding recombinant apoprotein. These results have also lead to a qualitative assessment of the solution-state conformation of the heme side chains for CcPCN.

EXPERIMENTAL PROCEDURES

Expression. The general method for expression and purification was as before (*57*). All expressions were carried out uniformly at 37 °C in a benchtop fermenter (BioFlow III; New Brunswick Scientific). Agitation was implemented by stirring at 400−600 rpm. Aeration was typically achieved by bubbling a gas mixture of O_2/Ar in a 3:7 ratio through the medium.

Recombinant CcP with a primary sequence identical to the native yeast-isolated enzyme (*4*, *6*, *56*, *57*) was the gene product that constitutes recNATCcP and both deuterated enzymes [recDCcP and recD(*H*is)CcP]. A plasmid, pT7CCPZf1, harboring a CcP gene with this native sequence, with T53 and D152, was obtained from Prof. Thomas Poulos (University of California, Irvine). The expressed enzymes contained no N-terminal alterations from the primary sequence of natWTCcP. For one expression, this CcP gene was excised from pT7CCPZf1 with *Nde*I and *Eco*RI and ligated into the pET24(a)+ plasmid cut with the same restriction enzymes. Both plasmid constructs, pT7CCPZf1 and pET24(a)+(NATCcP), were subcloned into BL21(DE3) *E. coli* cells and used for expression. *E. coli* [BL21(DE3)] harboring pT7CCPZf1was used to express recNATCcP and recDCcP, while pET24(a)+(NATCcP)-BL21(DE3) was used to express recD(*H*is)CcP. The correct native sequence of this CcP gene has been confirmed by completely sequencing the gene in both plasmids (*57*). Whether the recombinant enzyme product was deuterated or contained natural proton isotope abundance was controlled by the isotope composition of the medium, as described in detail in the following.

As previously demonstrated (*58*, *59*), it was necessary to adapt *E. coli* cells for successful growth in a predominantly deuterium-containing medium. pT7CCPZf1-BL21(DE3) cells and pET24a(+)-(NATCcP)-BL21(DE3) cells were adapted to D_2O by the same process of consecutive (overnight, 37 °C) growth in different solutions of LB medium prepared with 33%, 66%, and 97% D_2O, respectively. In each step, aliquots from the previous growth were used to inoculate subsequent medium. The final solution (97% D_2O) was used to make a glycerol stock, which was stored at −80 °C until used for expressions.

Recombinant native perdeuterated CcP (recDCcP) was expressed in 1 L of M9D(Martek)/Amp medium using pT7CCPZf1-BL21(DE3) cells. The culture was fermented

at 37 °C with 0.1 M IPTG added to induce CcP expression when the cell density had reached $OD_{600} = 0.6$. In an ultimately unsuccessful attempt to induce heme biosynthesis, δ-aminolevulinic acid (δ-ALA) and $FeSO_4$ were added to the fermentation mixture (*57*, *60*, *61*). CcP was expressed for an additional 17.5 h. Cells were harvested at $OD_{600} = 3.0$ (corresponding to 0.73 g of wet cells) by centrifugation. In the initial stages of purification, upon DEAE column purification (*57*) it became clear that holoenzyme had not been produced in vivo, as before (*57*). At this point, holoenzyme recDCcP was assembled by adding exogenous hemin (protoporphyrin IX chloride, Porphyrin Products) and continuing purification, as before (*57*).

Recombinant perdeuterated CcP incorporating natural proton isotope abundance histidine [recD(*H*is)CcP] was expressed in *E. coli* pET24a(+)-(NATCcP)-BL21(DE3) using essentially identical methods, in the presence of 4.5 mg/mL exogenous natural proton isotope abundance histidine.

The natWTCcP mimic, recNATCcP, was expressed using pT7CCPZf1-BL21(DE3) in 800 mL of rich LB medium at natural proton isotope abundance, employing analogous fermentation conditions. Heme biosynthesis was stimulated by adding δ-aminolevulinic acid and $FeSO_4$ to the fermentation mixture (*57*, *60*, *61*). Expression was again induced with IPTG, as above, at a cell density $OD_{600} = 0.75$. Cells were harvested at $OD_{600} = 9.1$, after 8 h of induction, which corresponded to 1.8 g of wet cells.

Isolation and Purification. A uniform general procedure for isolating pure enzymes was used, and is described in detail elsewhere (*57*). There were only minor differences in purifying the two deuterated enzyme preparations.

Yields and Purity Assessment. All recombinant enzymes produced in this work migrated (identically to yeast-isolated natWTCcP) as single lines on SDS−PAGE, which was carried out using a PhastSystem (Pharmacia Biotech) with PhastGel Homogeneous-12.5 gels (12.5% Polyacrylamide Gels). The yield of pure recDCcP was 14 mg per liter of minimal M9D medium. The purity index, PZ (*57*, *62*, and references cited therein), for recDCcP was 1.30. The total yield of recD(*H*is)CcP was 34 mg from 1.1 L of M9D medium (31 mg/L). Its PZ was 1.22. The yield of purified recNATCcP expressed in rich LB medium at natural proton isotope abundance was 25 mg from 800 mL of a culture (31 mg/L). This preparation of recNATCcP had a purity index value (PZ) of 1.27.

Physical Methods. UV−visible spectroscopy was carried out using a GBC Cintra 40 spectrometer. DNA sequencing employed an Applied Biosystems 373 fluorescence DNA automated sequencer in the WSU LBB-I. Mass determinations were achieved using matrix-assisted laser desorption ionization-time-of-flight spectrometry (MALDI-TOF; PerSeptive Biosystems Voyager DE-RP) in the WSU LBB-II, and by electrospray ionization with a Finnegan LCQ ion trap spectrometer. For MALDI-TOF, deuterated samples were taken from NMR solutions in 99.9% D_2O solvent/buffer, while nondeuterated samples were taken from solutions made from purified natural proton isotope abundance water/buffer (Barnstead E-Pure). For MALDI-TOF, the samples were desorbed/ionized from a sinapinic acid matrix (natural proton isotope abundance) with a 337 nm nitrogen laser. For ESI-MS, the sample was infused in 50/50 water/acetonitrile with 5% acetic acid. For MALDI-TOF measurements, both internal and external mass standards were employed for mass calibrations as previously described (*57*).

