Mutagenesis of the Conserved Lysine 14 of Cytochrome c-550 from *Thiobacillus versutus* Affects the Protein Structure and the Electron Self-Exchange Rate

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**Abstract:** The lysine residue K14 of cytochrome c-550 of *Thiobacillus versutus* has been mutated to a glutamine (Q) and a glutamate (E) residue. These mutations have a minimal effect on the $pK_a$ for replacement of the methionine ligand (the "alkaline transition"), indicating that a presumptive salt bridge between K14 and E11 does not help stabilize the native form. This is in contrast with mitochondrial cytochrome c, where the homologous K13 forms a structurally important salt bridge with glutamate 90. The NMR signals of protons close to the heme iron in wild-type and mutant ferricytochrome c-550 shift considerably with increasing ionic strength. These effects resemble those seen in mitochondrial cytochrome c upon addition of salt and upon complex formation with redox partners. It is likely that electrostatic screening of positive charges near the heme crevice leads to a slight redistribution of the electron density in the heme. At low ionic strength the NMR spectrum of wild-type cytochrome c-550 shows broad peaks. Line widths decrease upon addition of salt up to 200 mM. In K14Q and K14E cytochrome c-550 the line widths are much smaller at low ionic strength. Wild-type cytochrome c-550 may exist in two exchanging conformations, one of which may represent a more open (non-native) form, in analogy with cytochrome c. However, in the case of cytochrome c-550 this non-native form does not show ligand replacement. The electron self-exchange rates of wild type and mutants have been determined as a function of the ionic strength. At zero ionic strength these rates are $2.1 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ (wild type), $7.2 \times 10^3 \text{M}^{-1} \text{s}^{-1}$ (K14Q), and $1.2 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ (K14E); with increasing ionic strength the differences between these rates decrease. In the presence of 0.55 M NaCl the rate for wild-type cytochrome c-550 is $1.0\times 10^5 \text{M}^{-1} \text{s}^{-1}$.

Cytochrome c-550 (cytc-550) from *Thiobacillus versutus* was described for the first time by Lu and Kelly (1984). Its function was unclear until it was suggested in 1989 (van Wielink et al., 1989) that cytc-550 has a role in the methymaline dehydrogenase redox chain of *T. versutus*. The enzyme methymaline dehydrogenase can oxidize methymaline and transfer electrons to ferricytochrome c-550, either directly or via the blue copper protein amicyanin. Ferrocyan-c-550 can then be oxidized by a cytochrome c oxidase. Because the redox chain represents a good system for the study of biological electron transport at the molecular level, its constituent proteins are under intensive study (Ubbink et al., 1992, and references therein). Cytc-550 has been characterized with several spectroscopic techniques and was identified as a class I cytochrome c (Lommen et al., 1990). The gene encoding cytc-550 has been cloned and expressed. Holoprotein can be produced in *Escherichia coli* under semianerobic growth conditions and can be isolated from the periplasmic space (Ubbink et al., 1992).

Cytc-550 from *T. versutus* is similar to cytc-550 from *Paracoccus denitrificans* (Lommen et al., 1990; Ubbink et al., 1992). From the crystal structure of the latter protein (Timkovich & Dickerson, 1976) it emerged that cytc-550 is a close relative of cytochrome c from mitochondria. An interesting difference between cytc and cytc-550 from *T. versutus* is the $pK_a$ of the "alkaline transition." At high pH the methionine ligand of the heme iron is replaced by another residue, probably a lysine (Moore & Pettigrew, 1990; Pettigrew et al., 1993). The $pK_a$ for this transition is between 9 and 10 in mitochondrial cytochromes c but higher than 11 in cytc-550 (Lommen et al., 1990). In cytc the conserved lysine 13 (K13) plays an important role in maintaining the $pK_a$ at 9–10. Chemical modification of this residue lowers the $pK_a$ considerably (Osheroff et al., 1980). These authors attributed this to the loss of the salt bridge between K13 and E90. In both *P. denitrificans* and *T. versutus* cytc-550 K13 is conserved as K14, but E90 is replaced by an asparagine (N107) (see Figure 1), and thus the salt bridge K13–E90 is not conserved. However, examination of the crystal structure of *P. denitrificans* cytc-550 indicates that K14 is close to E11 (3.17 Å), although the relatively low resolution of the structure (2.45 Å) hinders accurate determination of salt bridges and hydrogen bonds (Timkovich, Miller & Dickerson, 1976). A salt bridge in this position could take over the function of the K13–E90 salt bridge. Still, the bridge between K14 and E11 would be "intrahelical", connecting two residues in the N-terminal α-helix (see Figure 1), unlike the K13–E90 salt bridge which is "interhelical" and connects the N-terminal with the C-terminal α-helix.

