crystallographic study. We conclude that a major structural change is induced in the C-terminal region of FB when it is bound to Fc. The present result is one of still relatively rare and interesting structural data, which demonstrate major structural changes between solution and crystal.

In the free FB, the three helical regions are located in close spatial proximity, interacting through the side chains of the hydrophobic residues that exist in each helix. It appears that, when FB is bound to the Fc portion of IgG through the two helices, I and II, the hydrophobic space surrounded by the three helices of FB in the free state is disrupted, resulting in a significant change in conformation of helix III comprising the C-terminal Ser42–Ala55 segment. It is also possible that the extended structure of the C-terminal Ser42–Ala55 segment has resulted from intermolecular interactions in the crystal. In order to pursue this point, a $^{15}N$ NMR study of the interaction between FB and IgG using uniformly $^{15}N$-labeled FB is under way in our laboratory.

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# Secondary Structure in Formylmethionine tRNA Influences the Site-Directed Cleavage of Ribonuclease H Using Chimeric 2′-*O*-Methyl Oligodeoxyribonucleotides†

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ABSTRACT: In order to cleave RNA at specific positions in *Escherichia coli* formylmethionine tRNA, RNase H and complementary chimeric oligonucleotides consisting of DNA and 2′-*O*-methyl-RNA (Inoue et al. (1987) *FEBS Lett. 215*, 327] were used. Specific cleavages in the D loop, anticodon loop, TΨC loop, anticodon stem, and acceptor stem were investigated. Virtually unique hydrolyses with RNase H were observed at the TΨC loop, anticodon stem, and acceptor stem when relatively longer chimeric oligonucleotides (20-mer) were used. An efficient cleavage at the anticodon was obtained with a chimeric 13-mer when the higher structure of the tRNA was broken by hybridization with a 20-mer at the acceptor as well as the TΨC stem region. It was found that stabilities of hybrids with chimeric oligonucleotides and the presence of minor nucleosides affect the cleavage of tRNA by this approach.

Sequence-dependent cleavage of DNA by restriction endonucleases are an essential procedure for gene manipulation (Nathan & Smith, 1975). However, methods for the site-directed cleavage of RNA have yet to be developed for structural and functional studies of RNA. Although self-cleaving RNAs have been shown to act as restriction RNases (Zang et al., 1986; Haseloff & Gerluch, 1988; Koizumi et al., 1989), those RNA enzyme reactions need further investigation. RNase H, which cleaves RNA in RNA–DNA heteroduplexes (Berkower et al., 1973; Crouch & Dirksen, 1982), has been used for the sequence-dependent hydrolysis of RNA in the presence of complementary DNA. Region-specific cleavages were observed (Donis-Keller, 1979; Stepanova et al., 1979; Lorenz et al., 1987; Berger, 1987). In contrast to those experiments, chimeric oligonucleotides consisting of a tetradeoxyribonucleotide and 2′-*O*-methyl oligonucleotide serve as splints which assist unique cleavages of RNA by RNase H at the complementary site of the 5′-end of the deoxy tract (Inoue et al., 1987a). The method was shown to be applicable to the cleavage in stem regions of a transcribed RNA (Shibahara et al., 1987). Atabekov et al. (1988) have reported an experiment that involved the use of a chimeric oligodeoxyribonucleotide with an internucleotide pyrophosphate bond in the site-specific cleavage of TMV RNA. In order to cleave the formylmethionine tRNA of *Escherichia coli* at

