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^{15}N and ^{1}H NMR Studies of *Rhodospirillum rubrum* Cytochrome c_2[†]

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Received September 17, 1987; Revised Manuscript Received November 17, 1987

ABSTRACT: ^{15}N-Enriched cytochrome c_2 was purified from *Rhodospirillum rubrum* that had been grown on $^{15}NH_4Cl$, and the diamagnetic iron(II) form of the cytochrome was studied by ^{15}N and ^{1}H NMR spectroscopy. ^{15}N resonances of the four pyrrole nitrogens, the ligand histidine nitrogens, the highly conserved tryptophan indole nitrogen, and some proline nitrogens are assigned. The resonances of the single nonligand histidine are observed only at low pH because of severe broadening produced by proton tautomerization. The resonances of exchangeable protons bonded to the nitrogens of the ligand histidine, the tryptophan, and some amide groups are also assigned. The exchange rates of the nitrogen-bound protons vary greatly: most have half-lives of less than minutes, the indolic NH of Trp-62 exchanges with a half-time of weeks, and the ligand histidine NH proton exchanges with a half-time of months. The latter observation is indicative of extreme exclusion of solvent from the area surrounding the ligand histidine and lends credence to theories implicating the degree of hydrophobicity in this region as an important factor in adjusting the midpoint potential. The dependence of the ^{15}N and ^{1}H NMR spectra of ferrocytochrome c_2 on pH indicates neither the Trp-62 nor the ligand His side chains become deprotonated to any appreciable extent below pH 9.5. The His-18 NH remains hydrogen bonded, presumably to the Pro-19 carboxyl group, throughout the pH titrations. Because neither deprotonated nor non-hydrogen-bonded forms of His-18 are observed in spectra of the ferrocytochrome, the participation of such forms in producing a heterogeneous population having different g tensor values seems unlikely. A single ionization, occurring with a pK of 6.8, causes the resonances of the pyrrole nitrogens and several groups near the heme to shift. This ionization, attributed to the protonation/deprotonation of His-42 or a His-42–heme propionate hydrogen-bonded pair, appears to affect a wide range of groups near the heme, perhaps by altering the packing of the Ω loops which cover that region of the protein.

The cytochromes c_2 are a class of electron-transport proteins found in the purple non-sulfur bacteria. The importance of their function in photosynthetic electron transport and their structural homology to the mitochondrial cytochrome c have made them the subject of a great deal of study in recent years. Many structural and functional parameters have been measured, and equally many attempts have been made to relate structure to function.

A property of obvious importance to the cytochromes is the midpoint oxidation–reduction (redox) potential of the heme iron. The character of the heme groups of the cytochromes is determined both by the *ligands* provided by the protein and by the *environment* created by the folded peptide chains. Several theories concerning the control and diversity of the midpoint potentials of the cytochromes have been proposed.

[†] This research was supported by NIH Grant GM-34194.
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0006-2960/88/0427-1949$01.50/0

A variation in the hydrophobicity of the protein near the heme was one of the earliest proposed explanations for the differences in the midpoint potentials of different cytochromes (Kassner, 1972, 1973; Schlauder & Kassner, 1979). Hydrogen bonding of the unligated nitrogen of the ligand histidine to a peptide carboxyl group has also been suggested to modulate the potential by providing differential stabilization of imidazole–iron coordination in the two oxidation states (Valentine et al., 1979). Different populations of cytochrome, some of which have protonated and some deprotonated ligand histidines, have been proposed on the basis of electron paramagnetic resonance (EPR) studies of several ferricytochromes and model compounds at different pH (Brautigan et al., 1977). These forms having different protonation states would clearly exhibit different properties and would be expected to be affected by the pH of the medium. The pH dependence of the physical and chemical properties of the cytochromes has been extensively studied by ^{1}H NMR spectra (Smith & Kamen, 1974; Smith, 1979) and by the redox potential (Pettigrew et al., 1976, 1978). Several ionizations have been observed with pK_a values of 6.2, 7.0, and 8.4 (Smith, 1979; Pettigrew et al., 1978). However, the origins of these ionizations and their effects on conformation and redox potentials are poorly understood. The difficulties in assigning these ionizations to particular groups arise most probably because most of the experimental methods employed are indirect and the properties that have been monitored vary as a secondary result of the ionizations; the ionizing groups themselves are not observed.

Many of these difficulties could be overcome and much knowledge could be acquired by ^{15}N NMR studies of the ^{15}N-enriched cytochromes because ^{15}N chemical shifts are sensitive to changes in protonation/deprotonation, hydrogen bonding, and metal ligation as well as to changes in the local environment. In the present paper, we report ^{15}N and ^{1}H NMR spectra for the reduced state of the cytochrome c_2 from *Rhodospirillum rubrum* as a function of pH and solvent composition. Many of the interesting nitrogen atoms are not protonated (ligands, proline nitrogens), or their protonation state is uncertain and perhaps even changes with conditions. Other nitrogen atoms, though protonated, do not exhibit J coupling because of proton exchange (Smith et al., 1987). Two-bond coupling constants between protons and nitrogen are small. Therefore, we have employed direct observation of ^{15}N rather than the proton-detected X-nucleus observation methods that are now available. Enrichment in ^{15}N enhances the sensitivity of the experiment and aids in the assignment of ^{15}N and ^{1}H resonances [e.g., see Richards and LeMaster (1985)]. ^{15}N chemical shifts give essential information regarding hydrogen bonding as well as protonation state and conformational changes, all of which have been proposed to play roles in pH-induced changes in properties of the cytochromes *c*.

The studies of the oxidized state of cytochrome c_2 by ^{15}N NMR and the functions of the Lys residues in both oxidation states are in progress.

Experimental Procedures

Materials. $^{15}NH_4Cl$ was obtained from MSD Isotopes. The G-9 (blue-green) mutant of *R. rubrum* was kindly provided by P. F. Weaver.