To establish the importance of the presumptive salt bridge K14–E11 in maintaining the high $pK_a$ of the alkaline transition, we have mutated K14 to a glutamine (K14Q) and a glutamate residue (K14E). Many investigations have indicated that K13 also plays a key role in the interactions with other redox proteins (Kornblatt et al., 1992; Hahn et al., 1992; Pelletier & Kraut, 1992; Roberts et al., 1991; Moore & Pettigrew, 1990; Pettigrew & Moore, 1987) and with small molecules (Rush et al., 1988) and in the electron self-exchange reaction (Concar et al., 1986).
The analogous lysine in cytochrome $c_2$ is involved in the complex formation with cytochrome $b_{5}$ (Bosshard et al., 1987) and the photosynthetic reaction center (Caffrey et al., 1992). In the present case the mutants of cytc-550 also permit the study of this aspect of the possible role of K14. We investigated with $^1$H-NMR the electrostatic and structural effects of these mutations on the heme and its direct surroundings. Furthermore, the electron self-exchange (ese) rates of wild-type, K14Q, and K14E cytc-550 have been measured as a function of the ionic strength. The results are reported here.

**MATERIALS AND METHODS**

**Site-Directed Mutagenesis.** To obtain mutants of cytc-550 the PstI–BamHI restriction fragment MU19 (Ubbink et al., 1992) was cloned into bacteriophage M13 mp18. On this fragment are present the coding sequence for cytc-550 and its Shine–Dalgarno sequence. Mutations were introduced by using uridine-enriched single-stranded DNA of this clone and synthetic oligodeoxynucleotides according to the method described by Kunkel et al. (1991).

The complete cytc-550 gene was sequenced in clones that contained the mutation to check that no other, unwanted mutations were present. The mutated MU19 fragment was then cloned into vector pUC19, and the HindIII site of this vector was filled like in the original expression vector for cytc-550, pMU19 (Ubbink et al., 1992). The vectors pMU19K14E and pMU19K14Q contain the codons GAG (glutamate) and CAG (glutamine) at the position of lysine 14, respectively.

**Production and Purification of Proteins.** Wild-type cytc-550 from *T. versutus* was isolated and purified as described (Ubbink et al., 1992). Mutant proteins K14E and K14Q were produced in *Escherichia coli* and isolated from the periplasmic space with osmotic shock treatment (Ubbink et al., 1992); purification of both mutants was performed as in the case of heterologous wild-type cytc-550 (Ubbink et al., 1992) except that chromatography on S-Sepharose was done at pH 5.0 in 10 mM sodium acetate instead of at pH 6.0 in phosphate buffer. This was necessary because the mutant K14E did not bind to S-Sepharose at pH 6.0.

**Determination of $pK_a$ Values.** Protein solutions of 60–70 $\mu$M in 10 mM sodium phosphate and 80 $\mu$M potassium ferricyanide (added to prevent autoreduction of cytc-550 at high pH) were used to measure the absorbance at 696 nm as a function of pH. The pH was altered by addition of small aliquots of NaOH solution. Total volume was 2.2 mL. The sample holder was kept at 24 °C.

**Preparation of NMR Samples.** Protein samples for NMR were prepared by repeated dilution and concentration in $D_2O$ in an Amicon stirred ultrafiltration cell with disc membranes from Millipore (PGLC025). Reduced samples were obtained by addition of a slight molar excess of sodium dithionite in NaOD solution followed by dilution–concentration cycles to remove the reductant. Tiny amounts of ascorbate/NaOD in $D_2O$ were added to maintain a reducing environment or to adjust the oxidized/reduced protein ratio in partially reduced samples.

