Characterization of pH-Dependent Conformational Heterogeneity in *Rhodospirillum rubrum* Cytochrome c_2 Using ^{15}N and ^{1}H NMR†

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ABSTRACT: The ^{15}N-enriched ferricytochrome c_2 from *Rhodospirillum rubrum* has been studied by ^{15}N and ^{1}H NMR spectroscopy as a function of pH. The ^{15}N resonances of the heme and ligand τ nitrogen are broadened beyond detection because of paramagnetic relaxation. The ^{15}N resonance of the ligand histidine π nitrogen was unambiguously identified at 184 ppm (pH 5.6). The ^{15}N resonances of the single nonligand histidine are observed only at low pH, as in the ferrocytochrome because of the severe broadening caused by tautomerization. The dependence of the ^{15}N and ^{1}H spectra of the ferricytochrome on pH indicated that the ligand histidine π NH does not dissociate in the neutral pH range and is involved in a hydrogen bond, similar to that in the reduced state. Because neither deprotonated nor non-hydrogen-bonded forms of the ligand histidine are observed in the spectra of either oxidation state, the participation of such forms in producing heterogeneous populations having different electronic g tensors is ruled out. Transitions having pK_a's of 6.2, 8.6, and 9.2 are observed in the ferricytochrome. The localized conformational change around the Ω loops is observed in the neutral pH range, as in the ferrocytochrome. Structural heterogeneity leads to multiple resonances of the heme ring methyl at position 8. The exchange rate between the conformations is temperature dependent. The transition with a pK_a of 6.2 is assigned to the His-42 imidazole group. The displacement of the ligand methionine, which occurs with a pK_a of 9.2, causes gross conformational change near the heme center. There are multiple conformations at high pH, only one of which is able to transfer electrons efficiently, as judged by saturation-transfer experiments. The N-terminus of the ferricytochrome has a pK_a of 8.6. In contrast to its partially restricted mobility in the reduced state, it is found to be very mobile, reflecting a looser structure of the ferricytochrome.

Rhodospirillum rubrum cytochrome c_2 is an electron-transfer protein and has been studied by NMR spectroscopy (Smith & Kamen, 1974; Smith, 1979; Smith et al., 1987; Yu & Smith, 1988a,b, 1990; Senn & Wuthrich, 1983), by redox potential measurements (Pettigrew et al., 1976, 1978; Moore et al., 1984), and by X-ray crystallography (Salemme et al., 1973; Salemme, 1977). The overall structures of *R. rubrum* cytochrome c_2 and horse heart cytochrome c are quite similar (Dickerson et al., 1971; Salemme et al., 1973), including the chirality of the ligand methionine sulfur (R configuration) (Senn et al., 1980; Takano & Dickerson, 1981a,b; Senn & Wuthrich, 1983). However, their midpoint potentials are different (Pettigrew et al., 1978; Moore et al., 1984). The occurrence of conformational heterogeneity in the heme pocket of these ferricytochromes is also different (Burns & La Mar, 1979, 1981).

There are multiple low-spin forms of these ferricytochromes c at neutral pH. The major population of horse heart cytochrome c, which has g-tensor components of 3.06, 2.25, and 1.25, has been assigned to a form in which the π nitrogen of the extraplanar ligand His-18 is protonated, whereas the major form of *R. rubrum* cytochrome c_2, which has g-tensor components of 3.17 and 2.05, is assigned to a form in which the π nitrogen of the ligand His-18 is unprotonated (Brautigan et al., 1977). The πNH of the ligand histidine is known to be a hydrogen bond donor, and its pH-dependent behavior has been characterized in the reduced state by ^{1}H and ^{15}N NMR studies (Yu & Smith, 1988a). This hydrogen bonding is proposed to be coupled to the change in oxidation states, thus linking the protein conformation with redox potential (Valentine et al., 1979). Therefore, the protonation state and hydrogen bonding of the ligand histidine in both oxidation states becomes an essential issue in understanding stability, conformational heterogeneity, and the regulation of redox potentials among cytochromes c. In the present paper, we investigate the pH dependence of *Rhodospirillum rubrum* ferricytochrome c_2 studied by ^{1}H and ^{15}N NMR spectroscopy.

