Structural Basis for the Variation of pH-Dependent Redox Potentials of *Pseudomonas* Cytochromes *c*-551†

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ABSTRACT: The redox potentials of many *c*-type cytochromes vary with pH over the physiological pH range. We have investigated the pH dependence of redox potential for the four homologous cytochromes *c*-551 from *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* strain 221, *Pseudomonas stutzeri* strain 224, and *Pseudomonas mendocina*. The pH dependence is due to an ionizable group that ionizes with pK_{ox} in ferricytochrome *c*-551 but with a higher p*K*, pK_{red}, in ferrocytochrome *c*-551. For *P. aeruginosa* cytochrome *c*-551 it has been shown that this ionizable group is one of the heme propionic acid substituents [Moore, G. R., Pettigrew, G. W., Pitt, R. C., & Williams, R. J. P. (1980) *Biochim. Biophys. Acta 590*, 261–271] but the values of pK_{ox} and pK_{red} are significantly lower in this protein than in the other three cytochromes. NMR and chemical modification studies show that for the two *P. stutzeri* cytochromes *c*-551 and *P. mendocina* cytochrome *c*-551, this propionic acid substituent is again important for the pH dependence of the redox potential. However, a histidine occurring at position 47 in their sequences hydrogen bonds to the propionic acid and thereby raises its p*K*. In *P. aeruginosa* cytochrome *c*-551, His-47 is substituted by Arg-47. Hydrogen-bonding schemes involving His-47 and the propionic acid are proposed.

The measurement of redox potential for an electron transport protein is essential to the understanding of its function since this parameter helps to define its thermodynamic relationship with other components of the electron transport chain. The redox potentials of *c*-type cytochromes are largely determined by the fifth and sixth iron ligands and by the nature of the polypeptide environment surrounding the heme (Kassner, 1973), but the precise features of this environment that can influence the redox potential are not yet fully understood.

Ionization of groups on the protein close to the heme might influence the redox potential, and in such cases, the heme would impose a separation in the p*K* values of any such group in the two redox states of the cytochrome. Effects of this kind are not observed in mitochondrial cytochrome *c* until pH values greater than 9 and are thus not considered to be physiologically relevant. However, in several bacterial cytochromes, the redox potential varies with pH between 5 and 9, and in the case of *Pseudomonas aeruginosa* *c*-551 the pH dependence has been interpreted as being due to the ionization of a heme propionic acid substituent with a p*K* of 6.3 in the oxidized form and 7.2 in the reduced form (Moore et al., 1980).

We consider these observations of importance for two reasons. First, the imposed separation of p*K* values results in a proton-linked electron transfer that may be of relevance to our understanding of more complex integral membrane cytochromes, such as mitochondrial cytochrome *b* and cytochrome oxidase, which have been proposed as proton pumps (Von Jagow & Engel, 1980; Wikström, 1977). Second, local fluctuations in pH at the respiratory membrane, by perturbing the redox potential of a cytochrome, may represent an important control mechanism over the rate of electron transport (Hashimoto & Nishimura, 1981).

A number of cytochromes *c*-551 have been sequenced (Ambler, 1963; Ambler & Wynn, 1973; R. P. Ambler, personal communication) and the crystal structure is known for *P. aeruginosa* cytochrome *c*-551 (Matsuura et al., 1982). Thus we can exploit the natural diversity of structure to investigate the important features of the heme environment that influence its redox properties. In the present paper we compare the effect of pH on the midpoint potential for three relatives of *P. aeruginosa* cytochrome *c*-551, namely, the cytochromes *c*-551 from *Pseudomonas stutzeri* 221, *Pseudomonas stutzeri* 224, and *Pseudomonas mendocina*, and we propose a structural model to explain the observed pH dependence.

Materials and Methods

Growth of Organisms. *P. aeruginosa* 10332 (Ambler, 1974; A.T.C.C. 10145), *P. stutzeri* strain 221 (Stanier et al., 1966; A.T.C.C. 17588), and *P. stutzeri* strain 224 (Mandel, 1966; A.T.C.C. 17591) were obtained as peptone-dried stubs from Dr. R. P. Ambler. The bacteria were grown anaerobically in 12-L batches for 12–24 h at 37 °C on the nitrate medium described by Lenhoff & Kaplan (1956). Cells were collected by centrifugation (Alfa-Laval LAB 102B-05 centrifuge).

Purification of Cytochromes. The cytochromes *c*-551 were purified from acetone-dried powders of the cells essentially as described by Ambler & Wynn (1973). Both strains of *P. stutzeri* contain two forms of cytochrome *c*-551 (designated forms I and II) that can be separated by ion-exchange chromatography. The two forms are identical except that form II has an N-terminal glutamic acid whereas in form I this appears to be converted to pyrrolidonecarboxylic acid (Ambler & Wynn, 1973). For both cytochrome forms, however, the level and pH dependence of E_m were virtually identical and the two forms were used interchangeably in subsequent experiments.

Initial experiments with *P. stutzeri* 221 cytochrome *c*-551 were carried out on cytochrome that was a gift from Dr. T. E. Meyer. Dr. R. P. Ambler kindly supplied *P. mendocina* CH 110 cytochrome *c*-551, purified according to Ambler & Wynn (1973).

Redox Potential Measurements. Midpoint oxidation–reduction (redox) potentials were determined spectrophotometrically for each cytochrome in the presence of ferricyanide–ferrocyanide solutions of known potential (Pettigrew et al., 1975; Davenport & Hill, 1952; Hanania et al., 1967).

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This method allows a single potential measurement to be made at a defined pH.

For each redox potential measurement, added potassium ferrocyanide was 0.5 mM and added potassium ferricyanide was 0.017, 0.003, or 0 mM. The cytochrome concentration was approximately 5×10^{-6} M. The total ionic strength was 0.007–0.009 mol/L. The redox mixture was buffered at a chosen pH value by addition of 2 mM acetic acid–sodium acetate (pH 4.5–5.8), 2 mM sodium phosphate (pH 5.8–7.5), 2 mM Tris-HCl (pH 7.5–8.8), 2 mM glycine–NaOH (pH 8.8–9.5), or 2 mM borate–NaOH (pH 9.3–10.7). The pH was measured before addition of sodium dithionite, which was used to achieve complete reduction.

