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Characterization of Mutant Met100Lys of Cytochrome *c*-550 from *Thiobacillus versutus* with Lysine–Histidine Heme Ligation†

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ABSTRACT: The heme iron in cytochrome *c*-550 from *Thiobacillus versutus* has a methionine and a histidine as axial ligands. In order to study the characteristics of a possible lysine–histidine ligation in a heme protein, the methionine has been replaced by a lysine. This residue acts as a ligand between pH 3 and 12. The midpoint potential of the mutant has shifted −329 mV compared to wild type, but apart from this shift the pH dependence of the midpoint potential is unchanged, suggesting that the large drop is caused by specific ligand effects and not by protein refolding. While the EPR spectrum of wild-type cytochrome *c*-550 shows one species with $g_z = 3.35$, in the spectrum of the mutant two species occur with g_z values of 3.53 and 3.30. The intensity ratio of both species depends on the presence of organic cosolvents. In the low frequency region (−4 to −1 ppm) of the ^{1}H NMR spectrum of mutant ferrocytochrome *c*-550, four one-proton peaks replace the resonances of the ligand methionine side chain protons. Using two-dimensional NMR spectroscopy (COSY and NOESY), these protons and five others have been assigned to the lysine ligand. The spectroscopic results obtained for this mutant show similarities with those observed for the alkaline form of cytochrome *c*, supporting the Lys–His ligation proposed for this protein. The data are consistent with the evidence for amine ligation in cytochrome *f*: the EPR spectrum of M100K cytc-550 is similar to that of cytochrome *f*. However, the NMR spectra show significant differences. At high pH wild-type cytochrome *c*-550 shows a complex EPR behavior: at pH >10 new species are observed, with $g_z = 3.45$ (possibly due to a lysine–histidine ligation) and $g_z \approx 3.2$. At pH >11 a species with $g_z = 2.93$, $g_y = 2.23$, and $g_x = 1.67$ is observed. This new form is also seen for the M100K mutant and may represent lysine–histidinate ligation of the heme iron.

Cytochrome *c*-550 (cytc-550)[1] from *Thiobacillus versutus* is a protein with a single, covalently bound heme and is involved in bacterial electron transport. When *T. versutus* is grown on methylamine, the expression of two proteins, methylamine dehydrogenase (MADH) and amicyanin, is induced. MADH catalyzes the oxidation of the substrate and donates electrons via amicyanin to cytc-550. The latter can reduce a cytochrome aa_3 (van Wielink et al., 1989). The gene encoding cytc-550 has been cloned, and an expression system has been developed in *Escherichia coli* through which holoprotein can be obtained (Ubbink et al., 1992). Mutations of a surface lysine (K14) have been made to study the importance of this charge in the electron self-exchange reaction (Ubbink & Canters, 1993) and in reactions with amicyanin and MADH (manuscript in preparation).

It was also considered of interest to change one of the axial ligands of the heme iron, the methionine 100 (see Figure 1).

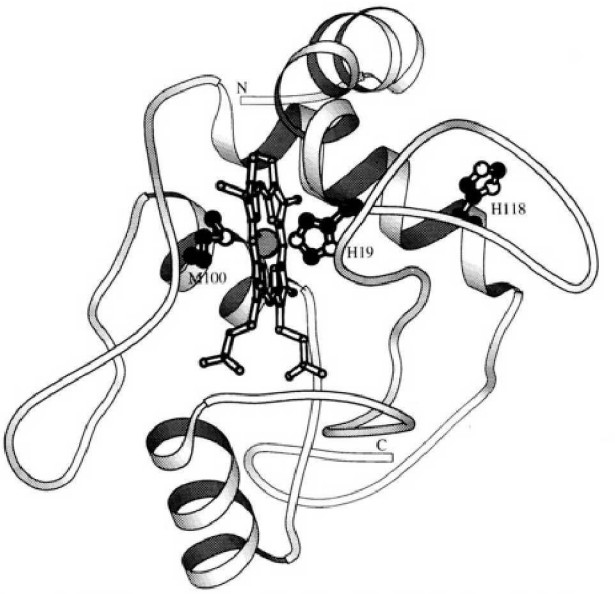

FIGURE 1: Ribbon representation (Kraulis, 1991) of a model of cytc-550 based on the protein structure of *P. denitrificans* cytc-550 (Timkovich & Dickerson, 1976). Heme and relevant residues are in ball-and-stick representation. N, and C indicate N- and C-terminus.

Cytc-550 of *T. versutus* is closely related to cytc-550 of *Paracoccus denitrificans* (84% of the residues is identical; Ubbink et al., 1992). The crystal structure of this protein has been solved and shows homology to mitochondrial cytochrome *c* (mcytc) (Timkovich & Dickerson, 1976). A wealth of information is available on mcytc, and many mutants have

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[1] Abbreviations: CV, cyclic voltammetry; cytc-550, cytochrome *c*-550; cytf, cytochrome *f*; EPR, electron paramagnetic resonance; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; M100K, mutation methionine 100 to lysine; MADH, methylamine dehydrogenase; mcytc, mitochondrial cytochrome *c*; Mops, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; Orn, ornithine; wt, wild type.

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been made (Moore & Pettigrew, 1990; Pettigrew & Moore, 1987; Mauk, 1991). However, few mutation studies have been aimed at the ligand methionine. Hampsey et al. (1986) have reported on two mutations of the Met ligand in yeast iso-1-cytochrome *c* to Arg and Ile in a wide range study on mutations of this protein. Semisynthesis has been used to specifically replace the Met80 of mcytc. Raphael and Gray (1989, 1991) synthesized a Cys, His, and Leu at position 80, while Wallace and Clark-Lewis (1992) introduced a variety of natural and nonnatural amino acids at this site. In these studies the characterization of the mutants was limited to optical properties, midpoint potential, and activity. Very recently a mutation of Met80 to Ala was reported (Lu et al., 1993). This form of mcytc has also been made via semisynthesis and has oxygen binding capability (Bren & Gray, 1993).