The uncorrected pH* of all samples was adjusted with DCI and NaOD solutions to 6.0. The ionic strength was altered with aliquots of 4 M NaCl in $D_2O$. Samples were flushed with argon to remove oxygen.

**NMR Measurements.** All measurements were performed on a 300-MHz Bruker WM300 spectrometer at 313 K. Free induction decays of samples containing oxidized cytc-550 were stored in 16K memory (spectral width 50 ppm); for reduced samples 8K was used (20 ppm). The HDO signal was suppressed by presaturation in all but the saturation-transfer experiments.

$T_1$ and $T_1^*$ (see below) measurements were done with a nonselective $\pi-\tau-\pi/2$ pulse sequence; the $\pi$-pulse consisted...
of a \((\pi/2)x-\pi_x-(\pi/2)x\) sequence (Leviti & Freeman, 1979). For each experiment 8–10 different values of \(\tau\) were used; for each \(\tau\) value 80–192 scans were acquired. Peak intensities from the spectra were used as data points for an exponential fit to calculate the \(T_1\) value. Spectra were resolution-enhanced for \(T_1\) determination of the broad M100 methyl peak of oxidized cytochrome-c-550. \(T_2\) values were determined from the line width at half-height.

Saturation-transfer experiments were performed on mixtures of reduced and oxidized cytochrome-c-550. A 4-σ specific saturation pulse was applied either on the M100 methyl peak of oxidized cytochrome-c-550 or off-resonance, followed by a \(\pi/2\)-pulse and signal acquisition. The delay between two scans was 2.5 s. The effect on the magnetization of this methyl group in the reduced protein was determined by integration of the corresponding 1H-NMR signal.

In mixtures of oxidized and reduced protein the concentration ratio of the two was determined by integration of methyl peaks of each species. After the NMR experiments, the sample was diluted and the total concentration of protein was measured optically. In esr measurements the concentration of ferrocytochrome-c-550 was circa 2 mM; the concentration of ferricytochrome-c-550 varied between 0.2 and 1 mM.

**Calculation of the Electron Self-Exchange Rate.** Several methods were used to determine the electron self-exchange (ese) rate: (a) saturation-transfer experiments, (b) \(T_1\) measurements, and (c) \(T_2\) measurements.

(a) From saturation-transfer experiments the first-order rate constant \(k\) was determined with (Lommen et al., 1990)

\[
I/I_0 = T_1^{-1}/(T_1^{-1} + k) \tag{1a}
\]

where \(I\) and \(I_0\) are the integrals of a particular peak in the NMR spectrum of reduced cytochrome-c-550 in on- and off-resonance experiments, respectively. \(T_1^{-1}\) is the reciprocal of the longitudinal relaxation time, \(T_1\), of the integrated peak. Subsequently the ese rate was determined from the experimentally determined \(k\) value with

\[
k = k_{\text{ese}}[\text{oxidized cytochrome-c-550}] \tag{1b}
\]

(b) The ese rate was also calculated from direct \(T_1\) measurements as follows. After a perturbation of its equilibrium magnetization, the longitudinal relaxation of a proton that is in exchange with a second, unperturbed site is described by a biexponential curve of the form

\[
M(t) = K_1 \exp(-\lambda_1 t) + K_2 \exp(-\lambda_2 t) + M(0) \tag{2}
\]

where \(M(t)\) and \(M(0)\) are the magnetization at time \(t\) and at equilibrium, respectively. \(\lambda_1\) and \(\lambda_2\) are the reciprocals of two apparent \(T_1\) times, and \(K_1\) and \(K_2\) are two preexponential factors (Dixon et al., 1989). Since the pulses applied for the measurements were nonselective, the magnetizations in both surroundings (oxidized and reduced) are perturbed. In that case eq 2 is valid only if the oxidized species can be regarded as instantaneously relaxed. It was found that this is true when the apparent \(T_1\) is more than 5 times the \(T_1\) of the oxidized species (4.3 ms).