The value of E_m obtained by this method was checked in a few cases by complete redox titration of the cytochrome. The values obtained by the two methods were in good agreement.

NMR Experiments. Cytochrome samples were prepared for NMR spectroscopy by passage through Sephadex G-25 packed into a Pasteur pipet and equilibrated in 10 mM NaCl–0.5 mM sodium phosphate, pH 7.0, in ^2H_2O (Merck Sharp & Dohme). Cytochrome concentrations were in the range 0.5–2 mM.

The pH of solutions in the NMR experiments were monitored by a glass electrode (Radiometer) that was inserted directly into the NMR tubes. The pH was adjusted by addition of small aliquots of concentrated NaO^2H or ^2HCl in ^2H_2O. pH values quoted for NMR experiments are direct meter readings, and since they are not corrected for any isotope effect (Glascoe & Long, 1960) they are denoted pH* (and pK values are denoted pK*).

^1H NMR spectra were obtained by using a Brucker 270 MHz or 300 MHz spectrometer as previously described (Moore et al., 1977). Chemical shifts are quoted in parts per million (ppm) downfield from the methyl resonance of 4,4-dimethyl-4-silapentane-1-sulfonate.

Chemical Modification of P. stutzeri (221) Cytochrome c-551. Ethoxyformic anhydride (EFA, Sigma) was prepared as a 110 mM stock solution by injection of 8 μL of 100% EFA into 0.5 mL of acetonitrile (Sigma).

To a solution of *P. stutzeri* (221) cytochrome *c*-551 in 50 mM sodium phosphate, pH 7.0, a single addition of EFA was made to give an 18× excess of EFA over cytochrome. The modification was allowed to proceed for 100 min at 20 °C, the extent of reaction being followed spectrophotometrically at 238 nm. The amount of *N*-(ethoxyformyl)histidine formed was calculated by using $\Delta\epsilon_{240} = 3200\ M^{-1}\ cm^{-1}$ (Ovadi et al., 1967). The modified cytochrome solution was then applied to a Sephadex G-25 column equilibrated in 2 mM sodium phosphate, pH 7.0, to remove unreacted EFA. Redox potential measurements were then made as described above.

The redox potential of the EFA-modified cytochrome at pH 7.2 was measured 2 h after the addition of EFA and then again after 8 h. The values were very similar, indicating that the modifying group remains attached over this period of time.

For NMR experiments, the cytochrome was modified in the same way as described above and then concentrated by adsorption to diethylaminoethylcellulose (Whatman) at pH 7.0, followed by elution with 0.5 M NaCl and desalting on Sephadex G-25 equilibrated in 10 mM NaCl–0.5 mM sodium phosphate, pH 7.0, in ^2H_2O.

Chemical Modification of Imidazole. EFA was added to 10 mM imidazole, pH 7.5, to give a 1:1 ratio of EFA:imidazole. This solution was used directly for NMR studies.

NMR spectra of the EFA-modified imidazole showed five resonances: three of these were assigned to the C-2, C-4, and C-5 protons of the modified imidazole, the other two being assigned to the C-2 and C-4 protons of residual unmodified imidazole. The reaction of EFA with only one of the imidazole ring nitrogens creates different environments for the C-4 and C-5 protons, which are equivalent in unmodified imidazole.

pH titration of the NMR spectra showed that the three resonances of EFA-modified imidazole had pH-dependent chemical shifts, titrating with a pK* of 3.6 in agreement with the figure quoted by Melchior & Fahrney (1970). The two resonances of unmodified imidazole titrated with a pK* of approximately 7.

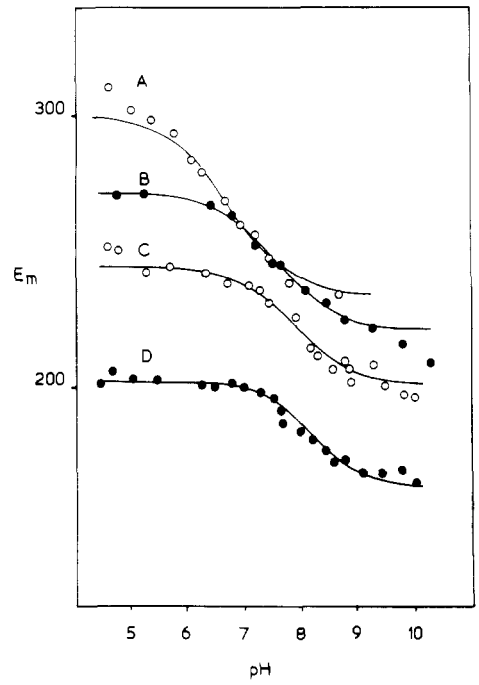

FIGURE 1: pH dependence of midpoint oxidation reduction potential (E_m) of the cytochromes *c*-551 from four species of *Pseudomonas*: (A) *P. aeruginosa* [data taken from Moore et al. (1980)], pK_{ox} = 6.2, pK_{red} = 7.3. (B) *P. mendocina*, pK_{ox} = 7.2, pK_{red} = 8.0. (C) *P. stutzeri* (221), pK_{ox} = 7.6, pK_{red} = 8.3; (D) *P. stutzeri* (224), pK_{ox} = 7.8, pK_{red} = 8.45.

Results

Redox Potential Measurements. Figure 1 shows the behavior of midpoint potential (E_m) with respect to pH for four homologous bacterial cytochromes, the cytochromes *c*-551 from *P. aeruginosa*, *P. stutzeri* 221, *P. stutzeri* 224, and *P. mendocina*. Since with all these cytochromes E_m is a function of pH, a proton must be involved in their redox reaction and their Nernst equation must include a proton concentration term. The appropriate equation that describes the curves observed in Figure 1 can be derived by using the methods of Clark (1960) and is

$$E_m = \tilde{E} + \frac{RT}{nF} \ln \frac{[H^+] + K_{red}}{[H^+] + K_{ox}} \quad (1)$$

where K_{ox} and K_{red} are proton dissociation constants for a chemical group that ionizes with a different pK in the two redox states of the cytochrome.