Deuterated phosphate buffer salts were made from K_2HPO_4 and KH_2PO_4 (Fisher) that were carried through three consecutive cycles of 100:1 (mass/mass) dissolution in 99.9% D_2O (Isotec) followed by lyophilization. Solution pH was measured using a standardized, calibrated Fisher combination electrode and an Orion model 310 meter and in D_2O solutions is reported as pH′, indicating the meter reading without adjustment for the isotope effect. Adjustments to CcP solution pH were made using DCl, NaOD, and D_3PO_4 (all Isotec).

Kinetic studies of the reaction between hydrogen peroxide and both recNATCcP and recDCcP were performed by stopped flow, with monitoring at 424 nm, using a model SX.17MV stopped-flow spectrofluorometer (Applied Photophysics Ltd.). The reaction was carried out in 50 mM potassium phosphate buffer, pH 6.5, 25 °C, employing enzyme concentrations of approximately 0.2 μM. Hydrogen peroxide concentrations ranged from 2.3 to 11 μM.

NMR experiments were carried out using a Varian Inova spectrometer operating at the nominal proton frequency of 500 MHz. Observed proton chemical shifts were internally referenced to the residual HDO peak, which was assigned a shift of 4.70 ppm at 21 °C. Several types of homonuclear proton two-dimensional experiments were carried out including Clean TOCSY (*65*) and NOESY (*66*). Variations of the basic NOESY experiment were also employed. These included DEFT-NOESY, in which a DEFT (*67*) sequence was substituted for the first NOESY $\pi/2$-pulse, and WET-NOESY, in which off-resonance water suppression was implemented using a WET sequence (*68*). WET-1D, 1D-DEFT, and standard S2PUL experiments were also used. NMR data were processed on an O2 computer (Silicon Graphics Inc.) and were edited using Photoshop 4.0 (Adobe Systems).

RESULTS AND DISCUSSION

Protein Production. Two extensively deuterated forms of CcP were produced for this study. One, recDCcP, used only highly deuterated medium (≥97% D) and resulted in a perdeuterated enzyme that was uniformly labeled, analogous to our previous results with a monomer hemoglobin (*58*, *59*). The second, recD(*H*is)CcP, incorporated histidine at natural proton isotope abundance that was added into the deuterated growth medium at initiation of expression. In this case, the objective was to create an enzyme in which all His contained natural proton isotope abundance, while the rest of the enzyme's polypeptide was deuterated. In addition to these two deuterated proteins, recNATCcP was expressed in rich medium containing natural proton isotope abundance. Yields of the purified enzymes were 14 mg/L for recDCcP and 31 mg/L for both recD(*H*is)CcP and recNATCcP. Production of both deuterated enzymes required induction ∼2−4 times longer than for recombinant native CcP (recNATCcP) expressed in a rich medium containing natural proton isotope abundance. The fact that high levels of expression were achieved in the His-supplemented growth medium, and that the His was incorporated without significant isotope scrambling, demonstrates the possibility for carrying out expression

Table 1: Physical Properties of Recombinant Deuterated and Nondeuterated Resting-State CcP

	natWTCcP	recNATCcP	recDCcP	recD(*H*is)CcP
Soret max[a]	408.8	408.9	408.7	409.2
PZ[b]	1.27	1.27	1.30	1.22
A_{408}/A_{380}	1.50	1.59	1.57	1.59
k_1[c]	$(6.6 \pm 0.2) \times 10^7$ [d]	$(6.1 \pm 0.5) \times 10^7$	$(5.6 \pm 0.2) \times 10^7$	

[a] In nm at pH 6.8, 0.1 M potassium phosphate, 22 °C. [b] Purity index, PZ, is the ratio of absorbance at 408 ± 1 nm (Soret band maximum) to the absorbance maximum at 280 ± 2 nm. [c] Reported in M^{-1} s^{-1} for the bimolecular reaction with hydrogen peroxide in 0.1 M potassium phosphate buffer. [d] Reference *27*.

with $^{13}C/^{15}N$-enriched His without resorting to His auxotrophs of *E. coli*.

All three of the cytochrome *c* peroxidases employed in this study were produced using a streamlined expression and purification protocol (*57*). In recent recNATCcP expressions (*57*), the growth medium was supplemented with the first committed precursor in the heme biosynthetic pathway, δ-aminolevulinic acid, in combination with $FeSO_4$. Such supplementation stimulated heme biosynthesis (*60*, *61*) so that holoCcP could be isolated directly from *E. coli*, without addition of exogenous heme. This in vivo holoCcP production also happened for recNATCcP expression in this work (natural proton isotope abundance), but deuterium-enriched cytochrome *c* peroxidases were only produced in the apoenzyme form, despite similar supplementation of the growth medium. Instead, both of these holoenzymes were assembled by subsequent addition of hemin solution following lysis [recD(*H*is)CcP] or after preliminary purification (recDCcP). We speculate that the use of minimal medium has a deleterious effect on the *E. coli* heme biosynthetic pathway.

Characterization. Physical characterizations of the perdeuterated CcPs were carried out in order to compare them to each other and to the undeuterated recombinant and yeast-isolated enzymes. Physical methods used included UV−visible spectroscopy, mass spectrometry, and kinetics. These measurements were carried out on resting-state and CN-ligated states of the enzymes, and the results are presented in the Supporting Information, as well as in the following.

The purity of resting-state CcP preparations has been historically judged by two UV−visible absorbance ratios (*60*): (i) the purity index, PZ, which is the ratio of UV−visible absorbances at 408 and 280 nm (A_{408}/A_{280}); and (ii) the A_{408}/A_{380} ratio. Values of these two ratios for the enzymes produced here (Table 1) are all very similar to each other and to literature values for undeuterated enzymes (*60*). Judged by these criteria the deuterated CcPs produced here are highly pure, and this is also borne out by their migration as single lines on SDS−PAGE.

Another indication of the purity and integrity of expressed CcP comes from functional evaluation, which in this case involves elucidating the rate constant for the bimolecular reaction with H_2O_2. This reaction is the hallmark of heme peroxidases and the first step in CcP's functional cycle (*3*, *4*). Pseudo-first-order kinetic plots are essentially superimposable for recNATCcP and recDCcP, and the corresponding rate constants are reported in Table 1.