At high pH the Met ligand of ferri-mcytc is replaced by another residue. This is probably a lysine (Moore & Pettigrew, 1990). The alkaline transition is accompanied by a number of spectroscopic changes. The 695-nm absorbance band, ascribed to Met ligation, disappears. In the NMR spectrum the methyl peak of the Met ligand at circa −25 ppm is lost, and the characteristic heme methyl peaks are replaced by two new sets of peaks, indicating that alkaline mcytc exists in at least two forms (Hong & Dixon, 1989). It has been proposed that both forms have a Lys ligand but that different lysines coordinate in each form. Recently, evidence has been presented that in one of these K79 is the ligating residue (Ferrer et al., 1993). In the EPR spectrum alkaline mcytc shows several species depending on the buffer and the presence of organic cosolvents like glycerol (Gadsby et al., 1987). The midpoint potential of alkaline yeast iso-1-cytochrome *c* shifts −460 mV compared to the native protein (Barker & Mauk, 1992). Also in cytc-550 the alkaline transition is observed. The 696-nm absorbance disappears, and in the NMR spectrum one (not two) new set of heme methyl peaks replaces the resonances of the native protein (Lommen et al., 1990). However, the pK_a of the transition is much higher: 11.2 in cytc-550 (Lommen et al., 1990; Ubbink & Canters, 1993) versus 9.0 in horse heart mcytc (Davis et al., 1974). In this study the Met100 of cytc-550 has been changed into a lysine. The latter residue was chosen to create a cytc-550 that would mimic the alkaline form observed for many *c*-type cytochromes. M100K cytc-550 is very similar to the alkaline form of mcytc but can be studied at neutral pH and in the reduced form. Reduced alkaline mcytc is not stable (Wilson & Greenwood, 1971; Lambeth et al., 1973; Barker & Mauk, 1992).

Furthermore, this mutant may provide additional structural and mechanistic information for amine ligation, as in cytochrome *f* (cytf), the only known naturally occurring *c*-type cytochrome that has an amine-bound heme (Martinez et al., 1994). This protein functions as an electron carrier in photosynthesis. It is relatively large (285 aminoacids) and membrane bound (Gray, 1992). The structure of soluble turnip cytf shows that the amino terminus of the polypeptide chain (in which a tyrosyl is the first amino acid residue) is one of the heme iron axial ligands. Several studies had already suggested an amine axial coordination, although it was suggested that it came from a lysine residue. The spectroscopic properties gave no indications for a Met ligand (Gray, 1978; Siedow et al., 1980; Davis et al., 1988; Rigby et al., 1988; Simpkin et al., 1989). The midpoint potential of cytf is quite high (365 mV) (Gray, 1978).

Finally, the properties of this mutant can be compared with those of ornithine 80 (Orn80) mcytc. This protein is one of the semisynthetic cytochromes *c* produced by Wallace and Clark-Lewis (1992). The ornithine residue is very similar to a lysine residue [$NHCOCH(CH_2)_3NH_2$ and $NHCOCH-(CH_2)_4NH_2$, respectively]. The Met to Orn mutation affects the optical spectrum and shifts the midpoint potential considerably, from 260 to −40 mV.

Thus, a Met to Lys mutation can in principle create a protein with a type of heme ligation that otherwise is rare and not easily accessible to spectroscopic study. Here, the results are reported of optical, NMR, EPR, and cyclic voltammetry (CV) studies of M100K cytc-550. The properties of this mutant are compared with those of alkaline mcytc, cytf, and Orn80 mcytc.

MATERIALS AND METHODS

Construction of Mutation M100K. Site-directed mutagenesis was performed as described (Ubbink & Canters, 1993). The expression vector pMU19M100K is identical to pMU19 (Ubbink et al., 1992) except for the codon on position 100 which has been changed from ATG to AAG.

Purification of Cytc-550. Wild-type (wt) cytc-550 was isolated from *T. versutus* and *E. coli* and purified as described (Ubbink et al., 1992). M100K cytc-550 was heterologously expressed in *E. coli*. Procedures for production, isolation, and purification were identical to those of wt cytc-550. Yield of purified protein was ca. 1 mg/g of wet cell paste for both wt and M100K cytc-550.

Optical Absorption Spectroscopy. Optical spectra at pH 7.4 were obtained with 5.3 and 19 μM protein samples in 20 mM Hepes/NaOH buffer at room temperature. Total sample volume was 3 mL. The sample was reduced after flushing with Ar to remove oxygen. Small aliquots of a 0.01 M dithionite/NaOH solution under Ar were added until the spectrum did not change anymore. For high and low pH spectra of oxidized protein small amounts of 1 M NaOH or HCl were added to adjust the pH.

Spectra of bis-pyridine cytc-550 were obtained by addition of NaOH (0.15 M), pyridine (25% v/v), and some sodium dithionite crystals to solutions of wt or M100K cytc-550. Using $\epsilon_{550} = 31.18 \times 10^3\ M^{-1}\ cm^{-1}$ (determined for pyridine ox heart cytochrome *c*; Bartsch, 1971), the concentrations and extinction coefficients of the native proteins were calculated.

Cyclic Voltammetry. Cyclic voltammetry (CV) was performed in a 1-mL standard electrochemical cell utilising an edge-plane graphite working electrode, a platinum gauze counter electrode, a pH electrode, and a saturated calomel reference electrode (Hill & Hunt, 1993). The protein concentration was 0.10–0.15 mM in 50 mM of various buffers (see Results). The pH was adjusted with concentrated solutions of HCl and NaOH. Oxygen was removed from the working compartment by passing humidified oxygen-free argon through the sealed cell. Voltammograms were obtained, at room temperature, by cycling between 50 and 450 mV (wt) and between −300 and 200 mV (M100K), with the use of a computer-controlled potentiostat (Oxsys Ltd., Oxford). All the potentials reported in this paper are with respect to the normal hydrogen electrode.

EPR. X-band EPR was performed on a Bruker ESP380 spectrometer at 10 K. The experimental conditions were as follows; microwave power, 2.4 mW; modulation amplitude, 1.5 mT; gain, 8×10^4; frequency, 9.444 GHz. A Cu(II) signal was observed in all samples both with and without protein. Spectra of buffer only were subtracted from spectra with buffer plus protein to correct for this artifact. Small residual copper signals are, however, still present in the spectra. Simulations

were carried out with a computer program, which includes the Aasa and Vänngård formalism (Aasa & Vänngård, 1975).

NMR. Samples for NMR were prepared by several cycles of concentration and dilution in D_2O using an Amicon stirred cell with Millipore disc membranes (PGLC025). For samples at neutral pH*, 20 mM deuterated potassium phosphate was added as a buffer, while for saturation transfer experiments (see below) the ionic strength was increased with 100 mM NaCl to enhance the electron self-exchange rate (Ubbink & Canters, 1993). Samples were flushed with Ar. Since M100K is oxidized very easily by air, fully reduced samples were obtained by addition of a tiny amount of solid dithionite under a constant flow of Ar. The pH* was determined after the NMR measurements.

NMR experiments were performed on a Bruker WM300 (300 MHz), a Bruker DPX (300 MHz), and a Bruker AMX600 (600 MHz) spectrometer. Temperature settings were checked with a sample containing HDO and TMA in D_2O (Hartel et al., 1982). FIDs with a sweep width of 50 ppm of oxidized samples were stored in 16K or 32K memory; for reduced samples 8K or 16K was used (sweep width, 20 ppm). Solvent suppression was achieved by presaturation of the HDO signal during 0.5 s. Exponential multiplication of the FIDs was performed to improve the signal to noise ratio.