Experimental Procedures

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

Growth of Rhodospirillum rubrum, Sample Preparation, and NMR Spectroscopy. ^{15}N-Labeled and unlabeled cytochrome c_2 was prepared for NMR spectroscopy from the G-9 mutant of *R. rubrum* as described in the preceding paper (Yu & Smith, 1990). ^{15}N NMR spectra were obtained at 50.7 MHz, and ^{1}H NMR spectra of the samples in H_2O were collected at 500 MHz as described in the same reference or at 360 MHz using a GE-NMR NT-360 spectrometer. Nitrogen chemical shifts are referenced to 1 M ^{15}N HNO_3 in $^{2}H_2O$ 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 214 composite pulse (Redfield et al., 1975) or the 1331 (Hore, 1983) water-suppression pulse was used for proton spectra taken in H_2O. The chemical shifts for ^{1}H were measured from sodium 2,2-dimethyl-2-silapentanesulfonate as reference. Other parameters are listed in the figure legends.

Results

pH Dependence of ^{1}H NMR Spectra of Ferricytochrome c_2. The low-field region of the ^{1}H NMR spectra of ferricytochrome c_2 in $^{2}H_2O$ at different pH values is shown in

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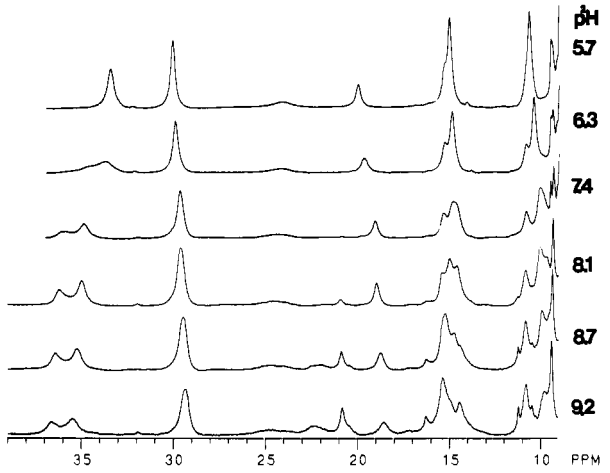

FIGURE 1: ^1H NMR spectra (low-field region) of a pH titration of ferricytochrome c_2 in ^2H_2O. The sample p^2H (direct meter reading) is indicated. The number of scans for each sample is as follow: 4608 at p^2H 5.7; 1024 at p^2H 6.3; 2048 at p^2H 7.4; 7040 at p^2H 8.1; 2048 at p^2H 8.7; 4224 at p^2H 9.2.

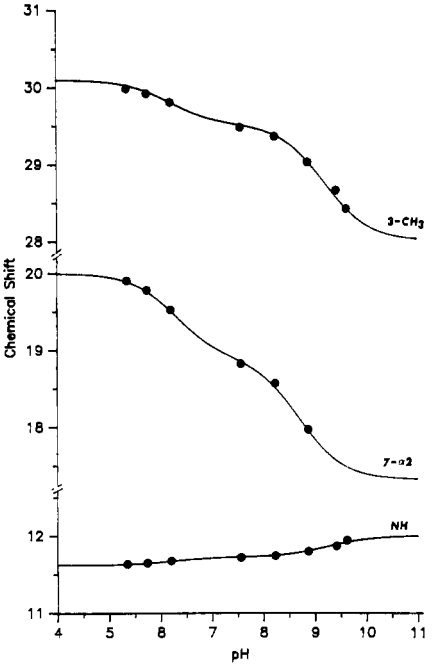

FIGURE 2: Dependence on pH of ^1H chemical shifts for the resonances of the heme 3-CH_3, the heme 7-propionate $\alpha 2$ proton, and an exchangeable NH proton for ferricytochrome c_2 dissolved in H_2O. The dots represent the experimental data. The solid lines represent the theoretical curves obtained with an equation containing two independent pK_a values and having the Hill coefficient set to 1.

Figure 1. The assignment of these low-field resonances has been described in the preceding paper (Yu & Smith, 1990). In general, the heme and ligand histidine resonances become broader with increasing pH, and the chemical shifts for these resonances are very pH dependent. The most striking effect is that the resonance of the 8-CH_3 group becomes split above neutral pH. The relative intensity of the elements of the doublet is pH dependent. In H_2O, the resonances around 15 ppm include the heme 5-CH_3 protons, the ligand histidine $\beta 1$ proton, and the ligand histidine NH proton (Yu & Smith, 1990). They form an envelope at low pH, but become resolved into multiple peaks as the pH increases. Other features are the same as in ^2H_2O.