Equation 1 was used to generate the theoretical curves in Figure 1 by selecting values of K_{ox} and K_{red} to give the best fit to the experimental data. For all four cytochromes the appearance of these curves is the same but the values of K_{ox} and K_{red} differ; in *P. aeruginosa* *c*-551 the pKs occur about 1 pH unit lower than for the other three cytochromes.

The existence of a heme-linked ionization with pK_{red} = 7.2 in *P. aeruginosa* ferrocytochrome *c*-551 was confirmed by

Table I: Aromatic Amino Acids Occurring in the Four *Pseudomonas* Cytochromes *c*-551

source of *c*-551	residue 7	27	34	47	56	77
P. aeruginosa	Phe	Tyr	Phe	Arg	Trp	Trp
P. stutzeri 221	Phe	Phe	Tyr	His	Trp	Trp
P. stutzeri 224	Phe	Leu	Asn	His	Trp	Trp
P. mendocina	Phe	Leu	Asn	His	Trp	Trp

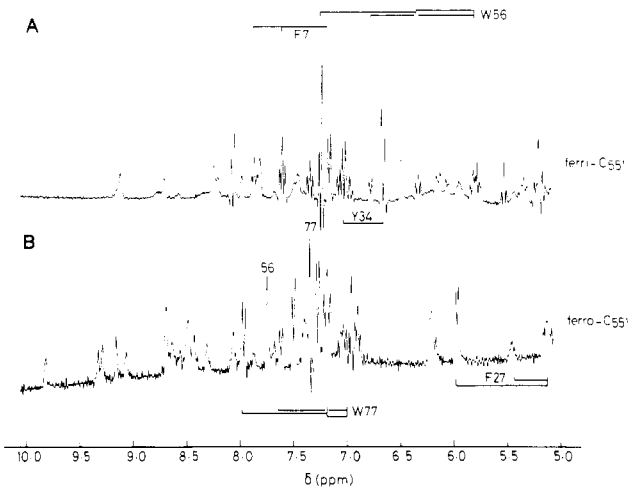

FIGURE 2: Aromatic region of the 300-MHz convolution difference spectrum of *P. stutzeri* (221) cytochrome *c*-551. The ferricytochrome spectrum was obtained at pH* 6.5 (27 °C) and the ferrocytochrome spectrum at pH* 7.7 (27 °C). The labeled resonances are F7 = Phe-7; F27 = Phe-27; W56 and 56 = Trp-56; W77 and 77 = Trp-77; Y34 = Tyr-34.

spectrophotometric pH titration in the region of the α-band (Moore et al., 1980). Red shifts and the appearance of asymmetry in the α-peak in alkaline solutions were also observed for the other *Pseudomonas* cytochromes studied here, but the changes were too small to allow quantitative analysis of p*K* values.

NMR Resonance Assignments for Cytochromes c-551. The aims of the NMR studies were to confirm that the tertiary structures of *P. stutzeri* (221) and *P. mendocina* cytochromes *c*-551 are similar to the *P. aeruginosa c*-551 structure, to identify the group ionizing with pK_{ox} and pK_{red} in each of the cytochromes, and to determine whether a conformational change accompanies ionization. The NMR spectra therefore first had to be characterized in terms of resonance assignments.

(*a*) *Aromatic Assignments.* The assignment of aromatic resonances of these cytochromes is facilitated by the small number of aromatic amino acids in their sequences and by fortuitous substitutions between the three cytochromes. From Table I it can be seen that *P. mendocina c*-551 contains no tyrosine and only one phenylalanine; *P. stutzeri* (221) *c*-551 and *P. aeruginosa c*-551 both contain one tyrosine and two phenylalanines, but only Phe-7 occurs at the same position in both sequences.

The aromatic regions of the convolution difference spectrum of *P. stuzeri* (221) ferricytochrome and ferrocytochrome are shown in Figure 2. These can be compared with the previously published spectra of *P. aeruginosa c*-551 (Moore et al., 1977). Assignments to amino acid types were made for the majority of resonances by using standard double-resonance techniques. However, for *P. stutzeri* (221) cytochrome *c*-551, a ferro–ferricytochrome exchange titration was required to complete the assignments. This is shown in Figure 3. The exchange titration was particularly useful for cross assignment of Trp-56

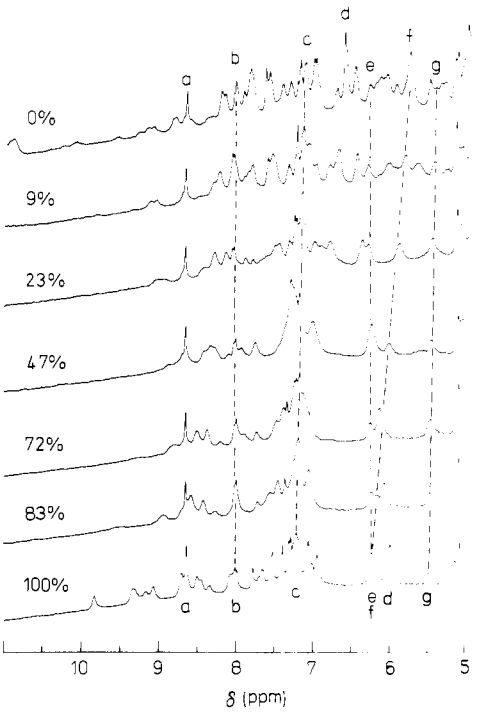

FIGURE 3: Titration of *P. stutzeri* (221) ferricytochrome *c*-551 with ascorbic acid. Figures on the left refer to the percent ferrocytochrome present. Labeled resonances are (a) His-47 C-2, (b) Trp-77 C-4 or C-7, (c) Phe-7 ortho, (d) Phe-27 ortho, (e) Trp-56 C-5 or C-6, (f) Trp-56 C-6 or C-5, and (g) Phe-27 para.