It can be shown that, under the conditions used, and with self-exchange rates between \(10^5\) and \(10^7\) M\(^{-1}\) s\(^{-1}\), \(K_2\) is insignificant and \(1/\lambda_2\) is very short. Therefore the second term in eq 2 can be neglected, and the relaxation can be described by a single-exponential curve with time constant \(1/\lambda_1\) (see also Figure 8 in the Results section). The apparent \(T_1\) (\(T_1^s\)) is equal to \(1/\lambda_1\), and thus (Dixon et al., 1989)

\[
1/T_1^s = (C_1 - (C_1^2 - 4D_1)^{1/2})/2 \tag{3a}
\]

\[
C_1 = k_A + k_B + K_{1A} + K_{1B} \tag{3b}
\]

\[
D_1 = (k_A + K_{1A})(k_B + K_{1B}) - k_Ak_B \tag{3c}
\]

where \(k_1\) is the reciprocal lifetime of species \(i\) and \(K_{1i}\) is the reciprocal \(T_1\) of species \(i\) (A or B).

Equations 3a–c are sometimes (Gupta et al., 1972; Leigh, 1971) approximated by

\[
1/T_1^s = 1/T_{1A} + k_A \tag{4}
\]

The approximation is valid when \(T_{1A} \gg T_{1B}\) and \(1/k_B \gg 1/k_A \gg T_{1B}\). These conditions imply that the concentration of oxidized cytochrome-c-550 (species B) is much lower than that of the reduced protein (A), which is not the case in our experiments. Although the \(k_{\text{ese}}\) values calculated with eqs 3a and 4 did not show large differences, eqs 3a–c were preferred since the extra parameters needed (\(T_1\) of oxidized protein and concentration of the reduced form) could easily be obtained. With \(k_A = k_{\text{ese}}[B]\) and \(k_B = k_{\text{ese}}[A]\), eqs 3a–c can be reformulated as

\[
k_{\text{ese}} = \frac{(T_1^s - T_{1A})(T_1^s - T_{1B})}{T_1^s T_{1B}[A](1 - T_1^s/T_{1A}) + [B](1 - T_1^s/T_{1B})} \tag{5}
\]

(c) Finally, \(T_2\) measurements were used as well to determine the ese rate. Also, transverse relaxation of a proton in exchange can be described with a biexponential curve (Leigh, 1971). Under the experimental conditions used, again, one apparent \(T_2\) is very short, leading to an extremely broad component in the peak, which was neglected. Then the solution for the apparent \(T_2\) is

\[
1/T_2^s = 1/2C_2 - (1/2E_2 + 1/2(E_2^2 + F_2^2)^{1/2}1/2 \tag{6a}
\]

\[
C_2 = k_A + k_B + K_{2A} + K_{2B} \tag{6b}
\]

\[
E_2 = (k_A + K_{2A} - k_B - K_{2B})^2/4 + k_Ak_B - \Delta \omega^2/4 \tag{6c}
\]

\[
F_2 = (k_A + K_{2A} - k_B - K_{2B})\Delta \omega/2 \tag{6d}
\]

where \(\Delta \omega\) is the chemical shift difference between the peaks of the oxidized and reduced species, and \(K_{2i}\) is the reciprocal \(T_2\) of species \(i\) (A or B).

Under restrictions similar to those for the \(T_1\) case (\(T_{2A} \gg T_{2B}\) and \(1/k_A \gg 1/k_B \gg T_{2B}\)) eqs 6a–d can be replaced by

\[
1/T_2^s = 1/T_{2A} + k_A \tag{7}
\]

By systematic variation of \(k_{\text{ese}}\) (using a simple computer routine) the observed \(T_2^s\) values were fitted to eqs 6a–d, with \(K_{2A} = 21\) s\(^{-1}\), \(K_{2B} = 405\) s\(^{-1}\), \(\Delta \omega = 19.3\) \times 10\(^3\) s\(^{-1}\). It turned out that eq 7 excellently approximates eqs 6a–d even though the restriction \(1/k_A \gg 1/k_B\) is not fully valid.

For low values of \(k_{\text{ese}}\) line broadening is small; thus the error in \(T_2^s\) is relatively large, while at high values of \(k_{\text{ese}}\) \(T_2^s\) becomes short (\(T_2^s < T_{1B}\)). Therefore, the \(T_2^s\) method was preferred for the determination of low \(k_{\text{ese}}\) values (up to \(3 \times 10^4\) M\(^{-1}\) s\(^{-1}\)), while for the determination of higher \(k_{\text{ese}}\) rates \(T_1^s\) results are used (Cansters et al., 1993).