Two independent pK_a values are needed in order to account for the chemical shift data (Figure 2). The pK_a values of 6.2 and 9.2 are obtained for the heme 3-CH_3 and an NH proton

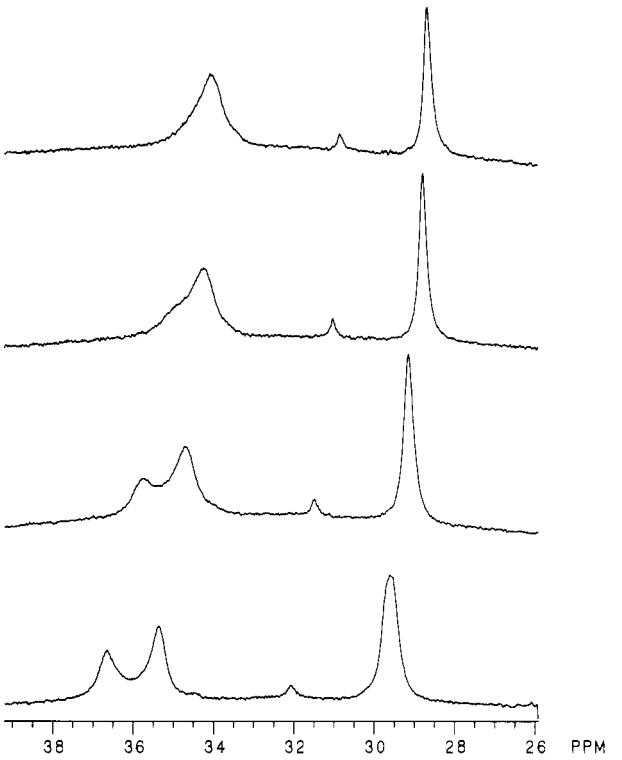

FIGURE 3: Temperature dependence of the splitting of the heme 8-CH_3 resonance at 360 MHz in ^2H_2O. Spectra were acquired at, from bottom to top, 20, 30, 40, and 45 °C, using a 90° pulse and a 0.5-s recycle time. The sample pH was 8.2, measured at 20 °C. The drift toward higher field at higher temperature results from the Curie law, which also obscures the relative movement of the two members of the doublet.

(in H_2O) at about 11.6 ppm. pK_a values of 6.3 and 8.7 are obtained for heme 7 propionate $\alpha 2$ proton. The latter resonance disappears above pH 8.8, placing a limit on the quality of the data at high pH and, ultimately, the accuracy of the pK_a obtained, perhaps producing the deviation of the pK_a of 8.7 from the value of 9.2 obtained from the other resonances. pK_a values of 6.3 and 9.2 are obtained for the lower field member, the heme 3-CH_3 doublet, and pK_a values of 6.8 and 9.1 are obtained for the other member of the doublet. The pK values are essentially the same whether the sample is dissolved in H_2O or ^2H_2O.

Temperature Dependence of the Splitting of the Heme 8-CH_3 Resonance. Raising the temperature of the sample (Figure 3) causes the degree of splitting of the 8-CH_3 resonance to decrease, until the two peaks merge at about 45°. The relative area of the two peaks, when resolved, depends only upon pH and not upon temperature, indicating that the splitting results from an ionization which leads to two forms in slow exchange, rather than from a temperature-induced conformational change. The splitting is not profoundly frequency dependent, as judged by a comparison of the spectra in Figures 1 and 3.

^{15}N NMR Spectra of Ferricytochrome c_2 in H_2O. The ^{15}N NMR spectra (low-field region) of ferricytochrome c_2 in H_2O at different pH values are shown in Figure 4. There are only two histidine residues, His-18, ligated to the heme iron, and His-42, ion-paired with and/or hydrogen-bonded to the heme 7 propionic acid group (Yu & Smith, 1988a). The ^{15}N resonances of the heme and ligand histidine are expected to be broadened and shifted by hyperfine and dipolar interactions in the ferricytochrome. Accordingly, the only relatively sharp pH-dependent resonance in the low-field region at 194 ppm at pH 5.4 is assigned to the His-42 imidazole group. The