To determine the resonance positions of the heme methyl protons in the reduced forms of wt and M100K cytc-550, saturation transfer experiments were performed on mixtures of oxidized (ca. 1.3 mM) and reduced protein (3–4.5 mM). Resonances of heme methyls of oxidized cytc-550 were saturated during 2 s before acquisition, and difference spectra of off- and on-resonance saturation were used to determine the peak positions of the heme methyls in reduced cytc-550.

Double-quantum filtered COSY of the reduced M100K cytc-550 was performed with the Bruker microprogram "cosydfprtp" with a relaxation delay of 1 s. The increment time was 41.6 μs, and 800 increments were used. Per increment, 32 transients were obtained in 4K memory with 20 ppm sweep width. NOESY spectra were recorded with the "noesyprtp" program with 400 increments of 41.6 μs and 24 transients in 2K memory. The mixing time was 100 ms.

RESULTS

Optical Absorption Spectroscopy. The optical spectra of oxidized and reduced M100K cytc-550 at neutral pH show some differences with spectra of wt protein. In Figure 2 the spectra of the mutant in both oxidation states are presented. The 696-nm band of wt ferri-cytc-550 is absent in the mutant spectrum, in agreement with the removal of the Met residue. The α-band in mutant ferro-cytc-550 still has its maximum at 550 nm, but it is somewhat more intense than in wt cytc-550, as is the Soret band. The extinction coefficients of wt and M100K cytc-550 have been determined using the pyridine hemochromogen spectra (Bartsch, 1971). The values obtained with this method for wt cytc-550 are slightly lower (13%) than those obtained from a redox titration (Lommen et al., 1990). The extinction coefficients are listed in Table 1.

At low pH the spectra of oxidized wt and M100K change significantly and become identical. In both spectra the Soret peak broadens, and the band at 525 nm disappears, while a new band at 622 nm arises. In the wt spectrum also the 696-nm band is bleached. These phenomena have also been observed in mcytc [state II to I transition; first reported by Theorell and Åkesson (1941)]. This is characteristic for a transition from low- to high-spin coordination of the heme

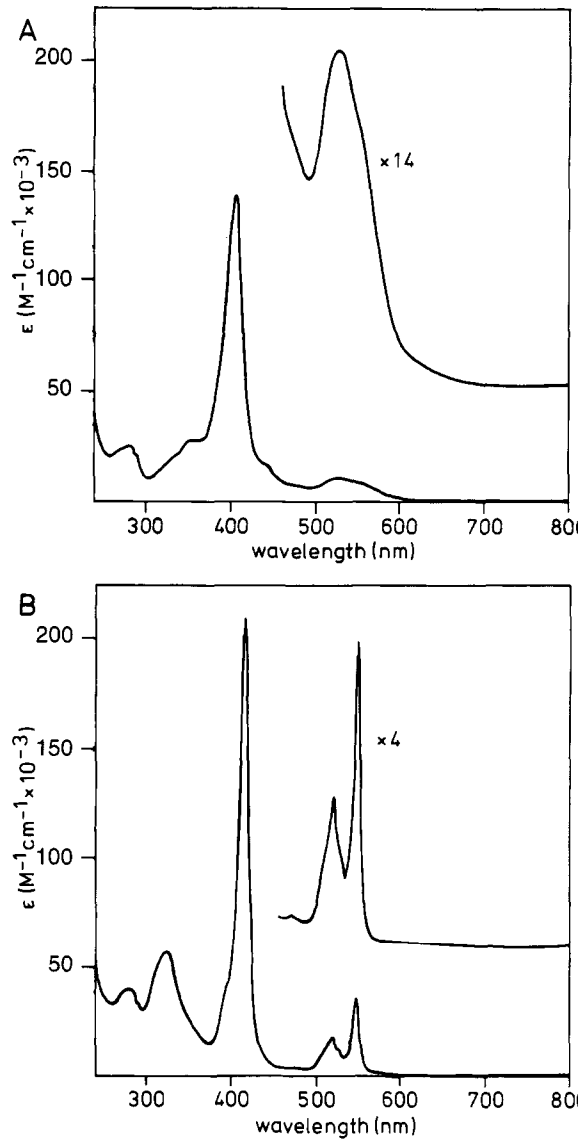

FIGURE 2: Optical spectra of M100K cytc-550. Protein concentration was 5.3 and 19 μM (enlargement in A) in 20 mM Hepes/NaOH buffer, pH 7.4. (A) Ferri-cytc-550; (B) ferro-cytc-550.

Table 1: Extinction Coefficients (M^{-1} cm^{-1} × 10^{-3}) of UV and Visible Absorbances of M100K and wt Cytc-550

oxidized				reduced			
wt		M100K		wt		M100K	
λ (nm)	ε	λ (nm)	ε	λ (nm)	ε	λ (nm)	ε
280	29	280	25	280	31	280	38
359	32	357	27	318	37	323	57
411	137	408	142	414	158	417	202
525	11.8	526	10.5	522	18	521	17
695	0.96			550	31	550	35

iron. The pK_a is 2.5 (0.1) for wt and 3.1 (0.1) for M100K cytc-550 (not shown).

The wt spectrum changes also at high pH. The 696-nm band disappears and the α/β-band broadens (the extinction coefficient decreases with 15%) and shifts from 525 to 532 nm with a pK_a of 11.2 (Ubbink & Canters, 1993). No changes are observed in the spectrum of M100K at this pH, but at higher pH a shift of the α/β maximum from 526 to 534 nm is seen, similar to the one in wt cytc-550. However, the extinction coefficient does not change noticeably. The pK_a of this shift has not been determined accurately but is approximately 12. The optical data indicate that the heme

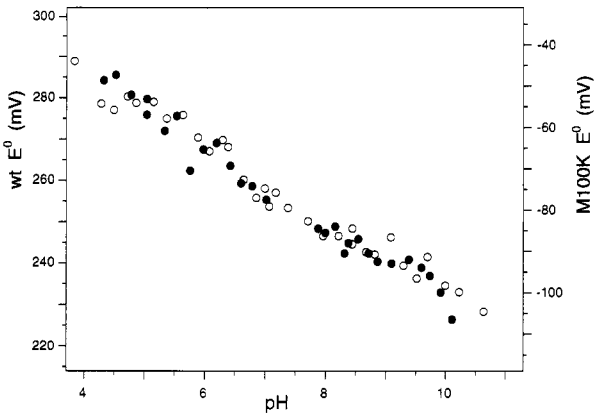

FIGURE 3: Midpoint potential as a function of pH. Data points (±3 mV) were obtained with CV. Protein concentration was 0.10–0.15 mM in 50 mM cacodylate/NaOH or Hepes/NaOH buffer. (Solid circles and left axis) wt cytc-550; (open circles and right axis) M100K cytc-550.

iron in M100K cytc-550 has a low-spin coordination between pH 3.1 and 12. Methionine is not one of the ligands.