**RESULTS**

**Absorbance at 696 nm.** At high pH many class I cytochromes \(c\) show a change in conformation and a concomitant displacement of the Met ligand, probably by a Lys residue.
The pKₐ of this alkaline transition can be determined from the decrease in absorbance at circa 695 nm, as this band is considered indicative of the presence of the Met ligand (Moore & Pettigrew, 1990).

Figure 2A shows the absorbance at 696 nm of wild-type, K14Q, and K14E cytc-550 as a function of pH. The change in absorbance is reversible over the whole pH range (7-11.8). The differences between the three curves are small but reproducible. It proved impossible to fit the decrease in absorbance at high pH satisfactorily by a standard titration curve. This could be due to the fact that the absorbance of this band is superimposed on the tail of the band at 525 nm (the a/β-band). With a change from neutral to alkaline pH this band shifts to 532 nm and broadens (Figure 2B). As fitting was difficult, the pKₐ was estimated by determination of the point on the curve with the steepest slope. Since the curves are asymmetric, this introduces a systematic error in the pKₐ values which we conservatively estimate as 0.2-0.3 pH units (i.e., the reported values can be slightly too high).

Table I gives the pKₐ values for wild-type, K14Q, and K14E cytc-550 at low and high ionic strength. The reported pKₐ values (given the method of determination) appear to be reproducible within 0.05 pK unit.

It is clear that the mutation of K14 to either a neutral or a negative residue has little influence on the pKₐ of the alkaline transition of cytc-550. Thus, even when a salt bridge is present between K14 and E11, it is unimportant for the stabilization of the protein and it can not be involved in maintaining the high pKₐ of the alkaline transition of cytc-550. This is in contrast with the effects of chemical modification of K13 in mitochondrial cytc. In that case, disruption of the K13-E90 salt bridge leads to considerably lower pKₐ values for the alkaline transition (Osheroff et al., 1980).

At high ionic strength an increase of the pKₐ of unmodified horse cytc with 0.5 mM unit has been observed, from pH 9.0 to 9.5 (Osheroff et al., 1980). It has been suggested that high ionic strength causes some structural reorganization which would lead to an increase in the hydrophobicity of the local environment of the salt bridge. This would then stabilize the salt bridge (Moench et al., 1991). A slight increase in pKₐ is also observed in cytc-550 under high ionic strength conditions in both the wild-type and the mutants. Again, this effect can not be attributed to the salt bridge.

**Ionic Strength Dependence of the ¹H-NMR Spectra of wt cytc-550.** To characterize the mutant proteins further, the dependence of the ¹H-NMR spectrum on the ionic strength was determined. In these experiments small aliquots of concentrated NaCl in D₂O were used so that the protein concentration did not decrease by more than 20%.

The spectrum of wt ferrocyanocytoc-550 was recorded at low (4 mM) and high salt concentration (580 mM) (not shown). At high ionic strength many peaks shift slightly to higher frequency (0.01-0.1 ppm). Two unassigned peaks shift to lower frequency, one from 8.08 to 7.87 and the other from 6.10 to 5.95 ppm.

A small, general, high-frequency shift is also observed in the wt ferricytocytc-550 spectrum (see, for example, Figure 3, f-i). Furthermore, specific changes can be seen. In the range of 0-200 mM a few peaks shift to lower frequency (Figure 3, a-d). The protons corresponding to these peaks are close...
be that also cyto-550 is partly in a different, perhaps more open form. Exchange between the conformations could cause the peak broadening.

Ionic Strength Dependence of the 1H-NMR Spectra of Mutant cyto-550. The NMR spectra of the ferrous form of the mutants K14Q and K14E cyto-550 show several effects connected with the mutations. At low salt concentration many peaks have a smaller line width than in wt cyto-550. Upon addition of salt their widths even decrease somewhat (see, for example, Table III). Some peaks have shifted slightly compared to the wt spectrum. Of the signals of the heme meso protons only the peak of the \( \alpha \)-proton (attached to carbon 5) shifts significantly (0.02 and 0.06 ppm in K14Q and K14E cyto-550, respectively). This meso proton is the one closest to residue 14 (see Figure 1). The ionic strength dependent shifts observed for wt are also present in K14Q and K14E cyto-550 spectra.