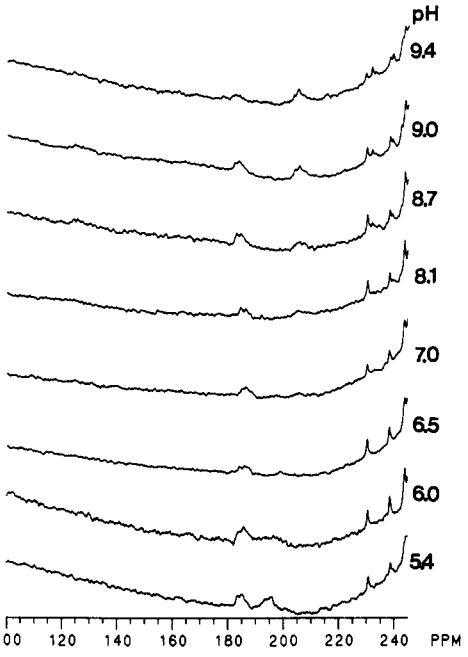

FIGURE 4: ^{15}N NMR spectra (low-field region) of a pH titration of ^{15}N-enriched ferricytochrome c_2 in H_2O. The sample pH is indicated. The number of scans for each sample is as follows: 13 440 at pH 9.4; 31 480 at pH 9.0; 10 300 at pH 8.7; 7408 at pH 8.1; 31 068 at pH 7.0; 52 760 at pH 6.5; 27 580 at pH 6.0; 8012 at pH 5.4. A recycle time of 0.5 s was used at pH 5.4, 5.0, and 7.0; a recycle time of 1 s was used for the other samples.

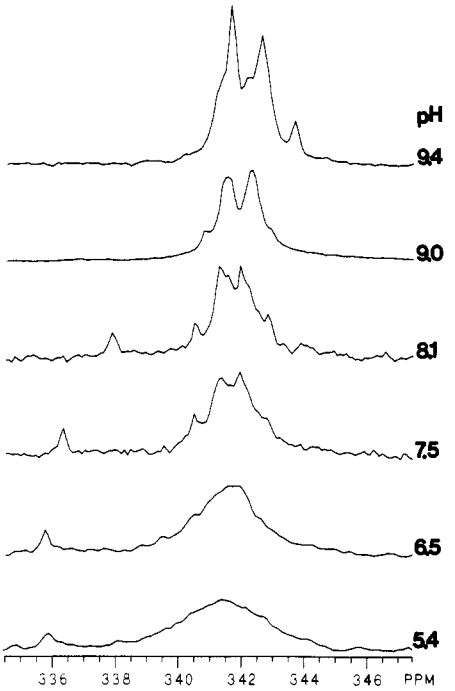

FIGURE 5: ^{15}N NMR spectra (high-field region) of a pH titration of ^{15}N-enriched ferricytochrome c_2 in H_2O. The sample pH is indicated. The spectra of the samples at pH 9.0 and 9.4 required about 10 000 scans, and the spectra for the other samples required about 1024 scans.

resonances of His-42 also occur at this chemical shift in the ferrocytochrome and are observed only at low pH (Yu & Smith, 1988a). The resonances are severely broadened above pH 5 due to intermediate chemical exchange, rendering the pK_a difficult to determine.

The broad ^{15}N resonance at about 183 ppm observed at all pHs has been firmly assigned to the ligand histidine πN on the basis of spin decoupling and saturation-transfer experiments (Yu & Smith, 1990). The ^{15}N resonances at about 231, 239, and 244 ppm arise from Pro-30, -74, and -85. Although they are singlets below pH 8.0, they appear as doublets at higher pH. The relative intensities change with pH, indicating a pH-dependent conformational change. Since Pro-74 and -85 are far from the paramagnetic center, based on the X-ray data, these results clearly indicate that there are at least two different, pH-dependent structures in solution. The shifts of the ^{15}N resonances at 231 and 239 ppm are also slightly pH dependent and consistent with two independent ionizations with pK_a values of 6.2 and 9.2.

The high-field region of the ^{15}N NMR spectrum of ferricytochrome c_2 in H_2O is shown in Figure 5. The resonance at 335.6 ppm at pH 5.4 arises from the N-terminus, which shifts upfield with a pK_a of 8.6. The appearance of lysine side chain ^{15}N resonances (about 341 ppm) at low pH is similar to that in ferrocytochrome c_2. However, when the pH is increased above 8.0, two groups of lysine side chain ^{15}N resonances are clearly observed. Since the lysines of the ferrocytochrome form a single envelope at this pH (Yu & Smith, 1988b), we suggest again that two forms of the ferricytochrome exist at high pH.