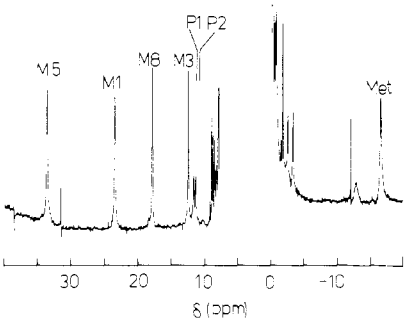

FIGURE 4: ^{1}H NMR spectrum of *P. stutzeri* (221) ferricytochrome *c*-551 obtained at pH* 6.3 (27 °C). The labeled resonances are M1, M3, M5, and M8 = heme methyls, P1 and P2 = the β-CH_2 protons of heme propionic acid 7, and Met = Met-61 methyl.

resonances since two of these coincidently overlap a thioether CH resonance in the ferrocytochrome.

From consideration of the identified coupling patterns, chemical shifts, and the amino sequences, the resonance assignments are deduced to be those shown in Table II.

(*b*) *Heme Substituent Assignments.* The heme substituent resonances in *P. stutzeri* (221) and *P. mendocina* ferrocytochromes were assigned by using standard double-resonance techniques (Keller & Wüthrich, 1978). Our assignments agree with those recently published by Senn & Wüthrich (1983).

The four heme methyl resonances of *P. aeruginosa* ferricytochrome *c*-551 have previously been assigned (Keller et al., 1976; Keller & Wuthrich, 1978) and the similarity in chemical shifts for these resonances in *P. stutzeri* and *P. mendocina* ferricytochromes *c*-551 indicates that their assignment is the same. The numbering of these is shown for *P. stutzeri* (221) *c*-551 in Figure 4. The resonance at −16.6 ppm arises from Met-61, the sixth iron ligand, and an analogous resonance appears at a similar chemical shift position in the spectra of the other cytochromes. Figure 5A shows the *P. stutzeri* (221) ferricytochrome spectrum at pH 8, between 5 and 20 ppm.

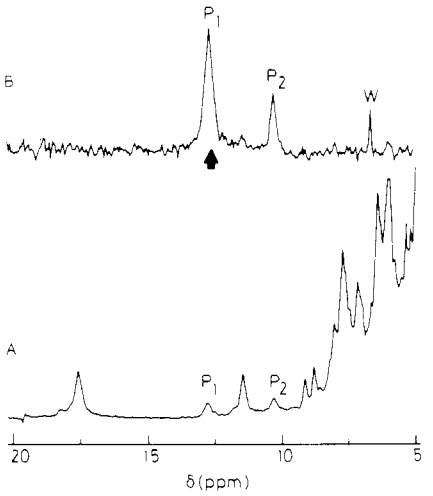

FIGURE 5: Low-field region of the 300-MHz NMR spectrum of *P. stutzeri* (221) ferricytochrome *c*-551 obtained at pH* 8 (27 °C). (A) Conventional spectrum; (B) the NOE difference spectrum obtained upon irradiation of P1 for 0.5 s prior to acquisition. The labeled resonances are P1 and P2 = the β-CH_2 protons of heme propionic acid 7 and W = Trp-56 C-2.

The two one-proton resonances P1 and P2 arise from one of the heme propionic acid substituents occurring at positions 6 and 7 on the heme ring (the "outer" and "inner" propionic acids, respectively). The NOE difference spectrum (Figure 5b) shows that irradiation at P1 causes an intensity change in P2 and in the C-2 resonance of Trp-56. From this it is deduced that P1 and P2 are the β-CH_2 protons of the inner propionic acid because the same experiment performed on *P. aeruginosa* *c*-551 gives an identical result and the X-ray structure of *P. aeruginosa* *c*-551 shows that N-1 of Trp-56 is hydrogen bonded to the inner propionic acid (Matsuura et al., 1982).

pH Titration of the Cytochrome NMR Spectra. (*a*) *Ferricytochrome c-551.* NMR spectra of *P. stutzeri* (221) ferricytochrome *c*-551 were obtained at a number of different pH values. Figure 6 shows that shifting resonances titrate with a pK* of 7.5–7.7, corresponding to the pK_{ox} of 7.6 observed in the E_m vs. pH curve for this cytochrome (see Figure 1). In the aromatic region of the spectrum only two resonances have pH-dependent chemical shifts, these being the resonances of the His-47 C-2 and C-4 protons. The direction of these shifts and their magnitude are typical for deprotonation of a histidine (Westler & Markley, 1979). Both resonances broaden considerably above pH* 6.5, although the C-2 resonance sharpens again beyond pH* 8.5, and their chemical shifts can only be followed in non resolution enhanced spectra. Broadening of histidine resonances midway through the course of a pH titration is a common occurrence (Bachovchin & Roberts, 1978) and is due to a relatively slow proton on–off rate (Sudmeier et al., 1980). The two titration curves are fitted to pK* values of 7.6 (Figure 7), and again this value agrees with the pK_{ox} value obtained from redox measurements. A few other resonances in the aromatic region show small shifts with pH, notably Trp-56 and Tyr-34 resonances, but these shifts are too small (<0.05 ppm) to be fitted to theoretical curves.

Qualitatively similar results were obtained for the *P. mendocina* pH titration, the same resonances shifting to about the same extent. All the titrating resonances could be assigned pK*s of 7.5. However, in this cytochrome the agreement with pK_{ox} predicted from redox measurements (pK = 7.2) was less good.

(*b*) *Ferrocytochrome c-551.* In the diamagnetic ferrocytochrome, the only resolved heme substituent resonances are

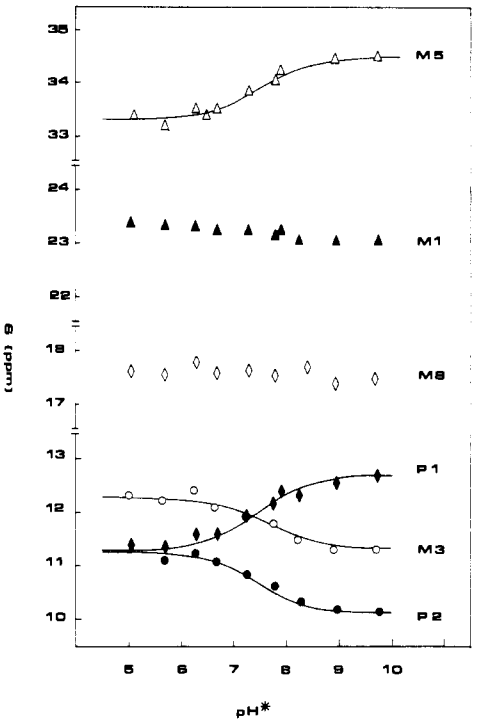

FIGURE 6: pH dependence of chemical shifts (δ) for heme-substituent resonances of *P. stutzeri* (221) ferricytochrome *c*-551. (○, ●, △, ▲, ◇, ◆) represent experimental points. Theoretical curves were calculated by using the following pK values: M5, pK = 7.5; M3, pK = 7.7; P1, pK = 7.5; P2, pK = 7.5. Labeling of resonances as for Figure 4.