Experimental masses for both recDCcP and recD(*H*is)-CcP are reported in Table 2. Comparison with calculated masses was also useful in determining the extent of deuterium incorporation (Table 2). The neutral enzyme form of apocytochrome *c* peroxidase harbors 2261 protons, of which 528 are N−H, O−H, and S−H, and thus are hydrogen-isotope exchangeable. These exchangeable protons are labile to isotope exchange with solvent water, and therefore may be either H or D, depending upon whether the solvent is H_2O or D_2O. The complement of exchangeable protons in a native protein structure, like that of cytochrome *c* peroxidase, exhibits a range of solvent isotope exchange rates (*69*−*71*), which complicates precise assessment of isotope incorporation levels. To estimate the extent of deuteration, we have calculated a set of CcP masses based upon whether all exchangeable and nonexchangeable (i.e., C−H) protons are H or D, also shown in Table 2.

Table 2: Calculated Masses and Mass Spectral Data for Recombinant Deuterated and Nondeuterated CcP

CcP form	nonexchangeable protons[a]	exchangeable protons[b]	calcd mass[c]	measured mass	
				MALDI	ESI-MS
recNATCcP	H	H	33532.5	33529	33531.3
recNATCcP	H	D	34063.7		
recDCcP	D	D	35807.4	35429	
recDCcP	D	H	35276.2		
recD(*H*is)CcP	D	D	35777.2	35389	
recD(*H*is)CcP	D	H	35246.0		

[a] Isotope composition of all C-bound protons; H in this column refers to natural proton isotope abundance; D in this column refers to 100% deuterium incorporation. [b] Isotope composition of all N-, O-, S-bound protons; H in this column refers to natural proton isotope abundance; D in this column refers to 100% deuterium incorporation. [c] The calculated masses are based on the neutral enzyme form and IUPAC Commission on Atomic Weights and Isotope Abundances (1993): H = 1.00794, D = 2.0141, C = 12.011, N = 14.00674, O = 15.9994, and S = 32.066. A previously reported mass for the charged form of recNATCcP at pH = 7 (*57*) differs slightly from the value reported here for the neutral enzyme form.

Comparing the measured masses with the calculated masses in Table 2 leads to the following observations. (i) The measured and calculated masses of recNATCcP (H_2O solvent) agree splendidly. (ii) The measured masses of the two deuterated CcPs are both larger than the masses calculated for each enzyme in which the nonexchangeable C−H are entirely replaced by C−D and the exchangeable hydrogens are entirely H. This is understandable because both of these samples were extracted directly from NMR samples in which the solvent was 99.9% D_2O. (iii) However, the measured masses of the two deuterated CcPs are both smaller than the masses calculated for each completely deuterated (exchangeable and nonexchangeable hydrogens) enzyme. This is an indication of incomplete deuterium incorporation into the enzyme, which could be due to isotope dilution in the original expression medium, or in the handling and preparation for MALDI-TOF mass spectrometry. We have not yet identified the reason. Using data from Table 2, the percent deuteration calculated for recDCcP and recD(*H*is)-CcP is 83.4% and 84.7%, respectively. This agrees well with the estimate of 85 ± 9% deuteration made by NMR (vide infra).

Proton NMR Characterization. (A) General. CcP is paramagnetic in both its resting-state and its cyanide-ligated state, and this situation makes proton NMR spectra more complicated than with diamagnetic proteins (*40*−*44*, *57*, *63*,

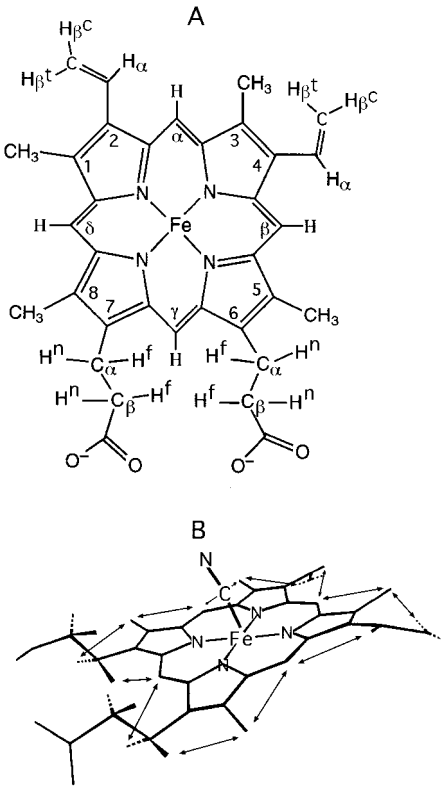

FIGURE 1: (A) Heme structure with stereospecific proton labels used in this study. (B) Heme structure derived from the X-ray crystal structure of CcPCN showing the side chain conformations and the primary NOEs between substituent groups identified at mixing times from 2 to 10 ms. Geminal connectivities for the propionate methylene protons were also observed in these early mixing time experiments, along with connectivities within each vinyl group, but are not shown on the figure for clarity reasons.

64). The heme-centered paramagnetism provides both advantages and disadvantages in analyzing enzymes of this size, as has been recently reviewed (*63*) and will be described in more detail below. One major advantage specific to the low-spin, CN-ligated, paramagnetic Fe(III)-heme is that it acts as an intrinsic nuclear shift and relaxation agent that imbues proton resonances of the active site with large hyperfine shifts (*63*, *64*), as illustrated by the spectra presented here. Resonances from the heme, heme-ligating amino acids, and other amino acids in close proximity to the heme are affected, although in the following we focus only on the heme proton resonances.

To provide a structural context for the following description of NMR results, Figure 1 presents heme structures that define the stereospecific labels we have used. Besides four methyl groups (Figure 1A), the heme peripheral substituents include two vinyl groups and two propionates. Vinyl group β-geminal protons are distinguished as cis (β^c) or trans (β^t) to the α-proton. Propionate groups possess two α-protons and two β-protons, and the extended propionate side chain can adopt a range of conformations. We classify these protons as to whether their conformation situates each of them closer to their nearest-neighbor methyl group, or further from it, compared to their geminal partner. For example, the 7-propionate has the 8-CH_3 as its closest methyl group. The label $7\alpha^n$ refers to the 7-propionate α-H situated nearest to the 8-CH_3, whereas $7\alpha^f$ represents the α-H oriented furthest from the 8-CH_3, similarly for the 7-propionate β-protons.