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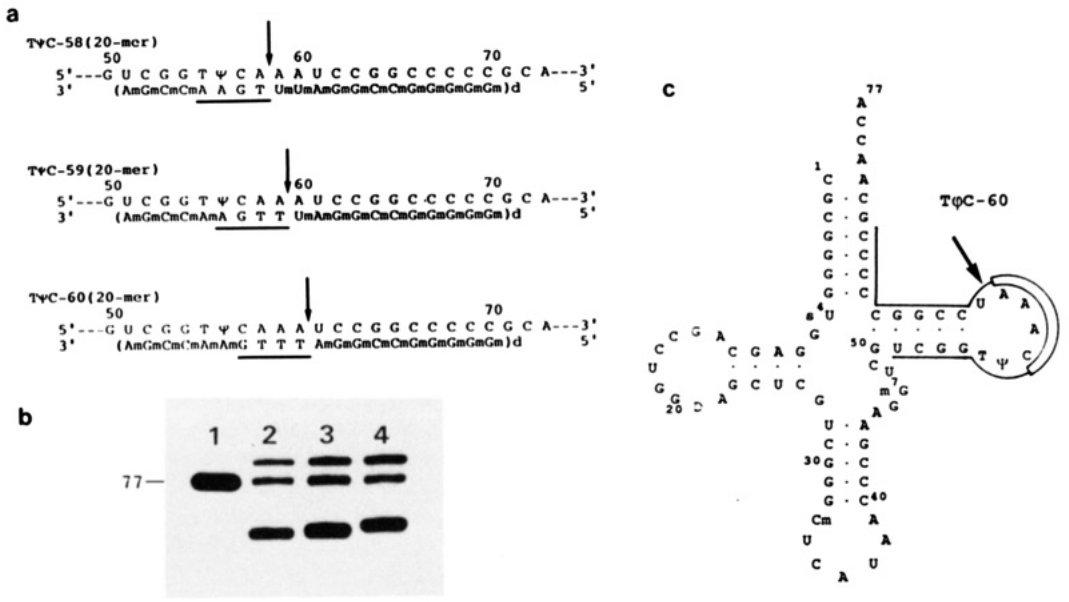


FIGURE 1: (a) Chimeric splints and complementary parts in $tRNA_f^{Met}$ for cleavage in positions 58, 59, and 60. 5′-Labeled $tRNA_f^{Met}$ (1 μM) was treated with RNase H (1 ng) by method 1. (b) Lane 1, no splint; lane 2, in the presence of TΨC-58; lane 3, in the presence of TΨC-59; lane 4, in the presence of TΨC-60. The slowest moving materials were identified as a hybridized complex with the tRNA and a chimeric splint (panel c). (c) Structure of a hybrid, $tRNA_f^{Met}$ and TΨC-60.

specific sites, the chimeric DNA–RNase H approach was employed in the present study. Chimeric DNAs containing 2′-*O*-methyl oligonucleotides (13-mers and 20-mers) were synthesized for this purpose. Methods for the dissection of tRNA in arbitrary positions are required for systematic modifications of tRNA. The present investigation provides a variety of information for application of this method to the field of RNA studies. The effects of the secondary structure of RNA and the presence of modified nucleosides in the RNase H reaction are discussed in this paper. Methods to overcome the effect of higher structure in the RNase H reaction are also described.

## Materials and Methods

*Enzymes.* *E. coli* RNase H was purchased from Takara Shuzo Co. Other enzymes for analysis were obtained as described previously (Koizumi et al., 1989).

*Oligonucleotides and tRNA.* 2′-*O*-Methyl oligonucleotides and chimeric oligodeoxyribonucleotides were synthesized on a DNA synthesizer (Applied Biosystems, Model 380A) with commercially available reagents and 3′-*O*-(methoxy *N*,*N*-diisopropylphosphoramidite) derivatives of 2′-*O*-methyl ribonucleosides (Shibahara et al., 1987), which were prepared from N-protected 2′-*O*-methyl nucleosides (Inoue et al., 1987b). Products were purified by reversed-phase and anion-exchange chromatography. Characterization of the products was performed as described (Inoue et al., 1987b).

*E. coli* $tRNA_f^{Met}$ was a gift from Dr. D. Söll of Yale University and was purified by 12% PAGE[1] in the presence of 7 M urea.