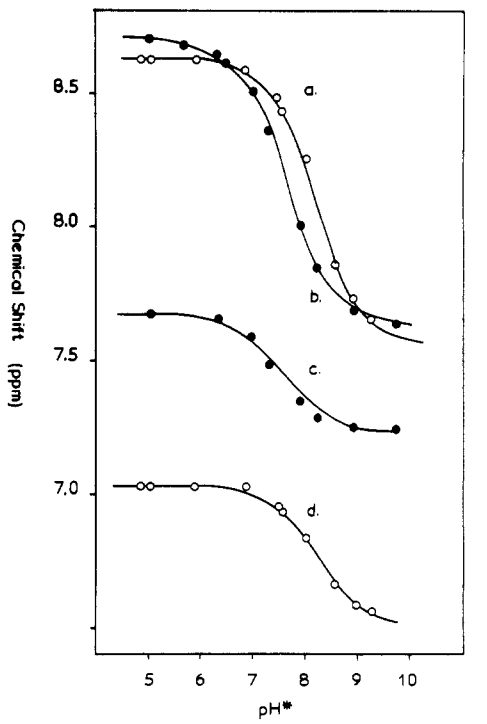

FIGURE 7: pH dependence of chemical shift for His-47 C-2 and C-4 resonances of *P. stutzeri* (221) cytochrome *c*-551. (○, ●) represent experimental points. The theoretical curves were calculated by using the following pK values: (a) C-2 (ferrocytochrome), pK = 8.2; (b) C-2 (ferricytochrome), pK = 7.6; (c) C-4 (ferricytochrome), pK = 7.6; (d) C-4 (ferrocytochrome), pK = 8.2.

those of the meso protons, which occur at the downfield end of the aromatic region between 9 and 10 ppm (see Figure 2B). With the exception of a small shift (<0.02 ppm) observed for the γ meso resonance, none of these resonances shifted with pH in *P. stutzeri* (221) ferrocytochrome *c*-551.

Table II: Comparison of Aromatic Resonance Assignments

	chemical shift (ppm)				
	P. aeruginosa cytochrome *c*-551		*P. stutzeri* cytochrome *c*-551		*P. mendocina* cytochrome *c*-551
resonance assignment	ferri[a]	ferro[a]	ferri[b]	ferro[c]	ferro[d]
Trp-56					
C-4 or C-7	6.70	7.36	6.76	7.4	7.72
C-5 or C-6	6.02	6.38	5.8	6.23	6.29
C-6 or C-5	6.34	5.81	6.32	6.23	5.74
C-7 or C-4	7.23	7.08	7.23	7.24	7.03
C-2	6.63	7.77	6.64	7.75	7.67
Trp-77					
C-4 or C-7	8.16	8.02	8.06	7.99	7.96
C-5 or C-6	7.43	7.28	7.34	7.2	7.24
C-6 or C-5	7.10	7.12	6.96	7.05	7.01
C-7 or C-4	7.22	7.61	7.07	7.65	7.54
C-2	7.13	7.28	7.21	7.34	7.34
Phe-7					
ortho	7.01	7.26	7.16	7.18	7.04
meta	6.81	6.94	7.61	6.93	6.69
para	7.15	7.12	7.87	7.05	6.56
Phe-27					
ortho			6.51	6.0	
meta			n.d.	5.11	
para			5.45	5.47	
Phe-34					
ortho	7.60	7.22			
meta	7.60	7.22			
para	7.60	n.d.			
Tyr-27					
ortho or meta	6.40	5.80			
meta or ortho	4.98	4.69			
Tyr-34					
ortho or meta			6.63	7.29	
meta or ortho			7.02	7.52	
His-47					
C-2			8.68	8.62	8.46
C-4			7.65	7.03	7.05

[a] Assignments made at pH 5.5 (27 °C). [b] Assignments made at pH 6.5 (27 °C). [c] Assignments made at pH 7.7 (27 °C). [d] Assignments made at pH 7.0 (57 °C).

As in the ferricytochrome, the His-47 C-2 and C-4 resonances are the only resonances in the aromatic region having marked pH-dependent chemical shifts. Figure 7 shows that in this oxidation state the titration curves have pK^* values of 8.2, agreeing well with the values of 8.3 obtained for pK_{red} in Figure 1B. Again, some of the resonances of Trp-56 and Tyr-34 show small shifts with pH but these are not fitted to theoretical curves.

The *P. mendocina* titration was very similar to the *P. stutzeri* titration in this oxidation state too, the only shifting resonances being those of His-47 and with pK_{red} being greater than pK_{ox} by at least 0.3 pH unit. This titration was less satisfactory than the *P. stutzeri* ferrocytochrome titration because reoxidation of the cytochrome and the subsequent electron exchange caused a marked deterioration in the spectral quality.

To summarize, the pH titrations of *P. stutzeri* (221) and *P. mendocina* cytochromes *c*-551 show that certain heme-substituent resonances and the His-47 resonances have pH-dependent chemical shifts. In the ferricytochrome these resonances titrate with the pK_{ox} value of 7.6 predicted from the redox measurements. The His-47 resonances titrate with a higher pK in the ferrocytochrome, this value agreeing with the predicted pK_{red} of 8.3. However, it is not clear from these data alone whether the ionization of His-47, which lies adjacent to the inner propionic acid, causes the observed shifts of the heme-substituent resonances or whether these are due to the ionization of the inner propionic acid itself (as in *P. aeruginosa* *c*-551).