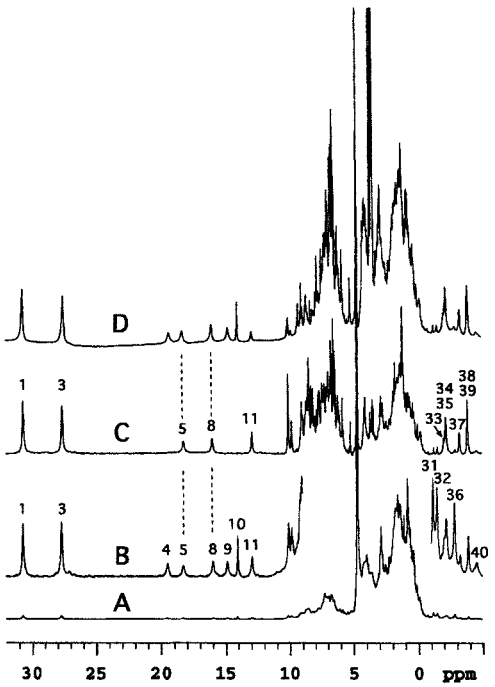

FIGURE 2: One-dimensional ^{1}H NMR spectra at 500 MHz, 21 °C, pH′ = 7.5, 0.1 M potassium phosphate, 99.9% D_2O. (A) recNATCcPCN; (B) recNATCcPCN, vertical expansion of resolved hyperfine shift regions; (C) recDCcPCN; (D) recD(*H*is)CcPCN. Spectra A and C are plotted at vertical expansions such that the tallest resonances in the aliphatic shift region of each are identical heights. Hyperfine resonances are numbered to correspond with assignments and peak numbers given in Table 3 and previous references (*42*, *43*).

For the 6-propionate group, the resonances are labeled with respect to their orientation to the 5-CH_3.

(B) Resting-State CcP. One-dimensional ^{1}H NMR spectra (data not shown) reveal essentially identical hyperfine shift patterns for the resting states of all three recombinant enzymes used in this study. In turn, spectra of resting-state recNATCcP and resting-state natWTCcP have been shown to have identical hyperfine shift patterns (*57*). The similarity in these spectra emphasizes the essentially identical nature of the heme active site in the resting state of all of these enzymes, and reinforces the concept of structurally and functionally intact active sites for both of the perdeuterated enzymes.

(C) CcPCN. Figure 2 shows one-dimensional NMR spectra in 99.9% D_2O buffers of the low-spin recNATCcPCN, recDCcPCN, and recD(*H*is)CcPCN. Due to the combination of contact and dipolar spin delocalization mechanisms, the hyperfine resonances shown in Figure 2A,B originate from protons of the heme, the axially coordinating His175, and neighboring amino acids whose side chains define the active site environment (*41*, *42*, *61*).

Figure 2A and Figure 2B show 1D spectra of recNATCcPCN. The hyperfine-shifted proton resonances, some of which were previously assigned, are designated by numbers (see Table 3) in Figure 2. Although not shown here, extensive work has revealed that proton NMR spectra (1D and 2D) of recNATCcPCN and natWTCcPCN (*40*−*43*, *57*) are essentially identical.

Figure 2C is a spectrum of recDCcPCN. Figure 2A and Figure 2C are plotted so that in each spectrum the tallest protein resonance has the same height. This is a convenient

Table 3: Complete, Stereospecific Heme Proton Resonance Assignments for CcPCN and Comparison with Previous Assignments

resonance assignment[a]	peak no.[b]	observed shift[c]	previously assigned[d]	previously misassigned[d]
methyl				
1		1.23		
3	1	30.73	40−44	
5		4.12		
8	3	27.71	40−44	
2-vinyl				
α		7.01	44	
β^c	38	−3.85	44	
β^t	37	−3.22	44	
4-vinyl				
α	8	16.08	40−44	
β^c	39	−3.85	40−44	
β^t	35	−2.15	40−44	
6-propionate				
α^n		−0.22		
α^f		6.09		
β^n	34	−2.22	41	
β^f	33	−2.01		
7-propionate				
α^n		6.26	41−44	
α^f	5	18.30	41−44	
β^n		4.18		43, 44
β^f		1.94		41−44
meso				
α		1.36		
β		5.90	42	
γ		2.75		
δ		7.43		42

[a] Vinyl group β-proton superscripts: c refers to the proton cis to the α-proton; t refers to the proton trans to the α-proton. Propionate group proton superscripts: In each geminal pair, n refers to the proton oriented nearest to the closest methyl group, and f refers to the proton oriented furthest from the closest methyl group (see Figure 1 and text). [b] As labeled in Figures 2−5 and in previous work from this lab. [c] In ppm, relative to internal residual water at 4.70 ppm, at 21 °C, 0.1 M potassium phosphate, pH′ = 7.5. [d] These two columns indicate the references for previous proton assignments that were correctly or incorrectly assigned. Note that only 12 of 22 individual heme protons have been previously assigned.

way to visually illustrate the extent of deuteration (Figure 2C). Figure 2A emphasizes the relatively low signal intensity of the resolved hyperfine-shifted proton resonances (11−30 and 0 to −5 ppm) as compared to the diamagnetic "envelope" (0−11 ppm) of remaining resonance intensity in recNATC-cPCN. An alternate, and illuminating, comparison is that in which the relative intensities of the two heme methyl resonances (peaks 1 and 3) at ∼27−31 ppm (*40*, *41*) are measured against the intensity of the diamagnetic "envelope". The prominent relative intensities of the heme hyperfine resonances in the 11−31 and 0 to −5 ppm regions shown in Figure 2C (compared to Figure 2A) further illustrate the extent of general proton resonance intensity reduction that we have achieved. Figure 2C further reveals which of the resolved hyperfine-shifted proton resonances in Figure 2A belong to amino acids situated close to the heme group since these resonances are absent in Figure 2C as a consequence of deuteration. Prominently missing in Figure 2C are resonances previously assigned (*41*−*44*) to two protons of the proximal histidine (His175) and one proton of the distal histidine (His52). The His52 (peak 10) and His175 (peaks 4 and 9) resonances are restored in the spectrum of recD(*H*is)-CcPCN (Figure 2D).