In the spectra of ferri K14E and K14Q cyto-550 several features can be observed. First, some peaks have shifted relative to the wild-type. These shifts are not limited to the close environment of K14 since the peaks of HM8 and the M100 methyl also shift. All affected protons are, however, in the neighborhood of the paramagnetic heme iron. The shifts are largest for HM8 and HM3, which are shown in Figure 4, panels B and C. They are qualitatively the same in both mutants but largest in K14E cyto-550.

In horse cyto the conformational change to the open form at low salt can be inhibited by conversion of the charge of residue K13 from positive to negative by CDNP modification (Rush et al., 1988). The mutation K14E has an effect on the charge similar to that of modification. Interestingly, the broadening of the peaks at low ionic strength is much smaller in the K14 mutant proteins than in the wt protein (Figure 5). This supports the hypothesis formulated above that the broadening of the peaks in the spectrum of wild-type cyto-550 is due to the presence of a second conformation, analogous to cyto. This form appears to exist up to 200 mM NaCl (Figure 5).

Second, the effect of salt on the positions of the peaks in the spectra of the mutant proteins is the same as observed for wt cyto-550: the HM8 and HM3 peaks shift and cross with increasing ionic strength (Figure 4, panels B and C). Also, the other low-frequency shifts seen for the wt protein are present in the mutant proteins. Again, a small, general, high-frequency shift is observed at high salt concentrations.

A third interesting feature is the partial splitting of the HM8 peak (Figure 4, panels B and C). The cause of this may be a local heterogeneity in the protein. Since it is observed only for this peak, it must be a small effect. It appears to be largely independent of the salt concentration and is seen in both K14 mutants, but the effect is larger in K14E cyto-550. Splitting of the HM8 peak has also been observed for *Rhodospirillum rubrum* cytochrome c\( _2 \) at higher pH values (Yu & Smith, 1990).
Electron Self-Exchange Measurements. The steady-state transfer rate of electrons in a mixture of oxidized and reduced large cytochromes c is low and increases with ionic strength [see Moore & Pettigrew (1990) for an overview]. Presumably the rate depends critically on the repulsion between the electron-transfer partners in the association complex by the positive charges around the electron-transfer site. At high ionic strength the repulsion is weakened, and the rate of electron transfer is enhanced. CDNP modification of the lysine residues 13 and 72 of horse heart cytochrome c leads to considerably higher electron self-exchange (ese) rates, although the ionic strength dependence is maintained (Concar et al., 1986).

The saturation-transfer technique (ST technique) is often used (Concar et al., 1986, 1991; Lommen et al., 1990) for the determination of relatively low ese rates (on the order of 10^2-10^4 M^{-1} s^{-1}). In the present work several methods have been used to establish the ese rates (k_{ese}) of wt and mutant cytc-550. ST experiments similar to those on wt protein (Lommen et al., 1990) were performed on K14E cytc-550, by using the peaks of the M100 methyl group at -2.86 ppm (reduced species) and -13.0 ppm (oxidized species). The k_{ese} obtained with ST is 1 x 10^3 M^{-1} s^{-1} and is nearly independent of ionic strength. However, when the line-broadening method was used (eqs 6a-c and 7), k_{ese} appeared to increase with ionic strength from 1 x 10^4 to 4 x 10^4 M^{-1} s^{-1}, as is illustrated in Figure 6. This is at variance with the ST-derived ese rates.