Growth of Rhodospirillum rubrum. A facultative photoheterotroph, *R. rubrum* can be grown photosynthetically under anaerobic conditions or fermentatively in air (Weaver, 1974). The growth media contained malate (73.3 mM), phosphate (9.6 mM), $^{15}NH_4Cl$ (7.5 mM), biotin (0.02 mg/mL), and minerals, pH 7.2. Yeast extract or other nitrogen-containing supplements are not required and were not added to ^{15}N-enriched cultures to avoid dilution of the label. At this level of $^{15}NH_4Cl$, the growth of the bacteria was found to be limited by the nitrogen source rather than by light penetration, ensuring maximum use of the ^{15}N-labeled material. Immediately after inoculation of the culture, the bacteria were allowed to grow fermentatively (in the dark) overnight to generate their own anaerobic environment. Then the cultures were grown in the light.

For cultures grown at natural abundance of ^{15}N, NH_4Cl was raised to 22.5 mM, and 2 g/15 L of yeast extract (Difco) was included to improve the yield of cells.

Both ^{15}N-enriched and unlabeled cytochromes c_2 were purified from *R. rubrum* according to the method described by Bartsch (1971) with minor modification (Smith, 1979). The purity of the protein obtained was ≥99% as determined on polyacrylamide gel electrophoresis using 8% gels. We estimate the isotopic enrichment to be at least 90%.

Sample Preparation. The cytochrome c_2 was desalted by passage over Sephadex G-25 equilibrated with doubly deionized water and concentrated by lyophilization. The protein was dissolved in either 99.98% ^2H_2O or 15% ^2H_2O in H_2O, depending on the experiments to be carried out. The final cytochrome c_2 concentrations were about 3.5 mM. The pH of the samples was measured directly in the NMR tube. The pH was adjusted by using 0.1 N NaOH or 0.1 N HCl for samples in H_2O, or 0.1 N NaOD or 0.1 N ^{2}HCl for samples in ^2H_2O. The values reported are uncorrected pH meter readings. To adjust the oxidation states of the protein, sodium hydrosulfite was added as a reducing agent.

NMR Spectra. The ^{15}N NMR spectra were obtained with GE NM-500 spectrometer at 50.7 MHz with a ^{15}N probe provided by Cryomagnet Systems, Inc. Chemical shifts are referenced to 1 N HNO_3 in ^2H_2O by taking the resonance of $^{15}NH_4Cl$ in H_2O to be 354.5 ppm. Sample temperature was usually controlled at 25 °C. The ^{1}H NMR spectra of the samples in H_2O were collected at 500 MHz on the same spectrometer equipped with a GE fixed-tuned probe, using either the 214 composite soft pulse (Redfield et al., 1975) or the 1331 (Hore, 1983) water suppression pulse sequences. The chemical shifts for ^{1}H were measured from sodium 2,2-dimethyl-2-silapentanesulfonate as reference. The frequencies used for ^{15}N decoupling during observation of protons were obtained through a minor modification of the NM-500 spectrometer. Other parameters are listed in the figure legends.

Results

^{15}N NMR Spectra of Cytochrome c_2. Typical 50.7-MHz ^{15}N NMR spectra of the protein at several pH values are shown in Figure 1. Three groupings of resonances are clearly observed: one group includes His and heme nitrogen resonances (120–210 ppm); a second group includes peptide nitrogens and resonances of the side chain nitrogens of Trp, Gln, and Asn (230–270 ppm); and the third group includes the N-terminus and Lys side chain nitrogen resonances (330–350 ppm). There is no arginine in this protein; otherwise, guanidino nitrogens would be visible as a fourth group of resonances (285–310 ppm). Differences in pH and in pulse repetition rate are responsible for minor differences between these spectra and those shown in a previous publication (Smith et al., 1987).

The molecular weight of this protein is 12.8 K (Bartsch, 1971). For this small protein, many individual resonances except in the peptide region are well resolved and relatively sharp. This clearly demonstrates the potential power of ^{15}N NMR studies of interactions involving His, Lys, Arg, N-terminal residues, and nitrogen-containing cofactors whose res-

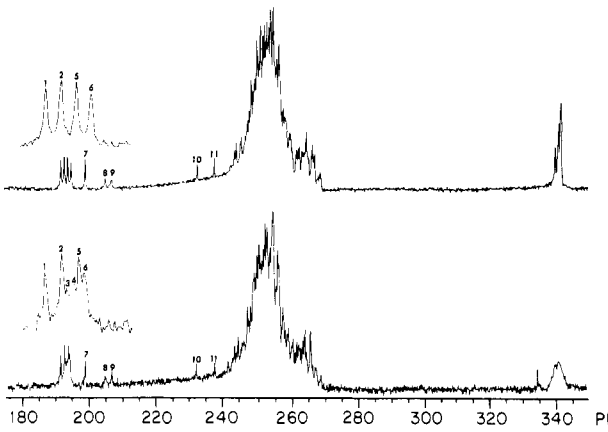


FIGURE 1: ^{15}N NMR spectra (50.7 MHz) of ^{15}N-enriched cytochrome c_2 in H_2O. The bottom trace represents about 6000 scans with a recycle time of 0.5 s; the pH of the sample was 4.9. The top trace represents about 9000 scans with a recycle time of 0.8 s with the sample at pH 8.8. Protons were not irradiated. The expanded regions between 190 and 197 ppm show the multiple peaks. The numbered resonances are referred to in the text.