The low-frequency hyperfine-shift region (0 to −5 ppm, Figure 2) can also be used to estimate the extent of deuteration that has been achieved in the following way. The two methyl proton resonances of the previously assigned Leu232, resonances 31 and 36 in Figure 2A, display dramatically reduced intensities in the perdeuterated enzyme (Figure 2C). Comparing their individual integrated intensities with that of each one of the bona fide single proton heme resonances, peaks 8 and 37 in Figure 2C, and explicitly accounting for the methyl and proton intensity ratio, leads to a calculated value for the extent of deuteration as 85 ± 9%, as described above.

Heme Hyperfine Resonance Assignments. The first heme resonance assignments for CcPCN were published 18 years ago using hemes with specific substituent groups deuterated (*40*). Subsequently, several NMR studies sought to completely and accurately assign the heme and active site amino acid protons, yet that goal has remained elusive (*41*−*44*). The complexity of the task is illustrated by the fact that despite those previous assignment attempts, only 12 of 22 heme proton resonances have been previously assigned correctly and several incorrect assignments have been proposed (Table 3). In several instances, the incorrect heme resonance assignments have subsequently resulted in confusion over active site amino acid assignments, which, in turn, has seriously impeded solution studies of the enzyme.

There are several specific reasons why unambiguously correct and complete hyperfine-shifted ^{1}H assignments have not been forthcoming.

1. The heme paramagnetism is a factor that eliminates straightforward chemical shift assignments using standard methodologies. Therefore, proton hyperfine shifts are not governed by rules of chemical type (*63*), as are observed proton shifts in diamagnetic molecules. Instead they are governed by more complex factors such as the heme electronic orbital ground state, the paramagnetic anisotropy of the heme metal ion, and the precise details of metal−ligand bonding (*63*).

2. Large hyperfine shifts are apparent in Figure 2, and whereas some are due to active site amino acid protons, less than half of the hyperfine-shifted heme protons appear resolved. Many of the hyperfine-shifted protons in CcPCN lie within the so-called "diamagnetic envelope" (11−0 ppm) where their individual intensities are overwhelmed by the combined intensities of ∼2225 other protons from the enzyme.

3. Although hyperfine-shifted ^{1}H resonances are highly temperature-dependent (*63*), no additional heme hyperfine resonances were revealed in variable-temperature NMR experiments of CcPCN over the comparatively limited temperature range of the enzyme's stability. In contrast to horseradish peroxidase-CN (*64*), which is stable at temperatures as high at 55 °C, CcPCN is stable in NMR solutions for only about 1 h at 37 °C.

4. As shown by the results presented here for CcPCN and the heme resonance assignments for HRPCN (*64*), a further complicating feature of ^{1}H hyperfine-shifted resonances is that even geminal protons are highly dispersed. For example, the following differences in observed proton (hyperfine) chemical shift ($\Delta\delta_{obs}$) occur for diasterotopic geminal CH_2 protons in CcPCN (21 °C): His(175) βH/βH′, $\Delta\delta_{obs}$ = 4.7 ppm; 7αH^n/7αH^f, $\Delta\delta_{obs}$ = 12.1 ppm; 6αH^n/6αH^f, $\Delta\delta_{obs}$ =

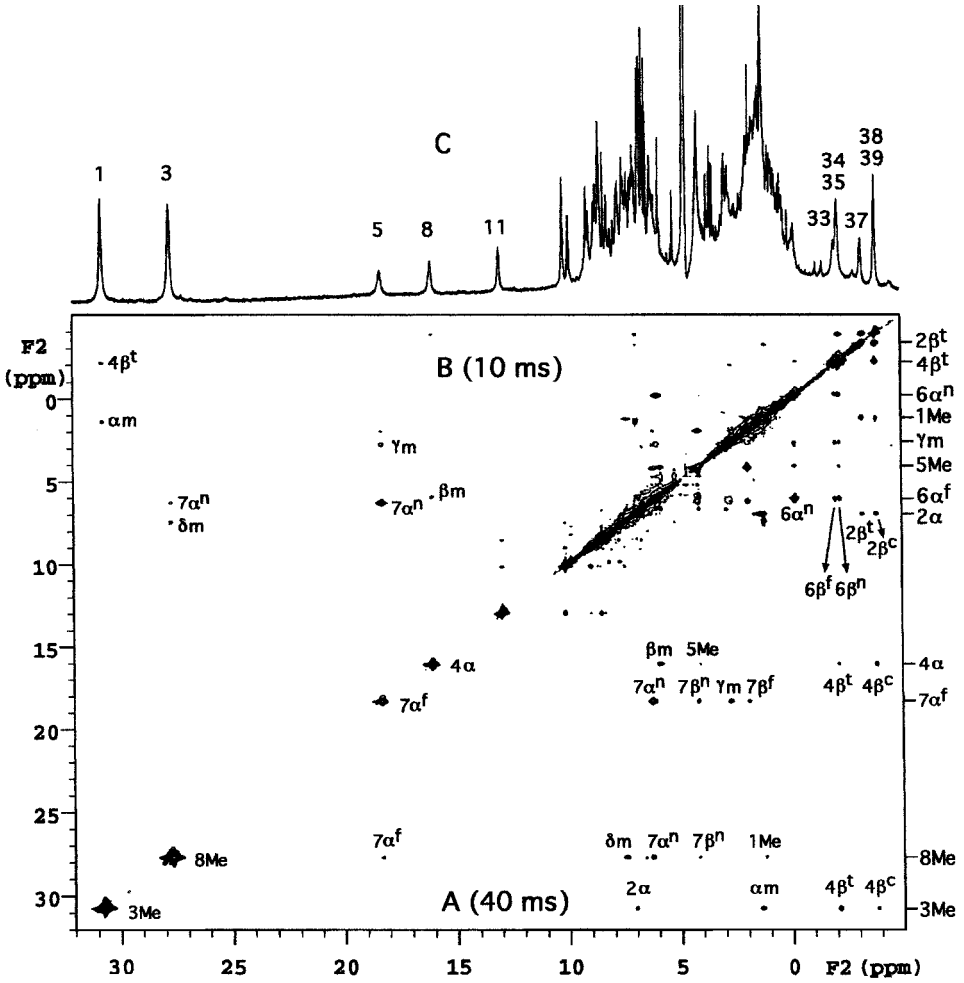

FIGURE 3: WET-NOESY ^{1}H spectra of recDCcPCN. Experimental conditions identical to Figure 2. (A) 40 ms mixing time; (B) 10 ms mixing time; (C) one-dimensional spectrum. Additional assignments are given in Figure 4.