To decide between these conflicting results, another ST experiment was performed: the peak of HM5 of the reduced species (at 3.55 ppm) was irradiated. A clear decrease could be observed in the HM5 peak of the oxidized species. The T_1 of this peak is much shorter (66 ms) than that of the M100 methyl group in the reduced species (1.3 s). This indicates that k is on the order of 10-100 s^{-1} (see eqs 1a,b), and since the concentrations of the oxidized and the reduced protein are in the millimolar range, k_{ese} (k/[oxidized protein]) must be much larger than determined in the first ST experiments. Unfortunately, because of spectral overlap in the irradiated region, precise quantification of the results was not possible. Therefore, k_{ese} was determined by the direct measurement of the apparent T_1 (T_1^a) of the M100 methyl group of the reduced protein as a function of the ionic strength for wt and mutant cytc-550. In Figure 7 an example of a T_1^a measurement is presented. Theory predicts a biphasic curve, but as discussed in the Materials and Methods section, under the conditions used one of the exponentials is expected to be negligible. Figure 7 clearly indicates that the measurements can indeed be fitted with a single exponential.

The values of k_{ese} calculated from these results (eqs 3a-c) are identical within error with those calculated from line-broadening data (Figure 8). The T_1 and T_2 values of fully reduced and oxidized proteins used in the calculations are listed in Table II.

Apparantly the ST method is not reliable in the present case, and previously reported literature values (Lommen et al., 1990) based on this method must be considered as
Lys14 Mutants of Cytochrome c-550

**DISCUSSION**

The pH-dependent change in ligation of the heme iron in oxidized cytc has been the subject of many studies [see Moore & Pettigrew (1990) for an overview; Hong & Dixon, 1989; Barker & Mauk, 1992; Ferrer et al., 1993]. It is assumed that this alkali line transition consists of a two-step process. Protonation with a pKₐ around 11 is followed by a structural rearrangement (Brandt et al., 1966; Davis et al., 1974). This gives rise to an apparent pKₐ (pKₐ') of 9–10. In cytc-550 a similar transition is observed since the changes in the NMR spectrum and in the absorbance at 696 nm for cytc-550 closely resemble those for cytc (Lommen et al., 1990; this work). However, in cytc-550 the pKₐ' is around 11. Several causes can explain this difference. The structural rearrangement may be less favorable in cytc-550, or the residue that is protonated in cytc-550 has a higher pKₐ.

Osheroff et al. (1980) found that chemical modification of K13 of horse cytc into a neutral or negative group decreased the pKₐ' from 9.05 to 8.10 and 8.20, respectively. This was ascribed to the rupture of the K13-E90 salt bridge causing a destabilization of the Met ligation.

Mutation of K14 in cytc-550 has only little influence on the pKₐ' of the alkaline transition. K14Q has a slightly higher pKₐ', while in K14E it is somewhat lower. It is clear that the mutations do not alter the stability of the native form considerably. The small differences can perhaps be attributed to a reorientation of the side chain of residue 14.

It can be concluded that a presumptive salt bridge between K14 and E11 has no stabilizing effect on the cytc-550 structure and can not be the cause of the high pKₐ' in cytc-550.

The replacement of the methionine ligand is observed in cytc not only at alkaline but also at neutral pH provided the ionic strength is low (Rush et al., 1988; Ångström et al., 1982). This phenomenon has been explained by assuming that cytc is in a more open conformation at low ionic strength which destabilizes the Met ligation (Rush et al., 1988; Ångström et al., 1982; Trewella et al., 1988). The presence of small ions or the removal of a positive charge (K13, K72) inhibits the change to the open form (Rush et al., 1988). Osheroff et al. (1980) also found that the pKₐ' of the ligand replacement increases with ionic strength.

At low ionic strength the NMR spectrum of oxidized wt cytc-550 shows relatively broad peaks. The line width decreases as a function of the salt concentration up to 200 mM and then becomes constant. The line widths are much smaller both in K14E and in K14Q. This is comparable to the results of Rush et al. (1988) and therefore is interpreted in the sense that the wt protein is partly in another, more open conformation, like cytc at low ionic strength. Removal of a positive charge by the mutations largely prevents this conformational change. In this case, however, the change to the open form does not promote ligand replacement, possibly because the Met ligation is more stable in cytc-550. High ionic strength does not stabilize the native form appreciably (only 0.1 pH unit increase in pKₐ').

It would be interesting to compare the form at low ionic strength with the conformation that cytc adopts upon complex formation with cytc oxidase. The latter differs from both the native and the alkaline form (Weber et al., 1987; Michel et al., 1989; Hildebrandt et al., 1990).