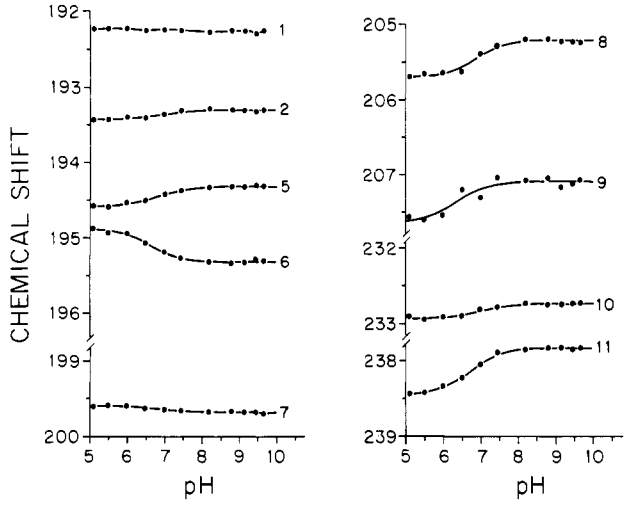


FIGURE 2: Dependence on pH of ^{15}N chemical shifts of the resonances numbered in Figure 1. The dots represent the experimental data. The solid lines represent theoretical curves obtained with the Hill coefficient set at 1.

onances are likely to be resolved. Their chemical shift changes are therefore relatively easy to follow.

Effect of pH on the Spectra of Cytochrome c_2. The ^{15}N-enriched cytochrome c_2 was studied in solutions of different pH. At low pH (between 4.8 and 5.5), there are 11 resolved ^{15}N resonances in the low-field region. However, when the pH of the solution is raised above 6.0, there are only nine resolved ^{15}N resonances observed in the low-field region (Figure 1). Some peaks exhibit qualitatively normal titration behavior. The nonlinear least-squares fit to the Hill equation [e.g., see Markley (1975)] of the peaks numbered 1–11 is shown in Figure 2. Peaks 3 and 4 are only observed at low pH. Peak 1 stays relatively constant with a variation of 0.06 ppm when the pH of sample changes from 5.1 to 9.6. Peaks 2 and 7 are also relatively constant, moving downfield by 0.13 ppm and upfield by 0.08 ppm, respectively. Peak 5 moves downfield by 0.27 ppm. Peak 6 moves upfield by 0.43 ppm. Peaks 8 and 9 move downfield by 0.50 ppm. Peak 10 moves downfield by 0.20 ppm. Peak 11 moves downfield by 0.63 ppm. The pK_a's observed for these resonances are indistinguishable from one another at about 6.8.

Figure 3 shows the ^{1}H chemical shift variations with pH in the low-field region for unlabeled cytochrome c_2. Their

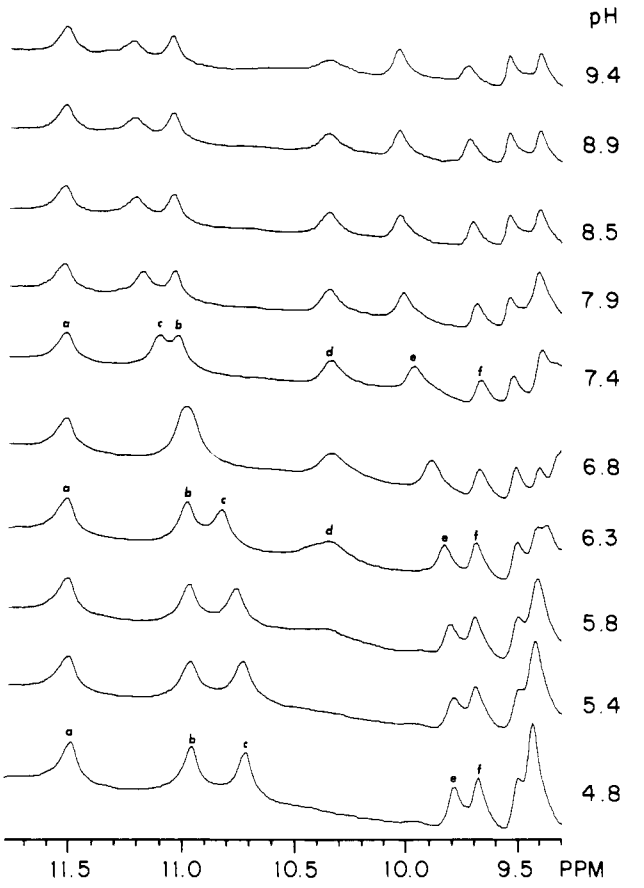


FIGURE 3: ^{1}H NMR spectra in H_2O of a pH titration of cytochrome c_2 at natural abundance of ^{15}N. The sample pH is indicated along each spectrum. The resonances designated a–f are referred to in the text. The spectra were collected by using the 214 sequence with a recycle time of 1 s. Each spectrum represents 1024 scans.

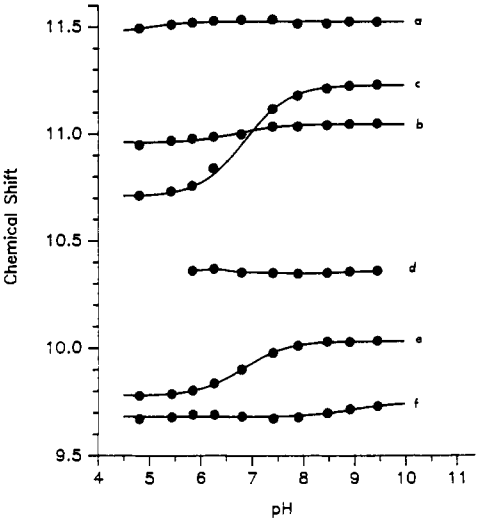


FIGURE 4: Dependence on pH of ^{1}H chemical shifts of resonances a–f (indicated in Figure 3). Conditions were described in Figure 2.

titration curves are shown in Figure 4. Six peaks labeled a–f in the low-field region (Figure 3) are observed. These resonances arise from slowly exchanging NH or OH protons. Peak a stays almost constant. Peak b moves downfield by 0.09 ppm and peak c by 0.52 ppm. Peak d stays almost constant, but it is not observed at low pH, and its peak height is reduced at high pH, suggesting both acid- and base-catalyzed exchange. Peak e moves downfield by 0.26 ppm. Peak f moves by 0.07 ppm. The peaks exhibiting the largest change in chemical shift were peaks c and e, both of which clearly exhibit a pK_a of 6.8.