Cyclic Voltammetry. The midpoint potentials of wt and M100K cytc-550 have been determined with CV as a function of pH. M100K cytc-550 gives an excellent response with an edge-plane graphite electrode, as has been observed for wt (manuscript in preparation). At pH 7.0 E^0 is 252 (3) mV for wt cytc-550 in cacodylate buffer (50 mM) for wt protein isolated from both *T. versutus* and *E coli*. The same value is found in Hepes buffer, but in sodium phosphate (50 mM) E^0 = 242 (3) mV. The midpoint potential determined with a potentiometric method has been reported to be 255 (5) mV (pH 7.0 in 100 mM Mops/KOH) (Lommen et al., 1990). M100K cytc-550 shows a shift of −329 (6) mV with E^0 = −77 (3) mV in cacodylate buffer.

Figure 3 presents the pH dependence of E^0. Wt cytc-550 shows a decrease from 285 (pH 4.4) to 228 mV (pH 10.1). The data do not allow the determination of pK_a values, but the curve is very similar to the potentiometrically determined curve (Lommen et al., 1990). Those data showed a sharp drop in E^0 above pH 10 due to the alkaline transition. Although few points are available in this region, a similar decrease appears to occur in the CV data.

M100K cytc-550 shows the same pH dependence between 4 and 10 as wt except for a shift of the whole curve by −333 mV. It is clear that introduction of the new ligand has lowered the E^0 dramatically. However, these results also indicate that the mutation has little effect on the protonatable groups that influence E^0, like, e.g., the heme propionate groups. We conclude that the overall conformation of the protein is not severely affected by the mutation.

EPR. Figure 4 shows the EPR-spectrum of wt (panel A) and M100K (panel B) cytc-550 at neutral pH. Table 2 lists the g values and the crystal field parameters V/λ, Δ/λ, and V/Δ (Blumberg & Peisach, 1972). Wt cytc-550 has crystal field parameters typical for a heme with Met–His ligation (wt form I) (Moore & Pettigrew, 1990; Palmer, 1985; Teixeira et al, 1993).

M100K cytc-550 shows two species with g_z values of 3.53 and 3.30 (M100K forms I and II). The intensity ratio of both species is independent of pH between 3 and 11 although the species at 3.30 shifts to 3.35 at pH <4. However, the ratio differs in different samples, and the 3.53 form nearly disappears when the sample contains 45% glycerol or 50% ethylene glycol (not shown). Both the occurrence of two species with these g values and their sensivity to organic cosolvents is remarkably

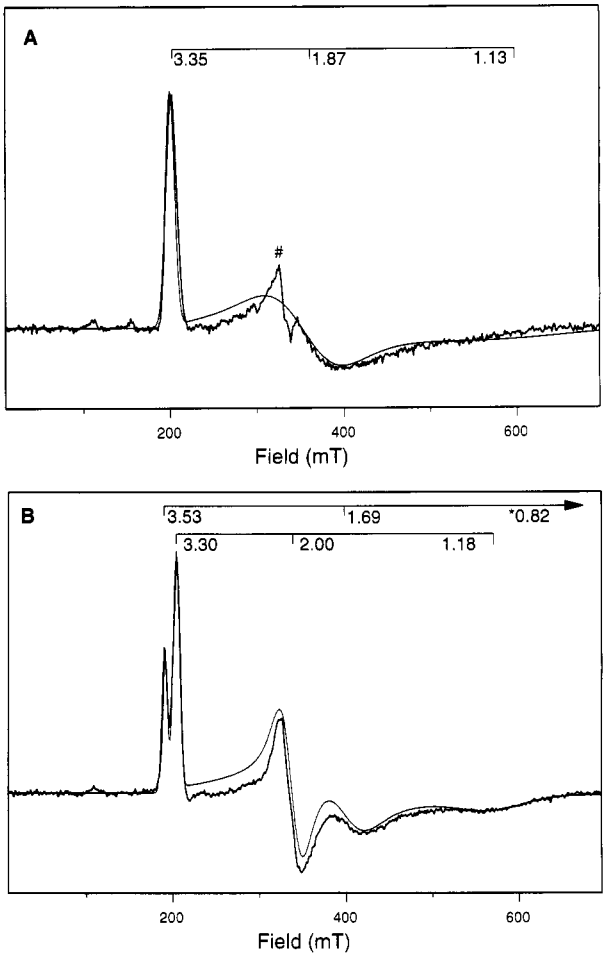

FIGURE 4: X-band EPR spectra of wt and M100K cytc-550. Protein concentration was 1–1.5 mM in 50 mM Mops/NaOH. Microwave power, 2.4 mW; modulation amplitude, 1.5 mT; gain, 8 × 10^4; frequency, 9.444 GHz; temperature, 10 K. Spectra of a sample with only buffer were substracted from the buffer + protein spectra to correct for Cu(II) signal. The residual Cu(II) signal is marked with a "#". The spectra were simulated (smooth line) using the g values indicated in the figure. (A) wt cytc-550 (pH 6.8); (B) M100K cytc-550 (pH 6.7). The g_x value labeled with an asterisk has been calculated with $\sum g_i^2$ = 16 using the experimental g_z and g_y values.

Table 2: g Values of Low-Spin EPR Species of wt and M100K Cytc-550 and of Alkaline Mcytc and Cytf.

EPR forms	g_x	g_y	g_z	pH range	V/λ	Δ/λ	V/Δ	proposed assignment
				Wild-Type Cytc-550				
I	1.13[a]	1.87	3.35	4–11.5	3.20	2.65	1.21	Met–His
II	nd[b]	nd[b]	≈3.45	10–11.9				Lys–His
III	nd[b]	nd[b]	≈3.2	10–11.9				
IV	1.67	2.23	2.93	>11.6	3.72	4.11	0.91	Lys–His⁻?
				M100K Cytc-550				
I	0.82[a]	1.69	3.53	5–11.6	3.45	2.51	1.37	Lys–His
II	1.18	2.00	3.30	5–11.6	3.08	2.73	1.13	Lys–His
III	1.67	2.23	2.93	>11.5	3.72	4.11	0.91	Lys–His⁻?
				Alkaline Mcytc[c]				
I	0.69	1.81	3.50	11	2.51	2.04	1.23	Lys–His
II	0.84	2.05	3.33	11	1.94	1.96	0.99	Lys–His
cytf[d]	<1.3	1.70	3.51	7.5	8.39	5.27	1.59	Lys–His
cytf[e]	1.6	2.07	3.48	8.0	7.77	5.63	1.38	Lys–His

[a] Assuming $\sum g_i^2$ = 16. [b] nd = not detected. [c] Gadsby et al. (1987). [d] Rigby et al. (1988). [e] Siedow et al. (1980).

similar to the EPR behavior of mcytc at high pH (Gadsby et al., 1987; see Table 2).