*5′-Labeled $tRNA_f^{Met}$.* $tRNA_f^{Met}$ (40 pmol) was dephosphorylated by bacterial alkaline phosphatase and treated with T4 polynucleotide kinase (10 units) in 10 μL of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol in the presence of 50 μCi of [γ-$^{32}$P]ATP (9 pmol) at 37 °C for 30 min. The mixture was desalted by passing it through NENSORB 20 (Du Pont) and purified by 12% PAGE containing 7 M urea. The product was precipitated by ethanol and mixed with unlabeled $tRNA_f^{Met}$ to give a 10 μM solution.

*3′-Labeled $tRNA_f^{Met}$.* $tRNA_f^{Met}$ (40 pmol) was ligated with 50 μCi of [5′-$^{32}$P]pCp in 10 mM $MgCl_2$, 10% DMSO, 3 mM DTT, 10 μg/mL BSA, and 15% glycerol in 20 μL by T4 RNA ligase (5 units) at 5 °C for 16 h (Uhlenbeck & England, 1978). The product was desalted and isolated by 12% PAGE containing 7 M urea as described above. A 10 μM solution was prepared.

*Cleavage with RNase H in the Presence of Chimeric Oligonucleotides.* Reactions with *E. coli* RNase H were performed in buffer A (40 mM Tris-HCl, pH 7.7, 4 mM $MgCl_2$, 1 mM DTT, 0.003% BSA, and 4% glycerol) or buffer B (same as buffer A but containing 5 mM NaCl instead of 4 mM $MgCl_2$). The loading solution for PAGE contains 10 M urea, 0.05% xylene cyanol, and 0.05% bromophenol blue.

*Method 1: Annealing in the Presence of $Mg^{2+}$.* A solution of tRNA and chimeric oligonucleotides was heated at 65 °C on a heating block for 10 min and left to cool to 30 °C in buffer A. Digestion was carried out at 30 °C for 1 h and stopped by addition of the loading solution.

*Method 2: Annealing without $Mg^{2+}$.* Annealing of a mixture of tRNA and chimeric oligonucleotides was performed as above except buffer B was used. Before the hydrolysis with RNase H, $MgCl_2$ was added to a final concentration of 4 mM.

*Analyses of Cleaved Products.* Aliquots of the reaction mixture were heated at 90 °C for 1 min and applied to 12% PAGE in 8 M urea (0.5 mm × 40 cm). Products of partial digestions with RNase $T_1$ or nuclease S1 were used as markers.

The 5′-termini of digestion products of 3′-labeled tRNA were analyzed by labeling the 5′-end with polynucleotide kinase and [γ-$^{32}$P]ATP after treatment with bacterial alkaline phosphatase. The labeled oligonucleotide was digested with nuclease P1 and applied to paper electrophoresis in 0.05 M triethylammonium bicarbonate using nucleoside 5′-phosphates as the markers.

The 3′-end analysis was performed by labeling the 3′-end with [5′-$^{32}$P]pCp and RNA ligase. The labeled product was

[1] Abbreviations: ACS, acceptor stem (ASC-7 refers to a chimeric splint designed for cleavage at position 7 of the tRNA); ANL, anticodon loop; ANS, anticodon stem; DIS, dihydro-U stem; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; TΨC, TΨC loop.