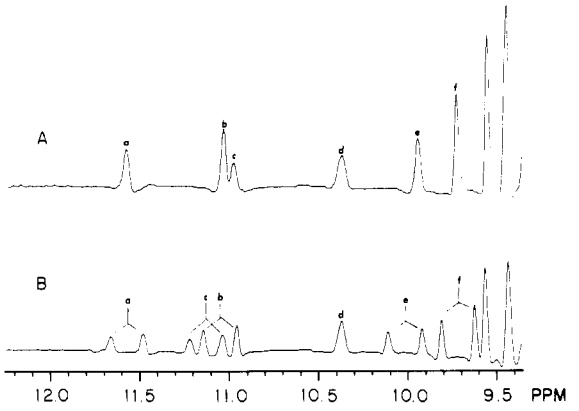


FIGURE 5: Comparison of ^1H NMR spectra (low-field region) of cytochrome c_2 at natural abundance of ^{15}N (trace A) with that of ^{15}N-enriched cytochrome c_2 (trace B) in H_2O. The pH of the sample was 6.82 for trace A and 7.58 for trace B. These spectra were treated with trapezoidal multiplication before transformation to improve the base line. The spectra were collected with a recycle time of 1 s. Each spectrum represents 1024 scans.

In order to identify these resonances, ^1H spectra have also been taken of ^{15}N-enriched cytochrome c_2. Comparison of ^1H spectra of a ^{15}N-enriched sample with that of a sample at natural abundance is shown in Figure 5. This comparison clearly indicates that resonances a, b, c, e, and f are NH proton resonances with exchange rates less than $\sim 0.35J = 35\ s^{-1}$ as evidenced by their appearance as doublets of $^1J_{NH} \sim 95$ Hz. However, resonance d is either a slowly exchanging OH proton or a NH proton with an exchange rate between 35 and 1000 Hz because it is a singlet and has not merged with the solvent water resonance.

Among the six slowly exchangeable protons in the low-field region, the exchange rates are found to be markedly different. In comparing spectra taken in ^2H_2O to those in Figure 5, it was found that that protons a, b, d, and f completely exchanged with deuterium but protons c and e were still visible as doublets in the ^{15}N-enriched sample. Storage of the sample at refrigerator temperature for 2 weeks caused the peak height of proton c to be reduced, but it was still seen clearly. Proton c was found to be essentially completely exchanged after a month's storage. In contrast, proton e was still present. Therefore, protons a, b, d, and f have half-life times ($t_{1/2}$) for exchange of hours or less, proton c has a $t_{1/2}$ of a few weeks, and proton e has a $t_{1/2}$ of months in the native protein at about 4 °C.

Spin Decoupling Experiments. Because of the rather small proton–nitrogen coupling (which was often diminished by exchange) and the relatively short nitrogen T_2, two-dimensional J-correlation experiments were found to be less sensitive than expected. However, since relatively few nitrogen resonances were of interest in this study, ^{15}N resonances and ^1H resonances could be correlated by conventional spin decoupling experiments. It was found that proton peaks a, b, c, e, and f correspond to ^{15}N resonances at 244.5, 251.6, 246.6, 206.1, and 252.8 ppm, respectively, for the sample at pH 8.8. When proton peak d is irradiated, the ^{15}N difference spectrum is essentially zero except that the irradiation of the proton peak d causes some perturbation of nearby proton resonances b and e which exerts some secondary effects on the ^{15}N difference spectra. In addition, the NOE experiments show that there is no significant effect on ^{15}N spectra when proton d is irradiated. These negative results, though not conclusive, suggest that proton d is a hydroxyl proton resonance, rather than an NH proton resonance. It was unexpected that among these low-field NH proton resonances, only one proton (peak e) is coupled to a nitrogen atom in the histidine region of the ^{15}N spectrum, which indicates that only one His NH proton is observed.

^{15}N-Decoupled ^1H NMR spectra have also been acquired. When the ^{15}N chemical shift at 206.52 ppm was irradiated between the doublet 8 and 9 with the sample at pH 6.2, decoupling was observed in the ^1H spectrum at proton e. Similarly, the ^{15}N resonance at 248.9 ppm was irradiated, proton c was found to be decoupled. All experiments confirm the results obtained from the selective ^1H-decoupled ^{15}N observation experiments.

Discussion

Assignment of His and Heme Resonances. There are two histidine residues in this protein. His-18 is ligated to the heme iron, and His-42 is located near the surface of the protein (Figure 6). As we have shown above, ^{15}N peaks 8 and 9 are a nitrogen resonance split by a proton with a coupling constant

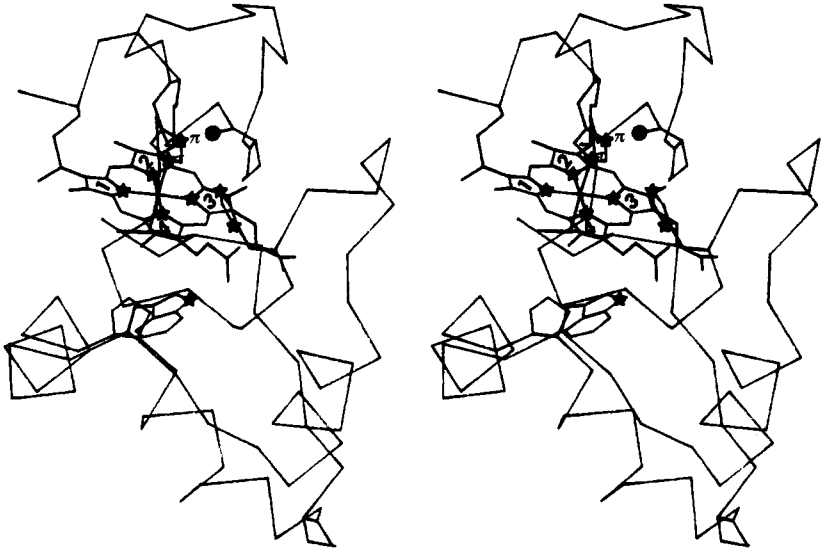
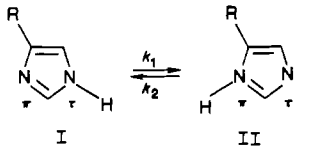


FIGURE 6: Stereo projection of the heme region and residues 13–93 of reduced cytochrome c_2. Side chains of Cys-14, Cys-17, ligand His-18, Pro-30, His-42, Trp-62, Pro-74, Pro-85, and ligand Met-91 are shown. The nitrogen atoms in the heme and in the side chains of ligand His-18, His-42, and Trp-62 are labeled with stars. The CO oxygen atom of Pro-30 is labeled with a black dot. The numbering for the four pyrrole rings is indicated. The four Ω loops (residues 18–34, 30–44, 41–57, and 74–90) are clearly shown.