6.3 ppm. One of these geminal partners may be resolved in a 1D spectrum, but the other may be buried in the spectrally dense diamagnetic region. Such lack of resolution, the high density of 2D cross-peaks within the diamagnetic envelope (*42*−*44*; vide infra), and the enhanced nuclear relaxation characteristics of highly hyperfine-shifted protons (next section) have conspired against achieving complete hyperfine resonance assignments.

5. Other factors that mitigate using typical NMR methods of assignment are that strongly hyperfine-shifted proton resonances also have short T_1s (typically ~100 ms or less) and T_2s (typically under 20 ms), which marginalizes nearly all NMR experiments higher than 2D that might be employed for assigning these protons (*63*, *64*). This situation even completely compromises employing some 2D coherence transfer experiments (*63*).

These complications are rare in diamagnetic systems and are a primary reason simply achieving proton hyperfine resonance assignments has been so very challenging for CcPCN. However, in the deuterated enzymes, the following strategy has led to straightforward, complete heme hyperfine resonance assignments.

For recDCcPCN and recD(*H*is)CcPCN, assignments of nearest neighbors and geminal partners were initially made using various ^{1}H-^{1}H NOESY experiments with short mixing times, up 10 ms. This initial set of assignments was subsequently expanded using a series of experiments that incremented the mixing time up to 70 ms to generate NOE rise curves. The following spin-system assignments were then confirmed with homonuclear Clean-TOCSY experiments: 4-vinyl, 2-vinyl, 7-propionate, 6-propionate. Some of our results are shown in Figures3 and 4. Together these figures and Table 3 present details of the complete heme proton assignments of CcPCN. These new assignments are highly certain and complete.

Several points bear discussion. Even though the 10−0 ppm region in the 1D ^{1}H spectrum of recDCcPCN looks overlapped (Figures 3C, 4C), resolution of the heme resonances in the NOESY spectra is superb, and has led to unambiguous connectivities and assignments (Figures 3A,B and 4A,B). By comparing the NOESY spectra in Figure 3A and 3B with similar spectra in refs *42* and *44*, it can be seen that the level of simplification in Figures 3 and 4 is exceptional. Comparing Figures4 and 5 illustrates the ambiguities of assigning hyperfine-shifted proton resonances in CcPCN that is at natural hydrogen isotope abundance, using the heme 6β geminal pair of protons as an example. Figure 5 is an "optimally adjusted" DEFT-NOESY experiment of recNATCcPCN (natural proton isotope abundance), designed to simplify spectra using relaxation filtering, which takes advantage of the T_1 differences between hyperfine-shifted ^{1}H resonances on the heme (or close to it), and those protons that are more distant. Figure 5 shows that even in an optimal situation with recNATCcPCN, unambiguously assigning

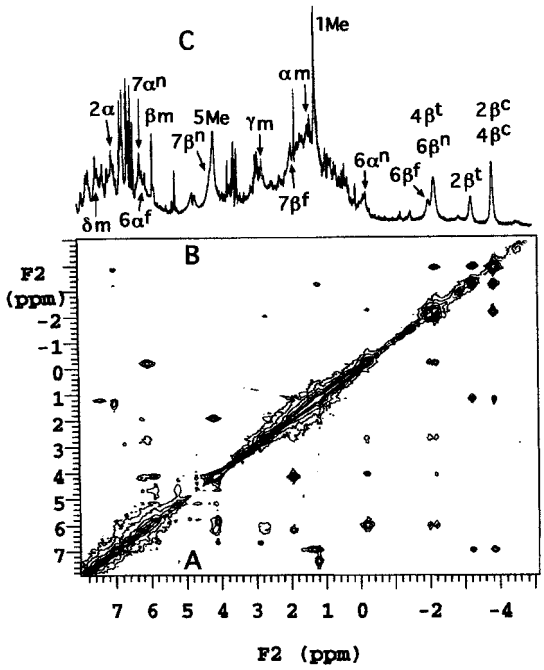

FIGURE 4: Expansion of the WET-NOESY ^{1}H spectra of recDC-cPCN shown in Figure 3. (A) 40 ms mixing time; (B) 10 ms mixing time; (C) one-dimensional spectrum.

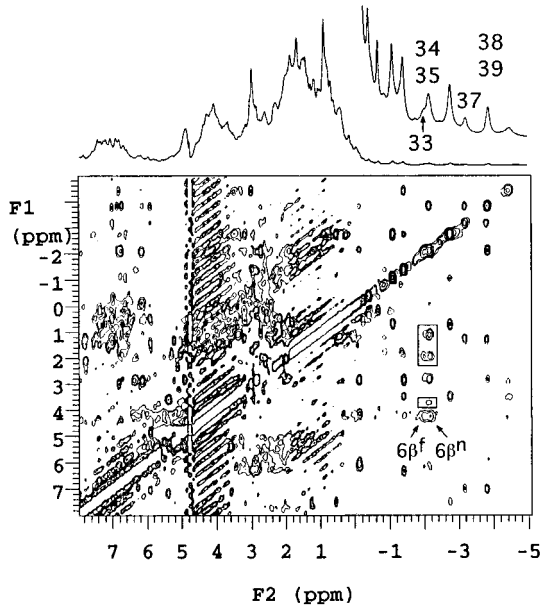

FIGURE 5: DEFT-NOESY and reference one-dimensional spectra of recNATCcPCN with experimental conditions identical to Figure 2.

cross-peaks to, e.g., the 6β pair of heme propionate protons would be impossible without deuteration (Figure 4). The boxed cross-peaks in Figure 5 (recNATCcPCN) show that in this case there are at least three possible alternate incorrect assignments for the cross-peaks assigned in Figure 4 (recD-CcPCN). An additional advantage of deuteration is that it allows the 6β^n/ β^f geminal proton cross-peak to be revealed, which is an unprecedented level of assignment detail in a paramagnetic heme protein of this size (see Supporting Information).