At pH 2 the EPR spectra of wt and mutant cytc-550 are identical, with characteristic high-spin ferriheme features (g_{max}

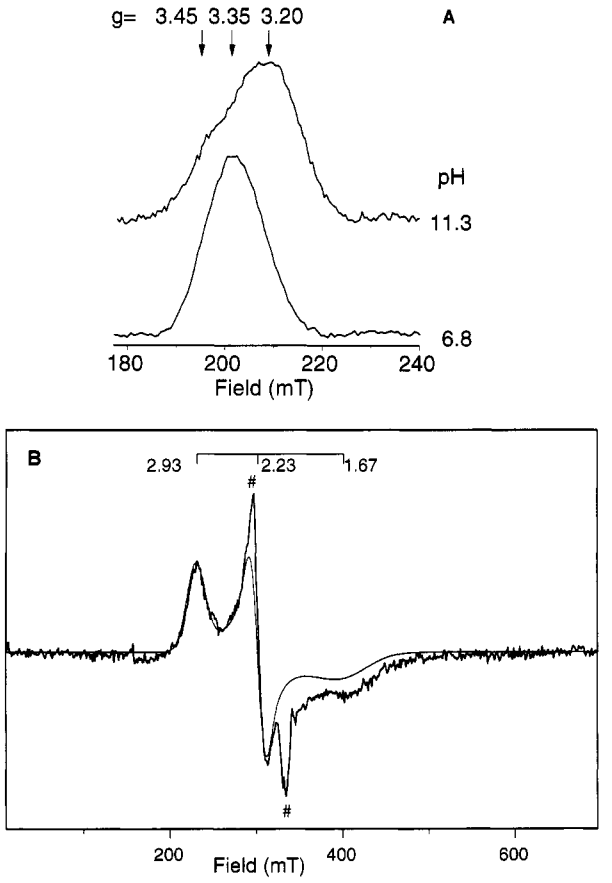

FIGURE 5: X-band EPR spectra of wt cytc-550. Protein concentration was 0.26 mM in water. The pH was adjusted with NaOH. Experimental conditions were as described in the caption of Figure 4. A spectrum with only buffer was subtracted from the buffer + protein spectrum to correct for Cu(II) signal. The residual Cu(II) signal is marked with "#". (A) High-frequency part of wt spectrum at pH 6.8 and 11.3. The numbers indicate g values. (B) Wt spectrum at pH 12.3. The spectrum was simulated (smooth line) using the g values indicated in the figure.

at 6.06, $g_{min} \approx 2$), in agreement with the optical high-spin properties.

The wt spectrum also changes at high pH. The behavior is complex: at pH >10 two new species appear (Figure 5A) with $g_z \approx 3.45$ (wt form II) and $g_z \approx 3.2$ (wt form III). Due to the proximity of the g values for these species, it was not possible to unambiguously deconvolute these spectra. These species may arise from the Met → Lys ligand replacement (alkaline transition). At pH >11 another species develops, with g_z at 2.93 (wt form IV). At pH 12.3 this is the major species (Figure 5B). M100K cytc-550 also shows this species with $g_z = 2.93$ (M100K form III) at a pH higher than ≈11.5. This species is tentatively assigned to a lysine–histidinate ligation of the heme iron, as is discussed below.

NMR. Figure 6A shows the ^{1}H NMR spectra of oxidized M100K cytc-550 (spectra a and b) and wt cytc-550 (spectrum c). The broad peak at −13 ppm in the wt spectrum, assigned to the Met100 methyl group (Lommen et al., 1990), is absent in the M100K spectrum. A relatively sharp one-proton peak (−5.41 ppm) and three broad peaks (−3.88, −9.50, and −12.93 ppm) appear in the low frequency region of the M100K spectrum. Very similar peaks are observed (at −5.23, −9.75, and −13.00 ppm) in the NMR spectrum of alkaline wt cytc-550 (Figure 8A, discussed below). The large shifts and line widths of these peaks indicate that the corresponding protons are located near the paramagnetic center and probably belong to one of the axial ligands. The heme methyl peaks in the

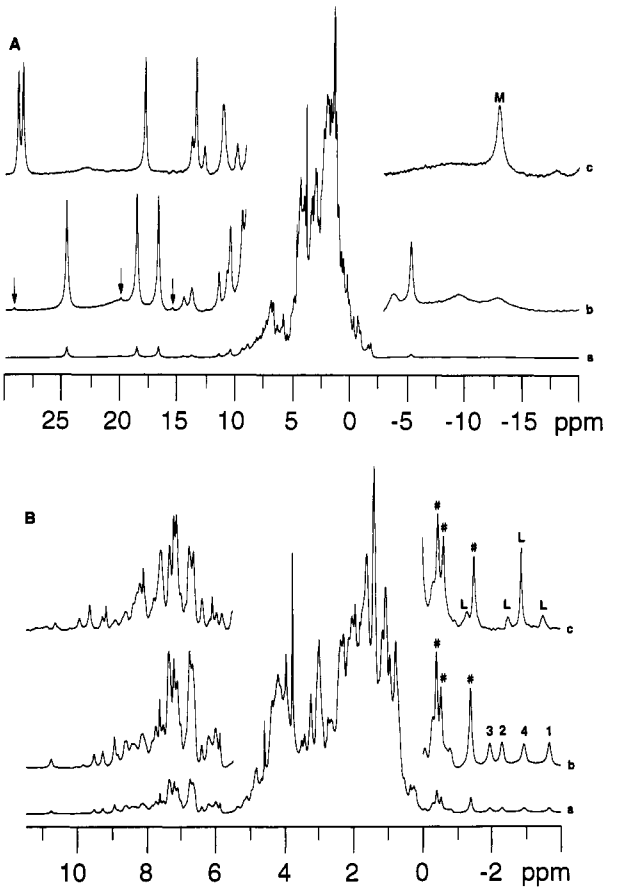

FIGURE 6: 300-MHz ^{1}H NMR spectrum of M100K and wt cytc-550. Protein concentration was 5.3 mM (M100K) and 1.9 mM (wt) in D_2O with 20 mM sodium phosphate, pH* 6.3. T = 313 K. (a) Full spectrum of M100K; (b) enlargement of resolved parts of the M100K spectrum; (c) resolved parts of wt spectrum. (A) Ferric proteins; the arrows indicate the positions of the heme methyls of the minor species (see text). M, M100 methyl resonance (Lommen et al., 1990). (B) Ferrous proteins; the numbers refer to Figure 7. The "#" indicate low-frequency peaks that are nearly unperturbed in the mutant spectrum. L, Met100 side chain proton resonances (Lommen et al., 1990). The sharp peak at 3.8 ppm derives from a contamination.

Table 3: Chemical Shift (ppm) of Heme Methyl Peaks in the ^{1}H NMR Spectrum of M100K and wt Cytc-550[a]

	reduced		oxidized	
heme methyl	wt	M100K	wt	M100K
18^1	2.31	2.35	28.76	16.65
7^1	3.24	n.o.	28.16	n.o.
2^1	3.51	3.42	13.05	18.68
12^1	3.58	3.51	17.38	24.80

[a] Samples contained 20 mM phosphate buffer, pH* 7, and 100 mM NaCl in D_2O. T = 312 K. n.o. = not observed.