Scheme I



I II

of 95 Hz. For a "normal" histidine residue in a protein, one of the two nitrogen atoms exhibits a large chemical shift ($\Delta\delta$) of about 65 ppm; the other has a $\Delta\delta$ of about 20 ppm during the course of the pH titration (Bachovchin & Roberts, 1978; Roberts et al., 1982). However, the chemical shift of the NH nitrogen of the His in our spectra changes very little ($\Delta\delta$ 0.5 ppm) when the pH of the sample changes from 5.1 to 9.6 (Figure 2). Therefore, this N resonance is assigned to the ligand His NH (πN) nitrogen, and the proton resonance e is then assigned to the ligand His NH proton.

Considering the similar but not precisely equivalent environments of the four nitrogen atoms in the heme ring, their ^{15}N resonances are found together, and the variations in their chemical shifts with pH should be small, since there is no mechanism to provide such a shift. Resonances 1, 2, 5, and 6 (Figures 1 and 2) are thus assigned to the four heme ring nitrogen atoms. These resonances are severely broadened and shifted downfield in the spectrum of the ferricytochrome (not shown). The chemical shifts of resonances 1, 2, 5, and 6 are similar to those observed in model compounds (Ozaki et al., 1977; Kawano et al., 1977). The resolved resonance remaining unassigned in the histidine chemical shift region is peak 7, a sharp singlet which experiences very little chemical shift change ($\Delta\delta$ 0.08 ppm) throughout the pH titration course. Therefore, it is assigned to the ligand His nitrogen atom (τN) which is coordinated to the heme iron atom.

Resonances 3 and 4 are resolved and observable at low pH, but are not observed at neutral to high pH. These two resonances then must be from the imidazole group of His-42. The difficulty in resolving His imidazole nitrogen resonances has also been encountered in the study of the catalytic triad of α-lytic protease by Bachovchin and Roberts (1978). However, their situation is quite different from ours. They were able to observe at least one (the hydrogen-bonded πN) resonance during the course of the titration. In our situation, we were unable to observe either of these two nitrogen resonances at neutral or high pH. The possibility that bound paramagnetic ions might be responsible for our failure to observe these resonances was eliminated by the use of buffers treated with Chelex-100 and the inclusion of ethylenediaminetetraacetic acid (EDTA) in the NMR tube. The paramagnetic metal ions in our Chelex-treated deionized water were found to be essentially zero (2.6 ppb of iron, 5.0 ppb of copper) by atomic absorption analysis. Chemical exchange of the proton from a *single* tautomer would not cause exchange broadening of *both* nitrogen resonances. Therefore, it is concluded that this kind of behavior must be due to tautomerization of the imidazole group of the His residue 42 at an intermediate rate. The process was not brought into the fast- or slow-exchange regime by increasing the temperature to 45 °C or lowering it to 6 °C, respectively. In free imidazoles in aqueous solutions, the tautomerization occurs but is fast and probably mediated by exchange with solvent.

In the following discussion, the form of histidine in which the proton tautomer has only the τ nitrogen (also called Nϵ_2) protonated is termed "state I", and that having only the π nitrogen (Nδ_1) protonated is referred to as "state II" (see Scheme I). The nomenclature employed by Bachovchin (1986) in which the uncharged ring N–H is called type α (resonating at ~210 ppm), the four-bonded N^+–H type α^+ (201 ppm), and an uncharged, unprotonated ring nitrogen type β (128 ppm) is also useful because observed chemical shifts are more characteristic of these classifications than of whether the nitrogen is at the τ- or π-position. Whether or not resonances of α^+-type nitrogens can be observed depends upon the rate of exchange with solvent protons, which depends on the accessibility to solvent as well as other factors. Whether or not type α and β resonances can be observed depends on the rate constants k_1 and k_2 of the tautomerization. Even in buried histidine side chains, this process may occur at an appreciable rate, and if the local environment is not too hydrophobic, the activation energy (i.e., temperature dependence) may be low. If the rate constants are very large compared to the chemical shift difference, number-averaged chemical shifts would be observed as is normally the case for protons. If the rate constants are very small or zero, then the individual nitrogen resonances in states I and II would be observed. However, if the rate constants are comparable to the chemical shift difference (~$0.35\Delta\delta$ = 1400 Hz at 50.7 MHz), no ^{15}N resonances may be observed because tautomerization broadening can be extreme. In the case of ^{15}N NMR, because of the greater chemical shift dispersion compared to protons, exchange must be faster to qualify as "fast exchange".