Straightforward connectivities in all of the NOESY and Clean-TOCSY spectra subsequently lead to stereospecific proton assignments, as follows. In early mixing-time NOESY spectra (not shown), cross-peak intensity differences are proportional to the corresponding internuclear distances (*63*, *64*). The relative cross-peak intensities in individual NOESY experiments in which the mixing time varied from 2 to 70 ms, confirmed by NOE rise curves (data not shown), lead to stereospecific assignments and also to qualitative conformational structural information. The heme side chain conformations that were determined from this NOESY data are completely consistent with the heme side chain conformations defined in the X-ray crystal structure of CcPCN, shown in Figure 1. In Figure 1B arrows indicate the primary NOEs among protons α to the heme that are observed at 2−10 ms mixing time in NOESY experiments. In addition, other geminal and vicinal connectivities were observed among the heme propionate methylene protons and among the protons of the two vinyl groups (not shown).

ACKNOWLEDGMENT

J.D.S. gratefully acknowledges Harold Goldberg, M.D., whose compassion, talent, and expertise made this paper possible. We thank Professor Thomas Poulos, University of California, Irvine, for kindly making available to us plasmid pT7CCPZf1, and the X-ray crystal coordinates of CcPCN. We also thank Derek Pouchnik for expert DNA sequencing in the Washington State University LBB-I.

SUPPORTING INFORMATION AVAILABLE

Four figures containing UV−visible spectra of deuterated and undeuterated, CN-ligated enzymes; pseudo-first-order kinetic plots for the reaction of recNATCcP and recDCcP reacting with hydrogen peroxide; MALDI-TOF mass spectra of recDCcPCN and recD(*H*is)CcPCN; and proton NMR spectra showing pH-dependent spectra (6 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Yonetani, T., and Ray, G. S. (1965) *J. Biol. Chem. 240*, 4503−4508.
2. Nelson, C. E., Sitzman, E. V., Kang, C. H., and Margoliash, E. (1977) *Anal. Biochem. 83*, 622−631.
3. Dunford, H. B. (1999) *Heme Peroxidases*, J. Wiley and Sons, New York.
4. Bosshard, H. R., Anni, H., and Yonetani, T. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., and Grisham, M. B., Eds.) Vol. 2, pp 52−78, CRC Press, Boca Raton, FL.
5. Everse, J., Everse, K. E., and Grisham, M. B., Eds. (1990) *Peroxidases in Chemistry and Biology*, Vol. I and II, CRC Press, Boca Raton, FL.
6. Poulos, T. L., and Kraut, J. (1980) *J. Biol. Chem. 255*, 8199−8205.
7. Poulos, T. L., and Finzel, B. C. (1984) *Protein Pept. Rev. 5*, 115−171.
8. Finzel, B. C., Poulos, T. L., and Kraut, J. (1984) *J. Biol. Chem. 259*, 13027−13036 (Protein Data Bank Crystal Structure ID: 2CYP).
9. Wang, J., Mauro, J. M., Edwards, S. L., Oatley, S. J., Fishel, L. A., Ashford, V. A., Xuong, N., and Kraut, J. (1990) *Biochemistry 29*, 7160−7173 (Protein Data Bank Crystal Structure ID: 1CPD).
10. Poulos, T. L. (1988) *Adv. Inorg. Biochem. 7*, 1−36.
11. Mauro, J. M., Miller, M. A., Edwards, S. L., Wang, J., Fishel, L. A., and Kraut, J. (1989) *Met. Ions Biol. Syst. 25*, 477−503.