M100K spectrum are shifted to lower frequency compared to the wt spectrum (Figure 6A and Table 3). Also in alkaline mcytc (Gupta & Koenig, 1971) and cytf (Rigby et al., 1988) the heme methyls show smaller chemical shifts than in c-type cytochromes with Met–His ligation. Three heme methyl peaks can clearly be distinguished; the fourth one is disguised by other peaks. Three very small peaks with large high-frequency shifts are also present at 29.08, 19.08, and 15.38 ppm, suggesting the presence of a second species of M100K in minor quantities (<2%).

The spectrum of reduced M100K cytc-550 (Figure 6B, spectra a and b) shows four new one-proton peaks in the low-frequency region (labeled 1–4), while the peaks assigned to Met100 (Figure 6B, spectrum c) (Lommen et al., 1990) are

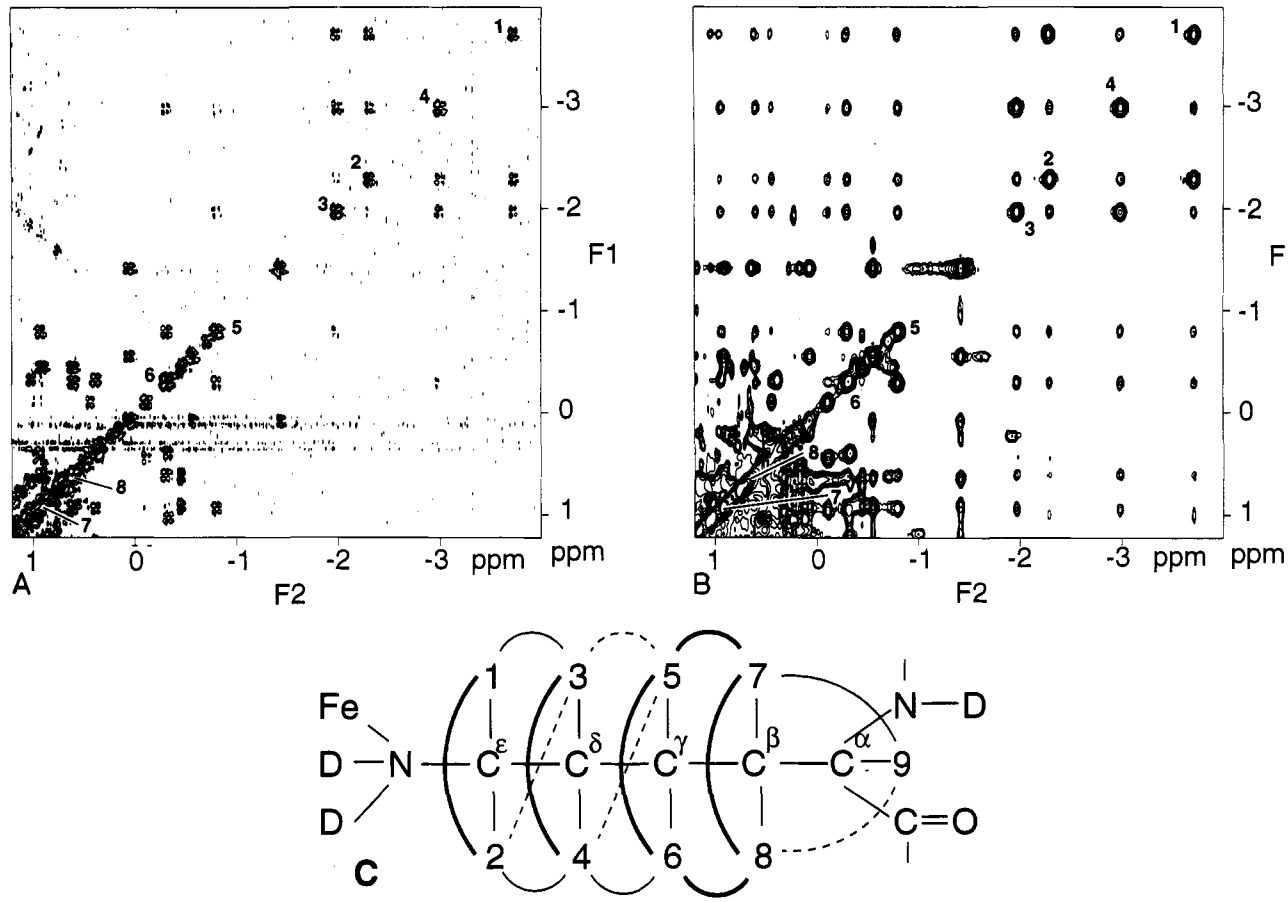

FIGURE 7: Two-dimensional 600-MHz ^{1}H NMR spectra of M100K ferro-cytc-550. The protein concentration was 5.3 mM in D_2O with 20 mM sodium phosphate, pH* 6.3. T = 313 K. (A) Low-frequency part of a COSY spectrum; (B) low-frequency part of a NOESY spectrum (mixing time 100 ms); (C) schematic representation of the lysine ligand in M100K cytc-550 showing the couplings observed in the COSY spectrum. The numbers represent the protons of the lysine and correspond with the numbered peaks in the spectra (see Table 4 for assignments). (Bold bows) Strong cross peaks; (thin bows) average cross peaks; (dashed bows and lines) weak cross peaks.

Table 4: Chemical Shifts (ppm) of the Ring-Current-Shifted Lysine Protons in the ^{1}H NMR Spectrum of Reduced M100K Cytc-550[a]

nr[b]	chemical shift (ppm)	nr[b]	chemical shift (ppm)
1 $C^{\epsilon}H$	−3.70	5 $C^{\gamma}H$	−0.79
2 $C^{\epsilon}H'$	−2.27	6 $C^{\gamma}H'$	−0.28
3 $C^{\delta}H$	−1.96	7 $C^{\beta}H$	0.94
4 $C^{\delta}H'$	−2.98	8 $C^{\beta}H'$	0.61
		9 $C^{\alpha}H$	4.27

[a] Protein concentration was 5.3 mM in 20 mM phosphate buffer in D_2O, pH* = 6.3, and T = 313 K. [b] See Figure 7C.