From this and other recent ^{15}N NMR studies of proteins, it appears that there are three classes of histidine residues in proteins. One class has zero or very slow tautomerization and is exemplified by the ligand His residues of cytochromes, the His residues involved in catalytic triads of serine protease (Bachovchin & Roberts, 1978; Bachovchin, 1986), and several His residues of *R. rubrum* ribulose-1,5-bisphosphate carboxylase (Domingues, 1987). The His residues in this class are usually involved in metal coordinations and/or very tight hydrogen bonding so that only one tautomer predominates and may be found in catalytic or functional sites. The chemical shifts should be about 130 ppm for β-type nitrogens and about 210 ppm for α-type nitrogens. However, the hydrogen bonding would modify the chemical shifts by up to 10 ppm. Members of the second class have intermediate rates of tautomerization. The His residues in this class seem to be located near the surface of the protein, where the environment is sufficiently polar to facilitate the transfer of the proton. Histidine residue 42 in this protein serves as an example of this class. The C2H proton resonance of this side chain was tentatively assigned to a pH-dependent resonance near 8.3 ppm (Smith, 1979). Members of the third class exhibit rapid tautomerization. The His residues in this class would certainly include those found on the surface of proteins. The chemical shifts are expected to be between 140 and 200 ppm at high pH, depending on the proportion of the two tautomers.

Assignment of Tryptophan Resonances. NOE and homodecoupling experiments have been carried out on the ^{1}H resonances in the low-field region of the ^{1}H spectra (Figure 3). Resonance c was found to be linked to a resonance at 7.7 ppm by an NOE effect at pH 5.0. Furthermore, the same resonance has been shown to be decoupled when the proton resonance c is irradiated during acquisition. Either histidine or tryptophan might exhibit an aromatic proton resonance and a low-field exchangeable NH proton resonance, but since the resonances of both histidine rings have been accounted for, the only reasonable candidate is the indole ring nitrogen of Trp-62. Therefore, the proton resonance c is assigned to the indole ring NH proton, and the resonance at about 7.7 ppm is assigned to the C2 proton of the indole ring. The resonance of the indole ring nitrogen atom of Trp residue 62 is found

to be at 246.6 ppm at pH 8.8 and at 249.5 ppm at pH 6.0 by the ^{1}H-decoupled ^{15}N observation experiments. Therefore, the indole ring nitrogen resonance, like the indole ring NH proton resonance c (Figures 3 and 4), undergoes a large downfield shift of about 3 ppm during the course of a pH titration.

Indole-containing model compounds were also studied by ^{15}N NMR for comparison to the protein. The ^{15}N resonance of indole in 90% ethanol/10% ^2H_2O (a hydrogen-bonding solvent) is at 246.4 ppm and in C^2HCl_3 is at 249.6 ppm, and is split by one-bond proton coupling of 98 Hz. The ^{15}N resonance of tryptamine in 90% ethanol/10% ^2H_2O, pH 9.7, is 246.0 ppm. The ^{15}N resonance of tryptophol in C^2HCl_3 is at 251.6 ppm and in 90% ethanol/10% ^2H_2O at 247.9 ppm. It can be seen from these studies that the indole ring nitrogen usually resonates at about 247 ppm in hydrogen-bonding solvents but at about 251 ppm in non-hydrogen-bonding solvents and that the substitution on the 3-position of the indole ring has a small effect on the chemical shift.

The X-ray crystallographic data indicate that Trp-62 is buried inside the protein, near one propionic acid side chain of the heme (Figure 6). Our model studies are consistent with the presence of a hydrogen bond to the carboxyl moiety of the propionate. The indolic NH proton of Trp-62 (resonance c) exchanges very slowly, as will be discussed below.

Assignment of Proline Resonances. The resonances of proline residues are unique among peptide nitrogen resonances in that they engage in imide instead of amide bonding. They are singlets and usually have ^{15}N chemical shifts at the low-field edge of the peptide nitrogen resonance envelope, about 230–245 ppm (Witanowski et al., 1981). At present, we tentatively assign the nitrogen resonance 11 (about 238 ppm) to Pro residue 30 and the nitrogen resonance 10 (about 233 ppm) to either Pro residue 74 or Pro residue 85. Model peptide studies and NOE measurements are in progress to confirm these assignments.

Ionization, Hydrogen Bonding, and Conformational Changes. The results from the chemical shift variations of both ^{15}N resonances and ^{1}H resonances with pH (Figures 1–4) clearly demonstrate that there is a transition which has a unique pK_a of 6.8. A similar pK_a of 7.0 has also been observed from the pH-dependent redox measurement and assigned to the reduced state of the cytochrome (Pettigrew et al., 1978). The possible origins for this pK_a are thiol SH groups, histidine residue side chains, carboxyl groups of extraordinarily high pK_a, or lysine side chains with extremely low pK_a's. In this protein, the only two Cys residues, 14 and 17, are involved in thioether linkages to the heme. There are only two histidine residues, 18 and 42. His residue 18 is ligated to the heme iron; its single πNH proton is present at all times throughout the pH titration, demonstrated clearly by the fact that both the proton resonance and its splitting of the ^{15}N resonance are invariant. The ^{15}N resonances of histidine-42 are not observed in the neutral to high pH range. The NH_2 group of the N-terminal glutamate residue is on the surface of the protein and has a typical pK_a value of 8.4 (not shown). The NH_2 groups of lysine side chains are all on the surface of the protein and have their pK_a's above 10 (not shown). There are 18 acidic groups including 9 Glu and 6 Asp side chains, 1 carboxyl-terminal COOH group, and two heme propionic acid groups. Among these COOH groups, only one propionic acid is completely buried inside the protein; the other propionic acid group is in contact with the protein surface. All the other COOH groups are on the surface of the protein and are expected to have the normal pK_a of about 4.5.