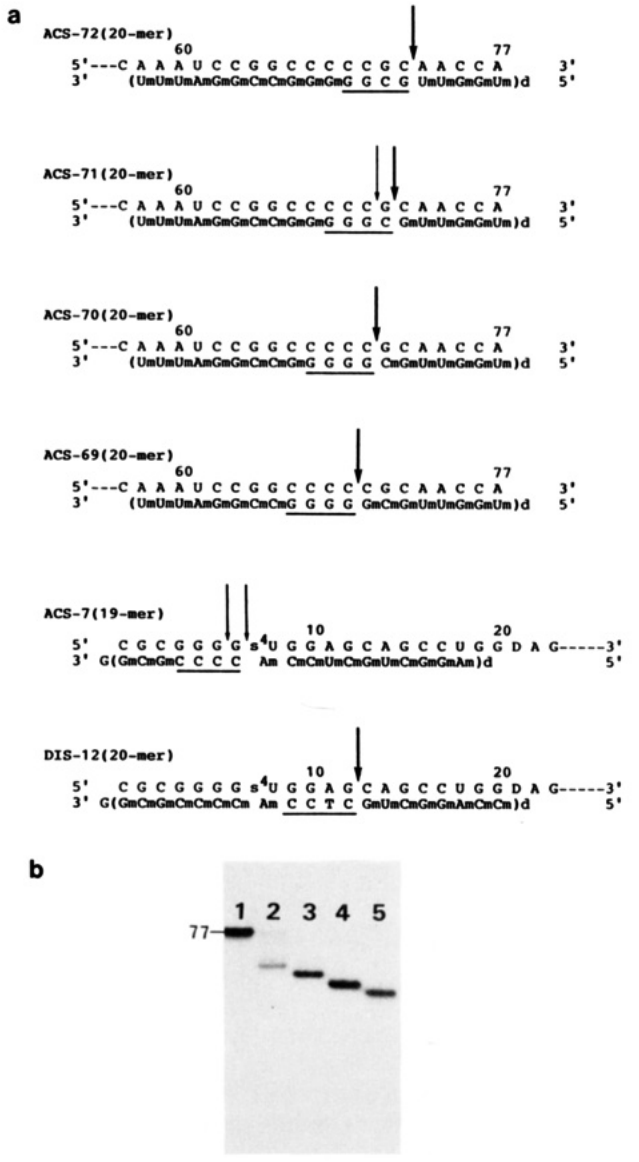


FIGURE 2: Cleavage of the acceptor and dihydro-U stems. (a) Cleavage sites and structures of chimeric splints. (b) 5′-Labeled $tRNA_f^{Met}$ (0.1 μM) and a splint (1 μM) were treated with RNase H under the same conditions given in Figure 1. Lane 1, no splint; lane 2, in the presence of ACS-72; lane 3, in the presence of ACS-71; lane 4, in the presence of ACS-70; lane 5, in the presence of ACS-69.

digested with RNase $T_2$ and analyzed by cellulose thin-layer chromatogrpahy (Avicell SF) using isobutyric acid–0.5 M ammonia (5:3 v/v) for the first dimension and 2-propanol–concentrated HCl–water (70:15:15 v/v) for the second dimension (Nishimura, 1972).

## RESULTS

*Site-Specific Cleavages in the TΨC Loop at Positions 58, 59, and 60: Formation of a Complex with a Splint.* The hydrolysis of $tRNA_f^{Met}$ with RNase H at position 58, 59, or 60 from the 5′-end was performed with a splint that was a complementary chimeric 20-mer consisting of a tetradeoxyribonucleotide and 2′-*O*-methyl oligonucleotides on both sides. The sequence of the splints and cleavage sites are shown in Figure 1a. The products were analyzed by PAGE (shown in Figure 1b) and found to have the expected lengths. The yield of cleavage was between 61 and 63%. The chimeric splints were designed for a specific hydrolysis at the 3′-end of RNA pairing with the DNA tract. Heteroduplexes consisting of tRNA and a chimeric 20-mer (e.g., Figure 1c) were observed in PAGE as a slower band (Figure 1b, lane 4). These