12. Goodin, D. B., and McRee, D. E. (1993) *Biochemistry 32*, 3313−3324 (Protein Data Bank Crystal Structure ID: 1CCA).
13. Fitzgerald, M. M., Musah, R. A., McRee, D. E., and Goodin, D. B. (1996) *Nat. Struct. Biol. 3*, 626−634.
14. Hirst, J., Wilcox, S. K., Williams, P. A., McRee, D. E., and Goodin, D. B. (2001) *Biochemistry 40*, 1265−1273.
15. Wilcox, S. K., Jensen, G. M., Fitzgerald, M. M., McRee, D. E., and Goodin, D. B. (1996) *Biochemistry 35*, 4858−4866.
16. Fülöp, V., Phizackerley, R. P., Soltis, S. M., Clifton, I. J., Wakatsuki, S., Erman, J. E., Hajdu, J., and Edwards, S. L. (1994) *Structure 2*, 201−208.
17. Summers, F. E., and Erman, J. E. (1988) *J. Biol. Chem. 263*, 14267−14275.
18. Vitello, L. B., Erman, J. E., Mauro, J. M., and Kraut, J. (1990) *Biochim. Biophys. Acta 1038*, 90−97.
19. Miller, M. A., Vitello, L., and Erman, J. E. (1995) *Biochemistry 34*, 12048−12058.
20. Kim, K. L., Kang, D. S., Vitello, L. B., and Erman, J. E. (1990) *Biochemistry 29*, 9150−9159.
21. Matthis, A. L., and Erman, J. E. (1995) *Biochemistry 34*, 9985−9990.
22. Vitello, L. B., Erman, J. E., Miller, M. A., Wang, J., and Kraut, J. (1993) *Biochemistry 32*, 9807−9818.
23. Palamakumbura, A. H., Foshay, M. C., Vitello, L. B., and Erman, J. E. (1999) *Biochemistry 38*, 25647−15652.
24. Mei, H., Wang, K., Peffer, N., Weatherly, G., Cohen, D. S., Miller, M., Pielak, G., Durham, B., and Millett, F. (1999) *Biochemistry 38*, 6846−6854.
25. Hahm, S., Durham, B., and Millett, F. (1992) *Biochemistry 31*, 3472−3477.
26. Hahm, S., Miller, M. A., Geren, L., Kraut, J., Durham, B., and Millett, F. (1994) *Biochemistry 33*, 1473−1480.
27. Erman, J. E., Vitello, L. B., Miller, M. A., Shaw, A., Brown, K. A., and Kraut, J. (1993) *Biochemistry 32*, 9798−9806.
28. Corin, A. F., Hake, R. A., McLendon, G., Hazzard, J. T., and Tollin, G. (1993) *Biochemistry 32*, 2756−2762.
29. Miller, M. A. (1996) *Biochemistry 35*, 15791−15799.
30. Zhou, J. S., and Hoffman, B. M. (1994) *Science 265*, 1693−1696.
31. Leesch, V. W., Bujons, J., Mauk, A. G., and Hoffman, B. M. (2000) *Biochemistry 39*, 10132−10139.
32. Fishel, L. A., Villafranca, J. E., Mauro, J. M., and Kraut, J. (1987) *Biochemistry 26*, 351−360.
33. Darwish, K., Li, H., and Poulos, T. L. (1991) *Protein Eng. 4*, 701−708.
34. Goodin, D. B., Davidson, M. G., Roe, J. A., Mauk, A. G., and Smith, M. (1991) *Biochemistry 30*, 4953−4962.
35. Miller, M. A., Geren, L., Han, G. W., Saunders, A., Beasley, Pielak, G. J., Durham, B., Millett, F., and Kraut, J. (1996) *Biochemistry 35*, 667−673.
36. Sigman, J. A., Kwok, B. C., Gengenbach, A., and Lu, Y. (1999) *J. Am. Chem. Soc. 121*, 8949−8950.
37. Bonagura, C. A., Sundaramoothy, M., Pappa, H. S., Patterson, W. R., and Poulos, L. (1996) *Biochemistry 35*, 6107−6115.
38. Wilcox, S. K., Putnam, C. D., Sastry, M., Blankenship, J., Chazin, W. J., McRee, D. E., and Goodin, D. B. (1998) *Biochemistry 37*, 16853−16862.
39. Palamakumbura, A. H., Vitello, L. B., and Erman, J. E. (1999) *Biochemistry 38*, 15653−15658.
40. Satterlee, J. D., Erman, J. E., La Mar, G. N., Smith, K. M., and Langry, K. C. (1983) *J. Am. Chem. Soc. 105*, 2099−2104.
41. Satterlee, J. D., Erman, J. E., and de Ropp, J. S.(1987) *J. Biol. Chem. 262*, 11578−11583.
42. Satterlee, J. D., and Erman, J. E. (1991) *Biochemistry 30*, 4398−4405.
43. Satterlee, J. D., Russell, D. J., and Erman, J. E. (1991) *Biochemistry 30*, 9072−9077.
44. Banci, L., Bertini, I., Turano, P., Ferrer, J. C., and Mauk, A. G. (1991) *Inorg. Chem. 30*, 4510−4516.
45. Satterlee, J. D., Teske, J. G., Erman, J. E., Mauro, J. M., and Poulos, T. M. (2000) *J. Protein Chem. 19*, 535−542.
46. Satterlee, J. D., Erman, J. E., Mauro, J. M., and Kraut, J. (1990) *Biochemistry 29*, 8797−8804.
47. Satterlee, J. D., Alam, S. L., Mauro, J. M., Erman, J. E., and Poulos, T. L. (1994) *Eur. J. Biochem. 224*, 81−87.
48. Alam, S. L., Satterlee, J. D., Mauro, J. M., Poulos, T. L., and Erman, J. E. (1995) *Biochemistry 34*, 15496−15503.
49. Castro, G., Boswell, C. A., and Northrup, S. H. (1998) *J. Biomol. Struct. Dyn. 16*, 413−424.
50. Charizanis, C., Juhnke, H., Krems, B., and Entian, K. D. (1999) *Mol. Gen. Genet. 262*, 437−447.
51. Pelletier, H., and Kraut, J. (1992) *Science 258*, 1748−1755.
52. Pappa, H. S., Tajbaksh, S., Saunders, A. J., Pielak, G. J., and Poulos, T. L. (1996) *Biochemistry 35*, 4837−4845.
53. Erman, J. E., Kresheck, G. C., Vitello, L. B., and Miller, M. A. (1997) *Biochemistry 36*, 4054−4060.
54. Wang, X., and Pielak, G. J. (1999) *Biochemistry 38*, 16876−16881.
55. Kaput, J., Goltz, S., and Blobel, G. (1982) *J. Biol. Chem. 257*, 15054−15058.
56. Takio, K., Titani, K., Ericsson, L. H., and Yonetani, T. (1980) *Arch. Biochem. Biophys. 203*, 615−629.
57. Teske, J. G., Savenkova, M. I., Mauro, J. M., Erman, J. E., and Satterlee, J. D. (2000) *Protein Expression Purif. 19*, 139−147.
58. Alam, S. L., and Satterlee, J. D. (1995) *J. Am. Chem. Soc. 117*, 49−53.
59. Alam, S. L., Dutton, D. P., and Satterlee, J. D. (1995) in *Nuclear Magnetic Resonance of Paramagnetic Macromolecules* (La Mar, G. N., Ed.) *NATO Adv. Res. Ser. B*, Vol. 457, pp 123−140, Kluwer, Dodrecht, The Netherlands.
60. Rivera, M., and Walker, F. A. (1995) *Anal. Biochem. 230*, 295−302.
61. Rivera, M., Qiu, F., Bunce, R. A., and Stark, R. E. (1999) *J. Biol. Inorg. Chem. 4*, 87−98.
62. Vitello, L. B., Huang, M., and Erman, J. E. (1990) *Biochemistry 29*, 4283−4288.
63. La Mar, G. N., Satterlee, J. D., and De Ropp, J. S. (2000) in *The Porphyrin Handbook* (Kadish, K. M., Smith, K. M., and Guillard, R., Eds.) Vol. 5, pp 185−298, Academic Press, San Diego, CA.
64. La Mar, G. N., Chien, Z., and De Ropp, J. S. (1995) in *Nuclear Magnetic Resonance of Paramagnetic Macromolecules* (La Mar, G. N., Ed.) *NATO Adv. Res. Ser. B*, Vol. 457, pp 55−74, Kluwer, Dodrecht, The Netherlands.
65. Griesinger, C., Otting, G., Wüthrich, K., and Ernst, R. R. (1988) *J. Am. Chem. Soc. 110*, 7870−7872.
66. Kumar, A., Ernst, R. R., and Wuthrich, K. (1980) *Biochem. Biophys. Res. Commun. 95*, 1−7.
67. Becker, E. D., Ferretti, J. A., and Farrar, T. C. (1975) *J. Magn. Reson. 19*, 114−117.
68. Smallcombe, S. H., Patt, S. L., and Keifer, P. A. (1995) *J. Magn. Reson., Ser. A 117*, 295−303.
69. Englander, S. W., and Kallenbach, N. R. (1984) *Q. Rev. Biophys. 16*, 521−655.
70. Paterson, Y., Englander, S. W., and Roder, H. (1990) *Science 249*, 755−759.
71. Yi, Q., Erman, J. E., and Satterlee, J. D. (1994) *Biochemistry 33*, 12032−12041.

BI0111000