On the basis of the fact that the Trp side chain NH proton has the largest chemical shift variation with pH (Figures 3 and 4) and that the indole ring nitrogen of Trp residue 62 is close to the deeply buried propionic acid group (2.97 Å) which in turn is close to the His-42 imidazole nitrogen (2.16 Å) but on the other side of the Trp residue (Figure 6), it is proposed that the ionization with a pK_a of 6.8 arises from the His residue 42 imidazole group. The difficulty in observing the ^{15}N resonances of this His residue imidazole group at neutral to high pH makes the verification of its pK_a difficult. This imidazole group likely forms a salt linkage at low to neutral pH with the nearby propionic COO^- group. Leitch et al. (1984) attribute an ionization with a similar pK_a in several cytochromes c_{551} to the propionic acid side chain itself. The presumed hydrophobic environment could destabilize the deprotonated form of the propionic acid and the protonated form of the imidazole so that the carboxylic acid is not very acidic and the imidazole is not very basic. However, in the absence of chemical modification evidence of the kind presented for cytochromes c_{551}, we prefer to propose that the carboxylate and imidazolium exist as an ion pair or hydrogen-bonded pair with no net charge and with each moiety having a relatively normal pK_a. The histidine of the catalytic triad of serine proteases is found to have a normal pK_a (Bachovchin & Roberts, 1978). The proton that is lost with the pK_a of 6.8 would formally belong to His-42, although it might be the proton shared in the hydrogen bond. If there is a hydrogen bond between this imidazole and the propionic COO^-, then the bonding must be relatively weak as evidenced by the following: (1) The imidazole NH proton is not observed during pH titration experiments, indicating fast exchange of the NH proton with water or moderate exchange into an environment with a distant chemical shift (including the other nitrogen atom of the same imidazole). The exchange rate would have to be more than 1500 s^{-1} based on the chemical shift difference between water and His imidazole NH protons ($\Delta\delta$ about 9 ppm). (2) The His imidazole nitrogen resonances are not observed due to the tautomerization, the rate of which is restricted by some mechanism, of which local hydrophobicity and hydrogen bonding are examples. Therefore, it is concluded that the effect of the ionization of this imidazole group on the Trp side chain NH resonances must be transmitted by the propionic COO^-. This interaction suggests the existence of a hydrogen bond between the COO^- group and the Trp side chain NH proton (2.97 Å between COO^- and the indole ring N). The presence of the hydrogen bond is consistent with other information: (1) The Trp side chain NH proton has a very low chemical shift, about 11 ppm, as compared to the chemical shifts of the Trp side chain NH protons at about 10 ppm from the ^{15}N NMR studies of bacteriophage T4 lysozyme (McIntosh et al., 1987); (2) the Trp side chain nitrogen resonance is at 246.6 ppm at pH 8.8, which is the same value for the indole ring nitrogen of the model compounds dissolved in hydrogen-bonding solvents.

The second largest proton chemical shift variation observed during the course of pH titration is the ligand His NH proton. The largest ^{15}N chemical shift variation observed in the low-field region is that of the proline-30 imide nitrogen ($\Delta\delta$ 0.63 ppm, Figure 2). Their chemical shift variations (though smaller than that of the Trp side chain NH resonances) with a pK_a of 6.8 suggest that the ionization of histidine-42 triggers the conformational changes of the Ω loops (Leszczynski & Rose, 1986) near the two propionic groups of the heme (Figure 6). The salt linkage between one propionic COO^- and the His residue 42 imidazole, the hydrogen bonding between the

propionic oxygen atoms and the Trp side chain NH proton, and also possible hydrogen bonding between the propionic oxygens and two peptide CONH protons (residues 41 and 42) would help transfer the effect throughout the Ω loops near the two propionic groups.

From the refined X-ray structure of the reduced cytochrome c_2 (Figure 6), it can be seen that the peptide CONH oxygen atom of Pro-30 is in proximity with the ligand His NH proton (2.59 Å between the CO oxygen and the πN). The relatively large chemical shift variations (though a secondary effect compared to Trp residue 62 indole ring NH resonances) of the ^{15}N resonance of the Pro-30 imide nitrogen and the resonance of the ligand His NH proton are consistent with the presence of a hydrogen bond between the peptide CONH oxygen of Pro-30 and the ligand His NH proton and suggest that its hydrogen-bonding strength is changed during the movement of the four Ω loops triggered by the ionization of the His residue 42 imidazole group.

The existence of this hydrogen bond can also be verified by the following evidence. The ^{15}N resonance of the ligand His NH nitrogen occurs at 206 ppm. The chemical shift for an α-type nitrogen (>N–H) is typically at 210 ppm (Bachovchin, 1986). Furthermore, this ligand His NH nitrogen would also experience the heme ring current effect which is estimated to be about 4.5 ppm (Giessner-Prettre & Pullman, 1971). Therefore, the expected value of resonance would be at about 214.5 ppm. The difference between the expected value and the experimental value is −8.5 ppm, which indicates the existence of a hydrogen bond (Bachovchin & Roberts, 1978; Bachovchin, 1986).

Therefore, we have direct evidence to confirm the suggestions made by several investigators (Takano et al., 1977; Salemme et al., 1973; Harbury et al., 1965) that the unligated nitrogen atom of His-18 is a proton donor in a hydrogen bond and to support the existence of several hydrogen bonds in this protein which were proposed solely on the basis of proximity in the X-ray crystallographic structure (Salemme et al., 1973). Brautigan et al. (1977), on the basis of the EPR studies of ferricytochromes *c*, proposed the existence of different populations of cytochromes, among which some molecules have a protonated ligand His and some have a deprotonated ligand His. However, only one state of reduced cytochrome c_2 has been observed from our studies; the ligand His τN is a singlet, and the πN is a doublet throughout the range of pH titration from pH 4.9 to 9.6.

The peptide NH proton resonances a, b, and f (Figures 3 and 4) are probably from inner sides of the four stable α-helices of residues 1–10, 64–71, 75–80, and 96–110 (Salemme et al., 1973). They appear to be involved in tight hydrogen bonding as evidenced by their low chemical shifts, low exchange rates, and small chemical shift variations with pH. We suggest that the peptide NH proton resonance b is probably from one of the two α-helices (residues 64–71 and 75–80), which are inside the Ω loops (Figure 6). The chemical shift variation of resonance b with pH shows a pK_a of 6.8, which, under this interpretation, would be a secondary effect of the conformational change of the Ω loops caused by the ionization of His residue 42 (as discussed above).