absent. Figure 7 shows parts of a COSY (A) and a NOESY (B) spectrum of reduced M100K cytc-550. Cross peaks are observed between the new one-proton peaks. Peaks 1 and 2 show a strong coupling and an intense NOE cross peak, just as peaks 3 and 4, while less intense cross peaks are present between peaks 1 and 3 and between 2 and 4. This suggests that protons 1 and 2 are geminal protons, as are protons 3 and 4. Peaks 1 and 2 show no further couplings and are therefore assigned to the $C^{\epsilon}H$ and $C^{\epsilon}H'$ protons of a lysine (see below). Peak 3 and 4 ($C^{\delta}H$ and $C^{\delta}H'$) give cross peaks with the peaks of the protons 5 and 6 ($C^{\gamma}H$ and $C^{\gamma}H'$) while these couple to peaks 7 and 8 ($C^{\beta}H$ and $C^{\beta}H'$). Peak 7 and 8 show a strong and a weak coupling to a peak at 4.27 ppm, respectively (not shown). The corresponding proton (9) is assigned to the $C^{\alpha}H$. All the couplings observed in the COSY spectrum for this spin system are shown in Figure 7C. Table 4 contains the chemical shifts of the nine protons. The residue is thus identified as a lysine. The shifts to low frequency increase along the side chain in the C^{α} to C^{ϵ} direction, analogous to the Met ligand protons. These data, together with the low-spin optical absorbance spectrum, are taken as evidence that a lysine is the sixth ligand in M100K. The N^{ζ}-protons are not observed; they have probably been exchanged for deuterons.

Other proton resonances in the high-frequency region of the spectrum of reduced M100K are relatively unperturbed, judged from their peak positions in mutant and wt spectra (see, for instance, Figure 6B, peaks marked with a "#"). Also, the resonances of the heme methyls show only small shifts in comparison to the spectrum of wt cytc-550. Table 3 gives the chemical shifts of the heme methyls for the oxidized and reduced proteins. The resonances of the heme methyls of wt cytc-550 have been assigned before (Lommen et al., 1990; M. Ubbink, unpublished results). The assignment of the heme methyl peaks of M100K cytc-550 is based on the similarity of their chemical shifts in the reduced protein with those of wt cytc-550 and is therefore tentative, especially for heme methyls 2^1 and 12^1 (IUPAC-IUB nomenclature). From the fact that the peaks at high frequency and the heme methyl resonances show only small shifts, it is concluded that the environment of the heme has not been changed drastically by the mutation M100 → K.

At high pH* (11.6) a new set of heme methyl peaks appears in the spectrum of wt ferri-cytc-550 due to the alkaline transition (Figure 8A, at 22.28, 21.82, and 18.92 ppm, labeled with a "#"). No evidence exists for more than one alkaline form of cytc-550, unlike the case of mcytc (Hong & Dixon, 1989; Ferrer et al., 1993). The M100K spectrum remains essentially unchanged up to pH* 12.0, apart from small pH*-

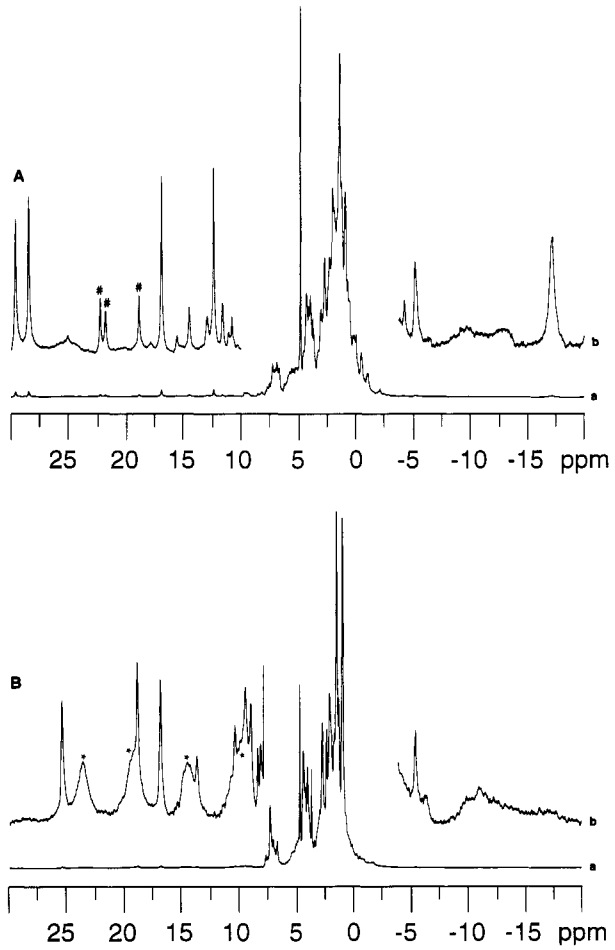

FIGURE 8: 300-MHz ^{1}H NMR spectrum of oxidized cytc-550 at high pH*. Protein concentration was ca. 2 mM in D_2O. (a) Full spectrum; (b) enlargement of resolved parts. (A) wt at pH* 11.6 and T = 292 K. The "#" indicate the heme methyl peaks of the alkaline form. (B) M100K at pH* 12.3 and T = 313 K. The asterisks indicate peaks belonging to the high-pH form of the protein. The sharp peak at 3.8 ppm derives from a contamination.

dependent shifts (0.3–0.5 ppm to higher frequency). At pH* 12.3 the heme methyl peaks are replaced by new, very broad peaks (Figure 8B, at 23.50, 19.15, 14.40, and possibly 9.8 ppm, labeled with an asterisk).

DISCUSSION

Lysine Ligation. The characteristic low-frequency resonances of the Met100 side chain observed in the ^{1}H NMR spectrum of wt cytc-550 have been replaced in the M100K spectrum by other resonances. These are assigned to a lysine side chain, strongly suggesting that a Lys acts as the sixth ligand of the heme iron in ferro-cytc-550.

Less direct evidence exists for lysine ligation in the oxidized form. The absence of both the 695-nm absorbance band and the characteristic −16 ppm ^{1}H NMR resonance strongly argue against Met ligation. Apart from the ligand histidine (His19) cytc-550 contains one other His residue (His118) that could in principle act as a ligand, but the NMR data and the high similarity between the pH dependences of E^0 of wt and M100K cytc-550 suggest only little conformational change in the mutant and not an extensive rearrangement as would be necessary for ligation by His118 (see Figure 1). However, the mutant has a low-spin heme Fe as indicated by the electronic absorbance spectrum, and thus a strong sixth ligand must be present. The optical extinction coefficients show the same increase (compared to wt) as those of Orn80 mcytc (Wallace & Clark-Lewis, 1992). Also the shift in E^0 is similar for M100K cytc-550 (−329 mV) and Orn80 mcytc (−300 mV). On the basis of these similarities and of the fact that the reduced form has Lys-ligation, the sixth ligand in oxidized M100K cytc-550 is also assigned as a lysine.

From mutagenesis studies it has emerged that a lysine side chain can be buried inside a protein, provided that it is unprotonated (Dao-pin et al., 1991; Stites et al., 1991). This would favor its coordination to the heme iron in the case of M100K cytc-550. At pH 3.1 the protein converts to high spin. The pK_a of the lysine is thus $\leq$3.1. This means a pK_a shift of at least seven orders compared to a solvated lysine side chain (p$K_a \geq 10$). The shifts in pK_a of buried lysines reported by Dao-pin et al. and Stites et al. are smaller (pK_a = 6.5) probably because these lysines are not involved in ligation.