^1H–^{15}N NMR NOE experiments were also performed on ferricytochrome c_2 at different pH values (not shown). In contrast to the observed immobilization of the N-terminus in the reduced state in the neutral pH range (Yu & Smith, 1988b), the resonance of the N-terminus in the oxidized state is observable from pH 5 to pH 9.5, indicating that it is relatively mobile, perhaps due to the looser structure of the oxidized state and possible loss of hydrogen bonding and/or salt linkages.

Table I: Individual Assignments and Chemical Shifts of ^{15}N NMR Resonances of *Rhodospirillum rubrum* Cytochrome c_2 at pH 6.0

resonance assignments	chemical shifts (ppm) reduced[a]	oxidized
heme pyrrole nitrogens	192.2, 193.4 194.5, 194.9	
His-18 τN	199.6	
His-18 πN	206.6	184
Trp-62 ring N	249.5	249.2[b]
Pro-30	238.3	238.7
Pro-74 or -85	232.9	230.7
His-42 imidazole	193.8,[c] 194.1[c]	194.4[d]
N-terminus (Glu) NH_3^+	335.3	335.9
lysine ϵNH_3^+	341.7	341.7

[a] From Yu and Smith (1988a,b). [b] From Yu and Smith (1990). [c] At pH 4.9. [d] At pH 5.4.

A summary of individual assignments and ^{15}N shifts for *R. rubrum* cytochrome c_2 appears in Table I.

pH Dependence of Electron-Transfer Efficiency. Successful saturation-transfer experiments depend upon the occurrence of electron exchange between the ferricytochrome and ferrocytochrome at a rate competitive with T_1 upon irradiation of a resonance in the oxidized state (Redfield & Gupta, 1971). For the *R. rubrum* cytochrome, it has been found (Smith, 1976) that the electron exchange rate is 1×10^3 s^{-1} and approximately constant between pH 6 and 9, and about 2-fold higher between pH 5 and 6. Therefore, the results from saturation-transfer experiments can be used to judge the electron-transfer efficiency of the cytochrome at different pHs.

When the heme 7 propionate $\alpha 2$ proton at about 20 ppm at p^2H 5.6 was irradiated, the corresponding resonance in the reduced state was observed at 3.7 ppm. At p^2H 8.1, the same protons exhibit two resonances at about 19 and 20.9 ppm. Irradiation of the resonances at 19 ppm produced saturation transfer to the reduced state at 3.7 ppm. However, no corresponding resonance was observed when the resonance in the

oxidized state at 20.9 ppm was irradiated, nor was a result obtained from this resonance at higher pH where the intensities of the two peaks are equal. This result suggests that among the multiple forms of the ferricytochrome at high pH, only one of them is able to transfer electrons rapidly enough to mediate saturation transfer. The irradiation of the heme 7 propionate α2 proton resonances and the multiple peaks at about 15 ppm in the oxidized state at higher pH yields the same result.

These results agree with those obtained by Greenwood and Palmer (1965) and Wuthrich et al. (1971). From the kinetic experiments, Greenwood and Palmer (1965) found that there are two structurally distinct forms of horse heart cytochrome *c* monomer at alkaline pH and that only one of these forms appears to be capable of reacting with redox agents in this potential range. On the basis of chemical modification studies of mammalian-type cytochromes *c*, Wuthrich et al. (1971) showed that the redox function of these cytochromes *c* is maintained only if the methionine ligand is bound to the heme iron in both oxidized and reduced forms, presumably because of thermodynamic as well as kinetic considerations.