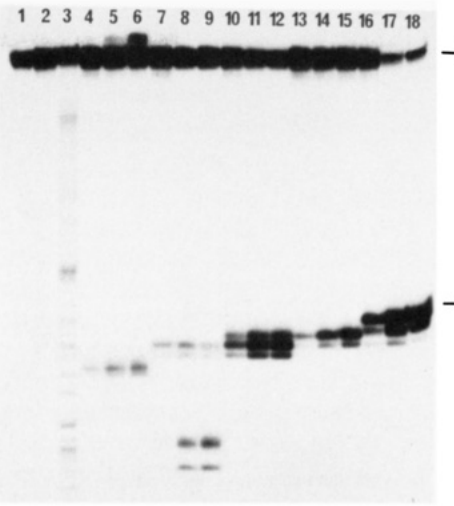


FIGURE 3: Autoradiogram (20% PAGE containing 8 M urea) of the products in hydrolysis of $tRNA_f^{Met}$ (5 pmol) by method 1 in a volume of 10 μL in the presence of a chimeric oligonucleotide 20-, 100-, and 500-fold excess. Lane 1, intact $tRNA_f^{Met}$; lane 2, plus nuclease S1; lane 3, alkaline ladder (Donis-Keller et al., 1977); lanes 4–6, in the presence of ANL-32; lanes 7–9, in the presence of ANL-34; lanes 10–12, in the presence of ANL-35; lanes 13–15, in the presence of ANL-36; lanes 16–18, in the presence of ANL-37.

slower moving materials were partially converted to the 77-mer by reapplication to PAGE. The cleaved products seemed to be denatured under the same conditions. Formation of heteroduplexes proved to be essential for hydrolysis by RNase H.

*Cleavage in the Acceptor Stem and D-Loop Regions.* The cleavage of the amino acid acceptor stem by this procedure also showed high specificities except at positions 7 and 71. Figure 2a indicates cleavage sites and the structure of hybridized splints and complementary parts of the $tRNA_f^{Met}$. Complete hydrolyses in the acceptor stem were obtained at positions 69, 70, and 72 specifically, as shown in Figure 2b. Also, in the D stem, the linkage in position 12 was cleaved efficiently in the presence of the splint.

With shorter splints such as d(UmCmGmUmCmGmG-mACCA) complementary to positions 11–21, the cleavage occurred at two sites (20 and 22) (data not shown).

*Cleavage in the Anticodon Loop and Stem.* Cleavage in the anticodon loop is an essential step for the replacement of anticodon bases or adjacent modified bases. When chimeric tridecamers were designed for cleavage in positions 32, 34, 35, 36, and 37, single hydrolyses were not obtained as shown in Figure 3. Although cleaved products, e.g., 5′-labeled 34-mer and 35-mer, could be isolated, the yield of the products was low, even in the presence of a large excess of splint, when the cleavage site was designed next to 2′-*O*-methylcytidine in the presence of ANL-32 (Figure 3, lanes 4–6). Hydrolysis of the 5′-phosphate ester of 33-Cm did not occur and ca. 2% of cleavage was observed at unexpected positions, 31-pG or 32-pG, to give the 30-mer or 31-mer. With a 13-mer, cleavage at position 37 occurred in a yield of 24%. These results were confirmed by using the 3′-labeled tRNA.

To improve the efficiency of cleavage, the tertiary structure of the tRNA was disrupted by hybridizing complementary 20-mers to the 3′-end. The hybridized structure of tRNA in the presence of the complementary 2′-*O*-methyl 20-mer (ACS-M) and the chimeric 13-mer (ANL-37, 13-mer) is shown in Figure 4a. Hydrolysis of the 5′-labeled tRNA occurred efficiently at position 37 as shown in Figure 4b (lane 4). When the chimeric 20-mer (ACS-69) was used to hybridize to the 3′-end, the 5′-labeled 37-mer was also detected