It can be questioned whether the ligand in M100K cytc-550 is K100 or one of the other Lys residues. The most obvious other candidate would be the lysine that replaces the Met ligand in the alkaline form of wt cytc-550. It is unknown which lysine coordinates the iron at alkaline pH, although mutagenesis studies have excluded Lys14 (Ubbink et al., 1992) and Lys99 (results to be published). In view of the structure model of *T. versutus* cytc-550, Lys84, Lys97, and Lys103 remain as candidates, all of which require a considerable rearrangement of the protein chain to coordinate the iron. For M100K cytc-550 no indications for such a rearrangement exist. Furthermore, the heme methyl peaks in the NMR spectrum of the alkaline form appear at 22.27, 21.82, and 18.92 ppm (Figure 8A, marked with an "#") while the heme methyl peaks in the spectrum of M100K cytc-550 occur at 24.80, 18.68, and 16.65 ppm (Table 3) and show only small pH-dependent shifts (to 25.31, 18.77, and 16.77 ppm at pH* 12.3; see Figure 8B). Therefore, it seems unlikely that in the alkaline form of wt and in M100K cytc-550 the same Lys is coordinating. It is tentatively concluded that K100 is the ligating residue.

Comparison with Similarly Coordinated Proteins. The low E^0 of M100K cytc-550 is similar to those of alkaline mcytc and Orn80 mcytc but unlike the midpoint potential of cytf. It is intriguing how cytf can have such a high E^0, while it also has an amine as sixth ligand. The low values in the other proteins may be a result of both the difference in the nature of the axial ligands (N-terminal amine in cytf and lysine amine in the other proteins) and a higher solvent accessibility of the heme.

The EPR of M100K cytc-550 is very similar to that of alkaline mcytc (Gadsby et al., 1987), exhibiting two species and a sensivity to organic cosolvents (see Table 2). The strong effect of glycerol and ethyleneglycol on the intensity ratio of the two species suggests that the occurrence of two forms may be a freezing artifact. It is conceivable that the change in temperature affects the ratio of two conformations of the protein that are in fast exchange at room temperature. From the NMR spectra it appears that at room temperature for oxidized M100K cytc-550 a second form is present at very low concentrations. It is noted that two forms of alkaline mcytc are observed in the NMR spectrum at room temperature, with different lysines ligated to the heme iron (Hong & Dixon, 1989; Ferrer et al., 1993). Probably, however, no relation exists between these two forms and the occurrence of two species in the EPR spectra, since alkaline K79A mcytc shows only one form in the NMR spectrum, while in the EPR spectrum still two alkaline species are observed (Ferrer et al., 1993). In any case, the high similarity in EPR data of M100K cytc-550 and alkaline mcytc strongly suggests that the axial

ligands in both proteins are identical. This would support the idea that in alkaline mcytc the iron in the heme group is ligated by a Lys and a His.

The NMR and EPR data of M100K cytc-550 and cytf also show similarity. The reduced form of cytf has two peaks in the −1 to −4 ppm range of the NMR spectrum (Rigby et al., 1988), while the spectrum of M100K cytc-550 shows four one-proton peaks, assigned to the C^{δ} and C^{ϵ} protons. The two peaks in the cytf spectrum can perhaps be assigned to the C^{β} protons of Tyr1. Since these protons are located near the heme plane in the cytf structure (Martinez et al., 1994), their resonances can be expected to show a large shift to lower frequency, in analogy to the resonances of the lysine side chain protons in M100K cytc-550. Two sets of g values have been reported for the EPR spectrum of cytf. The g values reported by Siedow et al. (1980) are very different from the ones of M100K cytc-550, while the reinterpretation of the cytf EPR spectrum by Rigby et al. (1988) gives g values very similar to form I of M100K cytc-550 (see Table 2), as would be expected for the similar amine coordination. The second species in the M100K cytc-550 spectrum (at $g_z = 3.30$) is not observed for cytf.

Spectroscopic Changes at High pH. Various spectroscopic changes are observed at high pH (>10) for wt cytc-550; in the optical spectrum the 696-nm band disappears ($pK_a = 11.2$) and the α/β-band broadens and shifts (Ubbink & Canters, 1993). In the EPR spectrum the species with $g_z = 3.35$ is replaced by two species with $g_z = 3.45$ and 3.2; at still higher pH these are converted to a form with $g_z = 2.93$. In the NMR spectrum one set of heme methyl peaks is replaced by another.

The loss of 696-nm band and the changes in the NMR spectrum reflect the alkaline transition (Met → Lys ligand replacement). The EPR form with $g_z = 3.45$ (wt form II) presumably represents the alkaline species (Lys–His ligation), by comparison with the alkaline mcytc spectra (Gadsby et al., 1987) and the neutral form of M100K cytc-550. The $g_z \approx 3.2$ species (wt form III) is presently unassigned and may be associated either with an intermediate form of the alkaline transition process and/or with another deprotonation step affecting the heme environment [see, e.g., Campos et al. (1993)].

In the optical spectrum of M100K cytc-550 no 696-nm band is present and in the NMR spectrum no replacement of the heme methyl peaks is observed up till pH* 12. Only at still higher pH* new, very broad peaks arise in the NMR spectrum, and the heme methyl resonances start to disappear. This is ascribed to a loss of low-spin coordination, perhaps due to protein denaturation. It is concluded that in M100K cytc-550 the coordinating Lys is not replaced by another lysine at high pH (no alkaline transition).

Still, at pH 12 the EPR spectrum of the mutant becomes identical to that of wt cytc-550. This EPR form (M100K form III/wt form IV) with g values of 2.93, 2.23, and 1.67 is tentatively assigned to a lysine–histidinate ligation of the heme iron, since the tetragonality [$\Delta/\lambda = 4.11$ in the Blumberg and Peisach formalism (Blumberg & Peisach, 1972)] of the species places it in a group of model heme compounds with histidinate coordination (Gadsby & Thomson, 1990; Moore et al., 1985). A more definite assignment may be obtained using magnetic circular dichroism in the near-infrared region on the high pH forms of wt and M100K cytc-550. It is noted that Gadsby et al. (1987) have mentioned very similar g values for mcytc at high pH (>12) without any assignment.

The pH dependence of the histidinate deprotonation (conversion to the EPR species with $g_z = 2.93$ of wt and M100K cytc-550) is approximately the same as that of the shift from 526 to 534 nm of the α/β-band in the optical spectra of wt and M100K cytc-550. This shift could therefore represent the same deprotonation process. Apparently, this shift of the optical band is not a result of the alkaline transition, since it occurs both in wt and M100K cytc-550.

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