Discussion

Ionization and Hydrogen Bonding of the Ligand Histidine. Brautigan et al. (1977) performed EPR studies on various eukaryotic and prokaryotic ferricytochromes *c* and suggested that the ligand histidine becomes deprotonated in the neutral pH range and that the protonation state of the πN is responsible for the multiple low-spin forms of these cytochromes *c* which exhibit different *g* tensors. In previous work (Yu & Smith, 1988a), we assigned the ligand histidine πNH proton of ferrocytochrome c_2 at 9.79 ppm at pH 5.6. In the preceding paper (Yu & Smith, 1990), we identified the same proton in the ferricytochrome at 14.7 ppm at pH 5.6. This resonance is clearly present between pH 5.3 and 6.2, and its intensity is almost constant within this pH range (Figure 1). The status of this resonance above pH 7.0 is not clear because of the presence of multiple peaks in this region at high pH. However, ^{15}N-decoupled proton spectra at pH 8.5 reveal that the ligand histidine πNH proton is still present. Therefore, it can be concluded that the ligand histidine does not become deprotonated in the neutral pH range.

In numerous studies, pK_a values near 6.2 have been found for ferricytochromes *c*, and pK_a values near 6.8 have been found in ferrocytochromes *c*. These pK_a's have been generally attributed to the same ionizing group in the two different oxidation states (Leitch et al., 1984; Moore el al., 1980, 1984). The pK_a has been said to be lowered in ferricytochromes by the charge repulsion between the metal ion center Fe^{3+} and the ionizing group. In previous work, we assigned the deprotonation occurring at pK_a 6.8 in ferrocytochrome c_2 to the His-42 imidazole group (Yu & Smith, 1988a, 1990). We attribute the pK_a of 6.2 in ferricytochrome c_2 to the same group, but have not been able to measure the pK_a of His-42 directly in the oxidized cytochrome.

Ionizations, Conformational Changes, and Electronic Structures in Ferricytochrome c_2. There are clearly at least three different effects involved in the pH-dependent phenomena. One effect is the continuous movement (i.e., fast exchange) of contact-shifted resonances occurring with a p*K* near 6.2. These include resonances of the methionine methyl and γ2 methylene protons, which move by about 2 ppm (Smith, 1979), the ligand histidine πNH proton, and the heme substituents (Figures 1 and 2), but also resonances of groups far from the heme, such as two proline α-imide nitrogens (Figure 4). In addition to these continuous shifts, there is a splitting of the resonances of some ring methyl groups, most notably the 8-CH_3, and of groups far from the heme, such as the nitrogen resonances of the prolines (Figure 4) and lysines (Figure 5). The third effect arises from the loss of the Met-91 coordination to the iron, which causes loss of the 698-nm iron–sulfur charge-transfer band (Pettigrew et al., 1978) and the upfield-shifted resonances of the ligand methionine (Smith, 1976, 1979).

Since it is known from previous studies that there is a conformational change involving the Ω loops in ferrocytochrome c_2, triggered by the ionization of the His-42 imidazole group (Yu & Smith, 1988a), it is reasonable that the ionization of the same group in the oxidized state would also alter the structure. Indeed, similar Ω-loop movement is clearly observed in ferricytochrome c_2 by the widespread variation in chemical shifts, to different degrees, but all having a pK_a of 6.2. The effect is even more pronounced in the oxidized state, probably for the following reasons. In the reduced state, the ligand histidine πNH proton exchanges with solvent with a half-life of about 1 month, and the Trp-62 indole NH, with a half-life of a few weeks (Yu & Smith, 1988a). In the oxidized state, however, these exchangeable protons have half-lives of hours or less (Yu & Smith, 1990), indicating much greater accessibility to solvent of the interior of the ferricytochrome. These comparisons suggest a looser structure in the ferricytochrome. Even the iron–methionine bond strength is known to be weaker in the ferricytochrome (Corradin & Harbury, 1971). It is therefore possible that when the His-42 imidazole ionizes at pH 6.2, the strength of the hydrogen-bonding network near the heme 7 propionate COO^- group changes to such a degree that disorder in the relative positions of the heme plane and surrounding groups is permitted. The electron density in the ring appears to be changed slightly, causing shifting and broadening of the resonances of the heme substituents.

The pH-induced shifts occurring at pH 6–7 are not observed in the mitochondrial cytochrome. Although there are two histidine residues in horse heart cytochrome *c* in addition to the ligand, both are located far from the heme propionic acid groups. The resonance of the 8-CH_3 group in the oxidized state is sharp and unsplit (Burns & La Mar, 1979, 1981). However, splitting of the heme 3-CH_3 group is observed and could indeed be triggered by one of the two histidine residues through a hydrogen-bonding network as discussed by Burns and La Mar (1981). This effect is quite localized, unlike the shifts observed in cytochrome c_2.