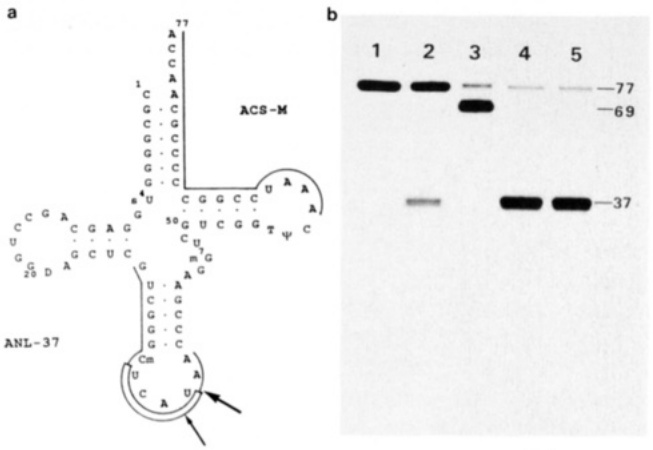


FIGURE 4: (a) A hybridized complex consisting of $tRNA_f^{Met}$ ANL-37 and a complementary 2′-*O*-methyl 20-mer (ACS-M) to the 3′-end. (b) Autoradiogram (20% PAGE containing 8 M urea) of the products in hydrolysis of $tRNA_f^{Met}$ (10 pmol) by method 1 in 10 μL. Lane 1, marker; lane 2, in the presence of ANL-37; lane 3, in the presence of ACS-69; lane 4, in the presence of ANL-37 plus the complementary 2′-*O*-methyl 20-mer; lane 5, in the presence of ANL-37 plus the chimeric 20-mer (ACS-69).

(Figure 4b, lane 5). These results may mean that hydrolysis of RNA with RNase H can be accelerated by disruption of tertiary structures of RNAs that hinder the hybridization with a complementary DNA.

This was further proved by experiments with a larger complementary oligonucleotide for the anticodon loop. A chimeric 20-mer [UmUmd(ATGA)GmCmCmCmGmAmCm] (ANL-37, 20-mer) was synthesized and used under two different conditions. When the splint was annealed without $Mg^{2+}$ ions (method 2) cleavage occurred efficiently. However, annealing in the presence of $Mg^{2+}$ ions before the enzyme reaction prohibited the reaction. With a shorter splint (13-mer), efficient hydrolysis was not obtained in either condition.

## Discussion

The formylmethionine tRNA of *E. coli* serves as the initiator in protein biosynthesis and is recognized by the formylation enzyme as well as its cognate aminoacyl-tRNA synthetase (Rich & RajBhandary, 1976). In order to investigate structural requirements for these recognitions, site-specific modifications of tRNA have been performed by replacement of a part of the molecule (Uemura et al., 1982; Ohtsuka et al., 1983; Schulman et al., 1983; Schulman & Peka, 1983; Doi et al., 1985; Seong & RajBhandary, 1987).

The site-specific cleavage of $tRNA_f^{Met}$ of *E. coli* by the present approach was shown to be feasible. Instead of using complementary DNA, shorter DNAs linked with 2′-*O*-methyl nucleotides were used for unique cleavages. It was known that, in the presence of these chimeric oligonucleotides, the 3′-side of RNA that is hybridized with the deoxynucleotide tract is cleaved most easily (Inoue et al., 1987a). When a DNA tract hybridized to a complementary RNA in a stem structure, RNase H seemed to act with the same efficiency as in a loop region. In comparison to reactions with other RNases, the present approach provides a more precise method to dissect tRNA. RNase A has been used to modify $tRNA_f^{Met}$ at the anticodon loop (Ohtsuka et al., 1983) and acceptor end (Uemura et al., 1982).

Limitations in this method were noted in the reaction at the D loop and anticodon loop when modified nucleosides were located near desired cleavage sites. The 2′-*O*-methyl group of cytidine seemed to hinder the hydrolysis of the 5′-phosphate ester. 4-Thiouridine in position 8 seemed to affect the cleavage of its 5′-phosphate to cause an extra hydrolysis in position 7. However, the TΨC sequence was recognized normally. These results indicate that the 2′-hydroxyl group in certain positions is recognized by the enzyme and that Watson–Crick-type hydrogen bonds may be required to be positioned in proximity to the substrate for hydrolysis. RNase H reactions with RNA–DNA duplexes containing modified bases either in the major or minor groove also suggested an interaction between the minor group of the heteroduplex with the enzyme (Y. Miura et al., manuscript in preparation).