Chemical Modification of P. stutzeri Cytochrome c-551. In order to determine the importance of His-47 for the pH dependence of redox potential in *P. stutzeri* *c*-551, the histidine was chemically modified with ethoxyformic anhydride (EFA). EFA has been shown to react quite specifically with accessible histidine residues in many proteins (Melchior & Fahrney, 1970; Miles, 1977). The progress of modification is followed by an increase in the cytochrome absorption at 238 nm, the absorbance maximum of *N*-(ethoxyformyl)histidine. Using the reaction conditions described in the legend to Figure 8, a value of 1.3 mol of histidine modified/mol of cytochrome was obtained. This value may be greater than unity because a bis(ethoxyformyl)histidine species can be formed in the presence of excess EFA and this species has a higher extinction coefficient at 238 nm (Avaeva & Krasnova, 1975).

Only two histidines are present in *P. stutzeri* (221) *c*-551, His-47 and His-16, the fifth axial heme ligand. The relative instability of *N*-(ethoxyformyl)histidine prevents the isolation of modified peptides but the modified histidine can be identified by the following arguments: (i) His-16 is buried in the center of the protein and is much less likely to be accessible to EFA than His-47, which lies near the surface of the protein; (ii) if His-16 was modified, it would have an impaired function as an iron ligand, which would certainly result in large changes in the visible spectrum but no such changes were observed; (iii) treatment of *P. aeruginosa* *c*-551 with EFA under the same conditions as the *P. stutzeri* cytochrome produced no increase in absorbance at 238 nm. *P. aeruginosa* *c*-551 has Arg-47 in place of His-47 but His-16 provides the fifth iron

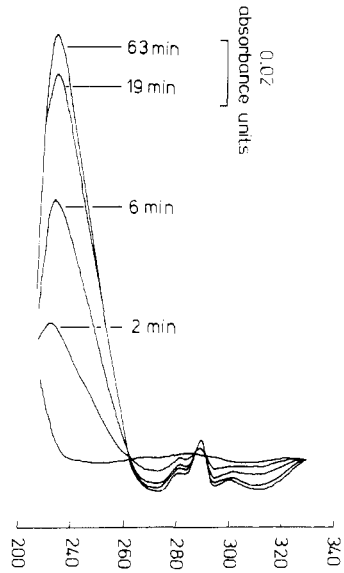

FIGURE 8: Chemical modification of His-47 of *P. stutzeri* (221) cytochrome *c*-551 by treatment with ethoxyformic anhydride (EFA). Cytochrome = 28 μM; EFA = 500 μM. Reaction was carried out in 40 mM sodium phosphate buffer, pH 7.0 (25 °C). Absorption maximum at 238 nm is due to *N*-(ethoxyformyl)histidine.

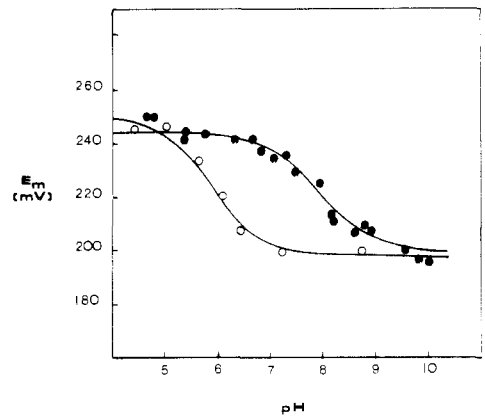

FIGURE 9: Effect of EFA modification of His-47 on the redox potential of *P. stutzeri* (221) cytochrome *c*-551. (●) Experimental points for unmodified cytochrome; the theoretical curve was fitted by using pK_{ox} = 7.6 and pK_{red} = 8.3. (○) Experimental points for modified cytochrome; the theoretical curve was fitted by using pK_{ox} = 5.45 and pK_{red} = 6.3.

ligand in this cytochrome also. The modified histidine is therefore concluded to be His-47.

Small absorption changes occur at 270–300 nm in parallel with those at 238 nm on treatment of *P. stutzeri c*-551 with EFA (Figure 8). The positions of the maxima at 280 and 290 nm are suggestive of perturbation of a tryptophan. It is possible that a small proportion of tryptophan becomes modified, although tryptophan in proteins is normally resistant to this reagent. Model studies with *N*-acetyltryptophan in ethanol showed that modification by EFA results in a decrease in tryptophan absorbance (Rosen et al., 1970). In view of the parallel nature of the absorbance changes at 238 nm and at 270–300 nm and in view of the possibility that Trp-56 and His-47 may both be hydrogen bonded to the inner propionic acid, the changes at 270–300 nm are more likely to be due to an alteration in the environment of the tryptophan. This alteration may be the ionization of the heme propionic acid as the histidine modification proceeds.

Figure 9 shows the effect of modification on the pH dependence of redox potential of *P. stutzeri* (221) cytochrome *c*-551. At low and high pH values, the redox potential of the modified and unmodified cytochrome is very similar. Since redox potential is a sensitive monitor of the heme environment, it is concluded that modification has not induced any significant structural rearrangements.

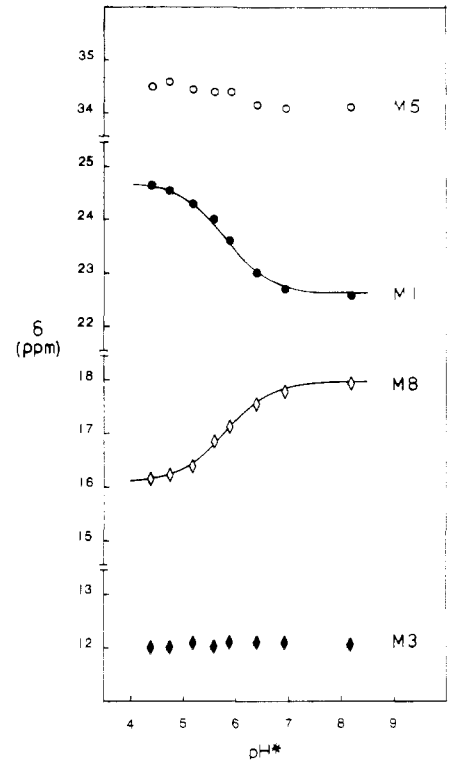

FIGURE 10: pH dependence of chemical shift (δ) for the heme methyl resonances M1, M3, M5, and M8 of EFA-modified *P. stutzeri* (221) ferricytochrome *c*-551. The theoretical curves were both fitted by using pK = 5.8.