Unlike the pH 6.2 transition, which behaves as a single deprotonation with a Hill coefficient of unity, the splitting of the 8-CH_3 and other groups covers a wide pH range, and the intensities do not follow the Hill equation. This, together with the fact that both members of the 8-CH_3 doublet exhibit their own chemical shift titration curves, indicates that at least two processes are occurring. The behavior would suggest that the deprotonated form which predominates above pH 6.2 is capable of existing in two forms which are in slow exchange. An alternate interpretation is that the sample itself is heterogeneous and the two forms have different pH dependences. The fact that the relative areas of the two members of the 8-CH_3 resonance are pH dependent, appear broadened by interconversion at pH 6.3 (Figure 1), and merge at higher temperature (Figure 3) supports the former interpretation. The splitting could not be followed above about pH 9.2 because of the third pH-dependent event, the loss of the methionine ligand.

The emergence of a different form of ferricytochrome c_2 is observed when the sample pH is above 8.2 as evidenced by

the formation of multiple peaks by the heme resonances and by an exchangeable NH proton at about 11.7 ppm (not shown), the splitting of the proline ^{15}N resonances, and the appearance of two groups of lysine side chain ^{15}N resonances at high pH. These pH-dependent multiple conformations are found to be in slow exchange on the NMR time scale, and only one of them is able to transfer electrons efficiently enough to transfer saturation. The pK_a of 9.2 obtained from the present pH titration experiments agrees well with the reported pK_a values of 9.1 from a near-infrared study of ligand displacement (Pettigrew et al., 1978) and of 9.2 from a ^1H NMR study of the loss of intensity of the ϵCH_3 resonance of Met-91 at high pH (Smith, 1979). Loss of the sulfur ligand would most certainly alter the redox potential and electron distribution in the heme. Furthermore, the iron–sulfur bond of the ferrocytochrome is known to be intact at pH 9. Since saturation transfer is mediated by a self-exchange reaction in which the products are identical with the reactants, an energetically symmetrical reaction coordinate is required by the principle of microscopic reversibility. The most reasonable means of achieving an energetically symmetrical reaction coordinate is through a structurally symmetrical transition state. It is thus reasonable to expect the electron exchange to be slower in forms of the ferricytochrome without methionine as the sixth ligand.

Redox Potentials and Structural Differences between Ferro- and Ferricytochrome c_2. Four pK_a values are found in this study: (1) one at 6.2 from the His-42 imidazole group in the oxidized state; (2) one at 6.8 from the His-42 imidazole group in the reduced state; (3) one at about 8.5 from the N-terminus of the protein in both oxidation states; (4) one at 9.2 from the displacement of ligand methionine in the oxidized state. The interior of the protein in the reduced state is very hydrophobic, as demonstrated by the absence of exchange of the ligand histidine πNH proton and other exchangeable protons inside the protein (Yu & Smith, 1988a). The interior of the protein in the oxidized state appears less hydrophobic because these deeply buried exchangeable protons can be exchanged very easily (Yu & Smith, 1990), suggesting also that the amino acid side chains in the oxidized state are more mobile, allowing solvent molecules in and out more freely. Therefore, the oxidized structure must be looser in this regard than the reduced structure.

Since redox potential is a measure of the differences between the free energies of the reduced and oxidized states, including energetically different structural differences between the two oxidation states, it might be expected that the deprotonation of the N-terminus with a pK_a of about 8.5 might be observed in the redox measurements at different pHs because the mobility of the N-terminus in the two oxidation states is known to be different. The reported pK_a value of 8.4 by Pettigrew et al. (1978) may be due to the ionization of the N-terminus instead of to the displacement of ligand methionine, which we find has a pK_a of 9.2. Therefore, for *R. rubrum* cytochrome c_2, an equation containing four pK_a values instead of the three pK_a values (Pettigrew et al., 1976, 1978; Moore et al., 1984) is needed to fit the E_m vs pH data. Three of these, at 6.2, 8.6 and 9.2, are detectable in the ferricytochrome and one, near 7.0, occurs in the ferrocytochrome.

Registry No. His, 71-00-1; Met, 63-68-3; cytochrome c_2, 9035-43-2.