Hydrophobicity. Proton exchange rates can be accounted for by three mechanisms: (1) the nature of the NH bonds and covalent structures of the compounds; (2) the H_2O (or ^2H_2O) concentration around the NH bonds; and (3) the strength of hydrogen bonding. Our studies of model compounds show that the imidazole NH protons of L-His are not visible in H_2O using 1331 or 214 pulse sequences over the accessible range of pH but that the L-Trp indole NH proton is observable at 10.2 ppm in H_2O and is relatively sharp at acidic to neutral pH. Above pH 9.4, the proton resonance is broadened, presumably by base-catalyzed exchange. Therefore, the His imidazole NH protons undergo more rapid exchange than Trp indole NH protons.

There are two very slowly exchangeable protons in the low-field region of the ^1H spectra of cytochrome c_2. The ligand His NH proton has been shown to undergo a much slower exchange with deuterium than the Trp indole NH proton. This low exchange rate must be due to the lack of accessibility to H_2O or ^2H_2O. Therefore, it is clearly demonstrated that there is an extremely hydrophobic environment around the heme crevice; there is simply no H_2O or ^2H_2O available for exchange. This extremely hydrophobic environment could play an important role for maintaining the structure of cytochrome c_2. Furthermore, we suggest that the measurement of exchange rates of the ligand His NH proton with deuterium could be employed as an approach to test the theory proposed by Kassner et al. (1972, 1973) and Schlauder and Kassner (1979) that the differences in hydrophobicity near the heme crevice are responsible for the differences in redox potentials in different cytochromes.

Heme Nitrogen Resonances. The four heme nitrogen resonances are clearly resolved from one another, reflecting the slightly different environments of four pyrrole nitrogens. This nonequivalence probably arises from the asymmetry of the heme ring itself and the hydrophobicity differences around the heme. The four heme nitrogen resonances can be divided into two groups: one (peaks 1 and 2) having very little chemical shift variation with pH and the other (peaks 5 and 6) having a larger chemical shift variation with a pK_a of 6.8. On the basis of the local dielectric constant, and its change with the presumed movement of the Ω loop, we make the following tentative assignments: resonances 5 and 6 are provisionally assigned to pyrrole nitrogens III and IV, respectively, while resonances 1 and 2 probably arise from the nitrogens of pyrrole rings II and I, respectively. Nuclear Overhauser effects have not yet led to unequivocal assignment of these resonances.

Metal Coordination Bonds. For a typical β-type nitrogen, the ^{15}N chemical shift would be about 128 ppm (Bachovchin, 1986). The ligand His nitrogen (τN), which is coordinated to the iron atom of heme, is a pure β-type nitrogen. The calculated ring current effect is about 8 ppm for this τN (Giessner-Prettre & Pullman, 1971). Its chemical shift position at 199.6 ppm suggests that the coordination shift would be 199.6 − 128 − 8 = 64 ppm (upfield). The small variation of the chemical shift ($\Delta\delta$ 0.08 ppm) with pH change from 5.1 to 9.6 indicates the stable linkage of the coordination bonds; that is, no replacement of the Met ligand has been observed, although it probably occurs in the oxidized state (Brautigan et al., 1977).

ACKNOWLEDGMENTS

The refined coordinates of both the reduced and oxidized states of cytochrome c_2 were kindly provided by Dr. J. Kraut.

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Localization of the Binding Site on Fibrin for the Secondary Binding Site of Thrombin†

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Received July 2, 1987; Revised Manuscript Received August 25, 1987

ABSTRACT: Affinity chromatography of active site inhibited thrombin on immobilized fragments derived from the central (desAB-NDSK) and terminal (D_1) globular domains of fibrinogen revealed that the site responsible for the binding of thrombin at its secondary fibrin binding site is located in the central domain. Chromatography of various domains of the central nodule (desAB-NDSK, fibrinogen E, and fibrin E) having nonidentical amino acid sequences showed that all of these fragments are capable of binding to PMSF-thrombin–Sepharose, suggesting that the thrombin binding site resides within the peptide regions common to all of these fragments: $\alpha(Gly_{17}–Met_{51})$, $\beta(Val_{55}–Met_{118})$, and $\gamma(Tyr_1–Lys_{53})$. Competitive affinity chromatography of the same binding domains revealed that there is no detectable difference in their binding constants to PMSF-thrombin–Sepharose, indicating that the $\alpha(Lys_{52}–Lys_{78})$, $\beta(Gly_{15}–Lys_{54})/(Tyr_{119}–Lys_{122})$, and $\gamma(Thr_{54}–Met_{78})$ peptide segments do not contribute significantly to the binding of thrombin. Chromatography of the isolated chains of fibrinogen E showed that the $\alpha(Gly_{17}–Lys_{78})$ peptide region itself contains a strong binding site for PMSF-thrombin–Sepharose. The location of the binding site suggests that the secondary site interaction may play an important role in determining the cleavage specificity of thrombin on fibrinogen and can affect the rate of release of the fibrinopeptides. Affinity chromatography of fragments prepared from polymerized fibrin showed that cross-linked DD (DxD) itself does not bind to thrombin, whereas the DxDE complex remained attached to the column, suggesting that the binding site on fragment E for thrombin is distinct from its binding site for DxD. Thus, the interaction between DxD and E during polymerization is not likely to release thrombin from fibrin. The possible consequences of the fibrin–thrombin interaction on the mechanism of fibrin polymerization and factor XIII activation are discussed.

Thrombin performs a regulatory function in thrombosis and hemostasis. In addition to its proteolytic action on fibrinogen, thrombin also activates factor V, factor VIII, factor XIII, and, in the presence of thrombomodulin, protein C. In each of these cases, activation involves limited proteolysis of the polypeptide chain by thrombin.

The major event in a series of controlled reactions is the activation of fibrinogen to fibrin monomers, which then polymerize spontaneously to form the fibrin network. During the conversion of fibrinogen to fibrin, thrombin is removed

† This work was supported by a research grant (HL-30616) from the National Heart, Lung, and Blood Institute of the National Institutes of Health.

0006-2960/88/0427-1956$01.50/0