However, the main point to note from Figure 9 is that the chemical modification of His-47 has not abolished the pH dependence of redox potential. Instead, the effect has been to lower the values of pK_{ox} and pK_{red} by 2 pH units. Therefore, we conclude that the principal ionization that affects the redox potential is that of the inner propionic acid. However, the interaction of this propionic acid with nearby His-47 affects the pKs at which the ionization occurs in the unmodified protein.

NMR Spectrum of EFA-Modified P. stutzeri Cytochrome c-551. The NMR spectrum of EFA-modified *P. stutzeri* (221) ferricytochrome *c*-551 was largely similar to that of the unmodified cytochrome with regard to the chemical shifts of the heme methyl and Met-61 resonances although all resonances were broadened. Figure 10 shows the chemical shifts of the heme methyl resonances at various pH* values, and it can be seen that the resonances that shift titrate with a pK* value of 5.8, in resonable agreement with the pK_{ox} of 5.45 calculated from the redox measurements described above. The propionic acid resonances were broadened in the modified spectra and the chemical shifts were not followed during the pH titration.

The NMR spectrum of EFA-modified imidazole shows the C-2 resonance to be located at 9.4 ppm at low pH, shifting to 8.3 ppm at high pH with a pK* of 3.6. EFA-modified *P. stutzeri* (221) cytochrome *c*-551 gave poor quality spectra, perhaps due to protein aggregation, but a singlet resonance was clearly discernible at 8.25 ppm in the ferricytochrome and at 8.4 ppm in the ferrocytochrome. This singlet has the approximate chemical shift expected for the C-2 resonance of neutral EFA-modified His-47. Titration of the cytochrome over the pH* range 4.4–8.2 produced no change in the

chemical shift of this resonance in either oxidation state.

No singlet resonance with a chemical shift comparable to the C-4 resonance of EFA-modified imidazole was observable in the NMR spectrum of either oxidation state during the pH titration.

From these data we conclude that the EFA-modified His-47 of *P. stutzeri* cytochrome *c*-551 has a pK of <4.4 and that in this form it probably does not interact with the inner propionic acid.

Discussion

Structural Comparison of the Four Cytochromes c-551. Published NMR data for *P. aeruginosa* *c*-551 suggested that the observed pH dependence of redox potential was a result of the ionization of one of the heme propionic acid substituents (Moore et al., 1980). A refined three-dimensional structure for *P. aeruginosa* *c*-551 has since become available (Matsuura et al., 1982), and together with further NMR data presented in this paper, it is now clear that the propionic acid occurring at substituent position 7 on the heme ring, the inner propionic acid, is the species ionizing with pK_{ox} in the ferricytochrome *c*-551 and with pK_{red} in the ferrocytochrome.

The *P. stutzeri* and *P. mendocina* cytochromes *c*-551 show the same general pattern of pH dependence of E_m as the *P. aeruginosa* cytochrome (Figure 1), but pK_{ox} and pK_{red} are shifted about 1 pH unit more alkali. Sequence comparisons of the four cytochromes suggest that they should have very similar three-dimensional structures and this is confirmed by the NMR assignments for heme substituent and aromatic resonances, the latter being given in Table II. The NMR pH titration data and the modification data demonstrate that the inner propionic acid is involved in the deprotonation event in *P. stutzeri* and *P. mendocina* cytochromes *c*-551 also, but with considerably more alkaline pK values. We propose that this alkaline shift in pK can be explained in terms of the immediate environment of the propionic acid in the different cytochromes.

From the *P. aeruginosa* *c*-551 crystal structure it is known that two amino acids, Arg-47 and Trp-56, are hydrogen bonded to the propionic acid. In the *P. stutzeri* and *P. mendocina* cytochromes, Trp-56 is conserved but Arg-47 is replaced by His-47. Precisely how this His substitution can lead to high pK_{ox} and pK_{red} values for the propionic acid is discussed below.

Interaction of His-47 with the Heme Propionic Acid Substituent. In *P. aeruginosa* *c*-551, deprotonation of the inner propionic acid permits salt bridge formation with Arg-47. However, in *P. stutzeri* and *P. mendocina* cytochromes *c*-551 the situation is more complex in that the deprotonation event involves two groups, His-47 and the inner propionic acid. The pK affecting these two groups is the same and is well above the normal pK for either group. We shall show that this pK represents a one-proton ionization and that it is higher than normal because of a hydrogen-bonding interaction between the histidine and the propionic acid.

Two types of hydrogen-bonding scheme can be envisaged, involving either two protons (Figure 11A) or only one proton (Figure 11B,C). The following arguments strongly support a one-proton scheme: (i) If eq 1 is rewritten to incorporate two deprotonations occurring with the same pK, then the equation becomes

$$E_m = \tilde{E} + \frac{RT}{nF} \ln \frac{[H^+]^2 + K_{red}[H^+] + K_{red}^2}{[H^+]^2 + K_{ox}[H^+] + K_{ox}^2} \quad (2)$$

In generating the theoretical curve for the *P. stutzeri* (221) cytochrome *c*-551 redox potential data of Figure 1C, we

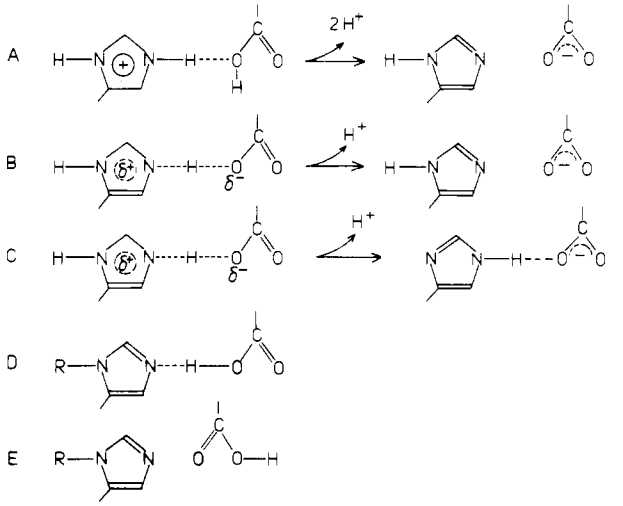

FIGURE 11: Possible hydrogen-bonding interactions between His-47 and the inner propionic acid: (A) two-proton scheme; (B) loss of hydrogen-bonded proton; (C) loss of non-hydrogen-bonded His proton; (D and E) cytochrome modified with EFA (R = ethoxyformyl).