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Structural Studies with the Uveopathogenic Peptide M Derived from Retinal S-Antigen†

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ABSTRACT: The 18-residue fragment of bovine S-antigen, corresponding to amino acid positions 303–320, is highly immunogenic and is known to induce experimental autoimmune uveitis. The solution conformation of this immunogenic peptide, known as peptide M, was studied by Fourier-transform infrared spectroscopy and by circular dichroism. In the pH range between approximately 4 and 9.5, peptide M has a strong tendency to form macromolecular assemblies in which it adopts an intermolecular β-sheet structure. The intermolecular β-sheets are stabilized by ionic interactions ("salt bridges") between the carboxylate groups and basic residues of the neighboring peptide molecules. These interactions can be disrupted by neutralization of either acidic (pH range below 4) or basic residues (pH range above 9.5) or by elevated hydrostatic pressure. The secondary structure of the peptide under conditions favoring the monomeric state appears to be a mixture of unordered structure and β-sheets. The present data are consistent with a recently proposed model [Sette, A., Buns, S., Colon, S., Smith, J. A., Miles, C., & Grey, H. M. (1987) *Nature 328*, 395–399], which assumes that certain immunogenic peptides adopt an extended β-type conformation in which they are "sandwiched" between the major histocompatibility complex and the T-cell receptor.

The S-antigen is a major soluble protein of the retina and pineal gland (Wacker et al., 1977) that binds to photo-excited–phosphorylated rhodopsin (Pfister et al., 1985) and is intimately involved in the visual cycle. The S-antigen is also a highly pathogenic protein, responsible for the induction of experimental autoimmune uveitis (Donoso et al., 1988) which has been characterized as a T-cell-mediated disease resulting in severe inflammation of the uveal tract, retina, and pineal gland (Mochizuki et al., 1985).

The specific region of S-antigen responsible for its pathogenicity has been recently identified (Donoso et al., 1987; Singh et al., 1988; Shinohara et al., 1988). Moreover, it has been shown that the synthetic octadecapeptide, known as peptide M, corresponding to amino acid positions 303–320 (Asp-Thr-Asn-Leu-Ala-Ser-Ser-Thr-Ile-Ile-Lys-Glu-Gly-Ile-Asp-Arg-Thr-Val) in bovine S-antigen is highly effective in inducing experimental autoimmune uveitis. Both clinically and histopathologically, the experimental autoimmune uveitis produced by the synthetic peptide is indistinguishable from the disease caused by the native S-antigen. The detailed understanding of the mode of action of peptide M may therefore provide essential clues for advancing our knowledge of autoimmune diseases. However, the molecular dynamic basis for the pathogenic action of peptide M and, in particular, the conformational properties of the peptide are still unknown.

In the present study, we have used infrared and circular dichroism spectroscopy to investigate the conformation of peptide M in solution. It is shown that the preferred conformation of the peptide in aqueous solution is β-sheet, although the detailed structural properties depend on the degree of peptide self-association.

Materials and Methods

Preparation of Peptide M. The octadecapeptide Asp-Thr-Asn-Leu-Ala-Ser-Ser-Thr-Ile-Ile-Lys-Glu-Gly-Ile-Asp-Arg-Thr-Val (peptide M) was synthesized by standard solid-phase chemistry in a commercial peptide synthesizer as described previously (Knight et al., 1988). In order to remove residual trifluoroacetic acid used in the synthesis of peptide M [the trifluoroacetate ion has a very strong infrared absorption band around 1670 cm^{-1} which interferes with the characteristic peptide bands (Surewicz & Mantsch, 1989)], the peptide was additionally purified by chromatography on an Amberlite IR-45 minicolumn. The column (5.5 × 80 mm) was preequilibrated with bidistilled water. The peptide was then applied as a solution in H_2O (5 mg/mL), followed by elution with bidistilled water, collection of fractions, and their lyophilization. About 80% of the starting material was recovered in the first 2-mL fraction. Peptide solutions at various concentrations and pH values were then prepared in D_2O buffer. The sequence and the concentration of the stock solutions of the octadecapeptide were confirmed by an amino acid analysis on a DURRUM 500 amino acid analyzer.

Infrared Spectroscopy. Infrared spectra under ambient conditions (room temperature and atmospheric pressure) were collected at a resolution of 2 cm^{-1} on a Digilab FTS-60 spectrometer equipped with a high-sensitivity deuterated triglycine sulfate detector. The samples were assembled into

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