Disruption of the tertiary structure of $tRNA_f^{Met}$ by hybridization of the complementary 20-mer at the acceptor stem area assisted in cleavage of the anticodon loop by RNase H in the presence of a chimeric 13-mer. The short splint (13-mer) was effective only with the assistance of the other complementary strand. However, when the chimeric 20-mer complementary to the anticodon area was hybridized by preincubation without $Mg^{2+}$ ions, two positions in the loop were hydrolyzed by RNase H. Although this method provides more controlled reactions than those by limited digestions with RNase A, anomalous hydrolyses with "chimera DNA–RNase H" seems to occur in the anticodon loop, presumably due to a characteristic structure of the loop.

The unique cleavage was performed most successfully at the acceptor stem and TΨC loop. The application of this technique to modifications at these regions by replacement of oligoribonucleotides seems to be particularly promising.

The present method is useful for the site-directed hydrolysis of tRNA and may be used for other RNAs when the sequence is available for designing the complementary chimeric oligonucleotides.

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# Comparative Proton NMR Analysis of Wild-Type Cytochrome *c* Peroxidase from Yeast, the Recombinant Enzyme from *Escherichia coli*, and an Asp-235 → Asn-235 Mutant†

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ABSTRACT: Proton NMR spectra of cytochrome *c* peroxidase (CcP) isolated from yeast (wild type) and two *Escherichia coli* expressed proteins, the parent expressed protein [CcP(MI)] and the site-directed mutant CcP(MI,D235N) (Asp-235 → Asn-235), have been examined. At neutral pH and in the presence of only potassium phosphate buffer and potassium nitrate, wild-type Ccp and CcP(MI) demonstrate nearly identical spectra corresponding to normal (i.e., "unaged") high-spin ferric peroxidase. In contrast, the mutant protein displays a spectrum characteristic of a low-spin form, probably a result of hydroxide ligation. Asp-235 is hydrogen-bonded to the proximal heme ligand, His-175. Changing Asp-235 to Asn results in alteration of the p*K* for formation of the basic form of CcP. Thus, changes in proximal side structure mediate the chemistry of the distal ligand binding site. All three proteins bind $F^-$, $N_3^-$, and $CN^-$ ions, although the affinity of the mutant protein (D235N) for fluoride ion appears to be much higher than that of the other two proteins. Analysis of proton NMR spectra of the cyanide ligated forms leads to the conclusion that the mutant protein (D235N) possesses a more neutral proximal histidine imidazole ring than does either wild-type CcP or CcP(MI). It confirms that an important feature of the cytochrome *c* peroxidase structure is at least partial, and probably full, imidazolate character for the proximal histidine (His-175).

Yeast cytochrome *c* peroxidase (CcP; EC 1.11.1.5) is the most thoroughly characterized of the heme peroxidases. It is a medium-sized ($M_r$ = 34K) ferriheme enzyme that is paramagnetic in its high-spin (resting state, native) and low-spin (ligated) forms. This heme-centered paramagnetism acts as an intrinsic shift and relaxation agent that facilitates proton NMR studies of the heme and its neighboring amino acids in the enzyme's active site (La Mar, 1979; Satterlee, 1986).

Elucidating specific structural features of CcP that imbue it with its characteristic chemistry and differentiate it from other classes of heme proteins has long been a goal (Finzel et al., 1984). On the basis of the refined wild-type CcP structure (Finzel et al., 1984), it was proposed that a hydrogen bond between His-175 $N_\delta$ (the proximal histidine bonded to heme iron) and Asp-235 occurs (Figure 1A). Circumstantial support for this type of proximal effect comes from experimental evidence involving other heme proteins (Peisach, 1975), horseradish peroxidase (HRP; de Ropp et al., 1985), and heme models (Traylor & Popvitz-Biro, 1988). The results from horseradish peroxidase are especially pertinent because, like CcP, HRP has oxidized intermediates that are low-spin ferryl species. The NMR results for HRP (de Ropp et al., 1985) clearly showed that full deprotonation of the proximal histidine (Figure 1C) occurs for low-spin, cyanide-ligated HRP (HRP-CN). Those results imply similar proximal imidazolate

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