Table III: $\Delta\delta$ for the Heme Substituent Resonances of the Cytochromes *c*-551[a]

source of cytochrome *c*-551	$\Delta\delta$ (ppm)					
	M5	M1	M8	M3	P1	P2
P. aeruginosa	1.1	−1.5	2.4	−1.0	−4.2	3.0
P. stutzeri 221	1.1	−0.35	−0.1	−0.8	1.4	−1.05
P. mendocina	0.6	−0.3	−0.05	−0.8	−1.3	1.25
P. stutzeri 221 (EFA modified)	−0.45	−2.1	1.8	0		

[a] $\Delta\delta$ is the difference in chemical shift value at alkaline and acid pH (i.e., $\delta_A - \delta_{HA}$).

substituted values of 7.6 for pK_{ox} and 8.3 for pK_{red} in eq 1. If these same pK values are used in eq 2, a markedly different theoretical curve is obtained and the redox data clearly do not lie on this line (not shown). By selecting values of pK_{ox} = 7.8 and pK_{red} = 8.1 for eq 2, it is possible to produce a theoretical curve that *will* fit the experimental data but ΔpK_{red-ox} is reduced from 0.7 to 0.3 pH unit. For this cytochrome, the NMR pH titration data yielded pKs that were very close to the redox pKs obtained by using eq 1, and ΔpK_{red-ox} was identical. (ii) With the one-proton scheme, it is easy to understand how His-47 and the propionic acid could be affected by the same deprotonation; the two-proton scheme offers no simple explanation.

Several different possibilities exist for hydrogen bonds involving one proton, two of which are shown in Figure 11B,C. In (B), the proton to be lost is involved in the hydrogen bond. In (C), the proton lost is not the one involved in the hydrogen bond, but as a result of the histidine deprotonation, the proton in the hydrogen bond is attracted more strongly to the histidine. This latter scheme is also the currently accepted form of the Asp–Ser–His interaction in the catalytic triad of the serine proteases (Steitz & Shulman, 1982, and references cited therein). In these composite schemes *both* groups carry a partial charge at low pH, but following loss of a proton, neutral histidine and fully charged propionate are generated. The pH dependence of the NMR chemical shifts supports a composite scheme. Table III shows the change in chemical shift, $\Delta\delta$, of heme substituent resonances during pH titration of *P. aeruginosa*, *P. stutzeri* 221, and *P. mendocina* *c*-551. For each of the *P. aeruginosa* *c*-551 resonances $\Delta\delta$ is the same or larger than for the corresponding resonance of *P. stutzeri* or *P.*

mendocina c-551. This is consistent with the propionic acid being partially charged at low pH in the *P. stutzeri* (221) and *P. mendocina* cytochromes as shown in parts B and C of Figure 11. The overall change in charge on the propionic acid on going to high pH is therefore larger in *P. aeruginosa* c-551 where the propionic acid is uncharged at low pH, and so its resonances experience bigger shifts.

At present it is not possible to distinguish which of the schemes B and C operates in *P. stutzeri* and *P. mendocina* c-551.

Possible interactions between chemically modified His-47 and propionic acid are shown in parts D and E of Figure 11. Above pH 4 modified His-47 is neutral, and the NMR pH titration data indicate that His-47 is unaffected by the propionic acid ionization. Scheme E is therefore likely to be the situation obtaining for the modified cytochrome.

Table III also shows $\Delta\delta$ for some of the heme resonances of chemically modified *P. stutzeri* cytochrome c-551. Since the propionic acid is uncharged at low pH, the magnitude of $\Delta\delta$ for any resonance is expected to be larger than the value for the corresponding resonance of the unmodified cytochrome and similar to the values observed for *P. aeruginosa* c-551. However, δ values for the propionic acid resonances of the modified cytochrome were not measured. Two of the methyl resonances, M1 and M8, do show larger shifts than any methyl resonance of the unmodified *P. stutzeri* cytochrome and also show similar values of $\Delta\delta$ to M8 and M1 to *P. aeruginosa* c-551.

Effect of Ionizable Groups on Redox Potential. NMR data published for *P. aeruginosa* cytochrome c-551 (Moore et al., 1980) and data presented in this paper for the other two cytochromes c-551 indicate that no conformation changes accompany ionizations occurring in the ferricytochromes or the ferrocytochromes. The change in chemical shift position observed for the heme-substituent resonances can be explained in electrostatic terms if an ionization occurring close to the heme affects the electronic distribution within the heme ring.

We propose that the deprotonation of the heme inner propionic acid substituent in each of the four cytochromes c-551 discussed, and the coupled deprotonation of His-47 in three of these cytochromes, causes the observed fall in redox potential at alkaline pH. That the lowering of potential can be explained by a change in the net charge carried by His-47 and the propionic acid (or the propionic acid alone in *P. aeruginosa* c-551) is rationalized as follows: in ferricytochrome c-551 the heme iron carries a net positive charge whereas in the ferrocytochrome it has zero net charge. The presence of the positively charged His-47 close to the iron at low pH will tend to destabilize the ferricytochrome with respect to the ferrocytochrome while the presence of the negatively charged propionate at high pH will tend to stabilize the ferricytochrome with respect to the ferrocytochrome. This change in the relative stabilities of the two oxidation states will cause the redox potential to fall with increasing pH.

Acknowledgments

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Registry No. Cytochrome c-551, 9048-77-5; ascorbic acid, 50-81-7; ethoxyformic anhydride, 1609-47-8; histidine, 71-00-1; *N*-(ethoxyformyl)histidine, 27932-76-9.

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