Some specific resonance shifts are observed in the NMR spectra of oxidized wt cytc-550 as a function of ionic strength. Largest are the low-frequency shifts of HM8 and other protons near the iron and the high-frequency shift of HM3. The mutations K14Q and K14E also cause shifts of HM8 and HM3. This effect is stronger in K14E. Although HM8 is much farther away from residue 14 than HM3 is, the former shifts more than the latter.

Concomitant shifts of HM8 and HM3 have been observed before. They occur in cytc, also as a function of ionic strength (Moench et al., 1991), as a consequence of chemical modification (Falk et al., 1981), or as a result of complex formation with cytc peroxidase (Moench et al., 1992; Satterlee et al., 1987), cytochrome b₅ (Eley & Moore, 1983), or plastocyanin (Bagby et al., 1990; King et al., 1985). Interestingly, similar shifts have also been described for HM1 and HM5. Saraiva et al. (1992) observe these shifts in the spectra of cytc-550 from Bacillus halodentriticas, as a function of pH. Since this cytochrome has $S$ rather than $R$ chirality, in contrast with cytc and cytc-550, HM1 and HM5 are the two heme methyls that exhibit the positions at higher frequency in the NMR spectrum (Moore & Pettigrew, 1990). They are therefore equivalent to HM3 and HM8 of cytc and cytc-550. The authors suggest that a deprotonation followed by a reorientation of the ligand histidine causes a redistribution of the electron density in the heme, which gives rise to the shifts. In a similar way, it can be suggested that changes in the electrostatic forces near the heme crevice also influence

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**Figure 8:** Ese rates of wt and mutant cytc-550 as a function of ionic strength. Ese rates were determined both with $T_{1s}$ measurements (solid symbols) and with line-broadening methods (open symbols) using the M100 methyl group of reduced cytc-550: circles, wild-type; squares, K14Q; triangles, K14E. The linear parts of the curves are lines of the fits up to 0.3 M.

**Erroneous.** A possible explanation for this is insufficient saturation of the peak of the oxidized species. A high irradiation power was used during a long period (4 s), and no peak was visible any more. Yet, since the M100 methyl peak is quite broad (129 Hz in wt protein), possibly some magnetization was still present though undetected. In that case less ST is observed, and the calculated $K_{eq}$ is too low.

The ese rates of the K14Q and K14E mutant proteins are different from that of wild-type cytc-550 (Figure 8). In the absence of salt the ese rates (at 313 K and pH 6.0) are $7(2) \times 10^3$ M$^{-1}$s$^{-1}$ (K14Q), $1.2(0.4) \times 10^3$ M$^{-1}$s$^{-1}$ (K14E), and $2(1) \times 10^2$ M$^{-1}$s$^{-1}$ (wild-type cytc-550). The slopes of the lines in Figure 8 represent the dependence of the ionic strength. For wild type this slope is 0.13 mM$^{-1/2}$, which is higher than for the mutants (0.086 and 0.069 mM$^{-1/2}$ for K14Q and K14E). The rates start to deviate from the linear relationship of log $k_{eq}$ and the square root of the ionic strength at salt concentrations above 0.3 M.

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is used, it is important to check whether a single exponential is a valid approximation for the magnetization decay or whether a double-exponential fit should be used.

An esr rate of $2(1) \times 10^7$ M$^{-1}$ s$^{-1}$ is found for wt cyto-550 at zero ionic strength, while at 547 mM $k_{es}$ is $1.0(0.3) \times 10^5$ M$^{-1}$ s$^{-1}$. The esr rate of cyto-550 from *P. denitrificans* is 1.6 $\times 10^8$ M$^{-1}$ s$^{-1}$ (100 mM NaCl, pH$^+$ 7.5, 25 °C) (Timkovich et al., 1984) and is higher than for cyto-550 from *T. versutus* [4(1) $\times 10^7$ at 100 mM NaCl, pH$^+$ 6.0, and 40 °C]. It is not likely that the differences in experimental conditions (pH$^+$, presence of phosphate) can account for the discrepancy, especially when the temperature difference between the two measurements is considered. The differences in esr rates could be due to small structural differences between the two cytochromes c-550. After all, the mutation of K14 also has a large effect on the esr rate. Both mutants, K14Q and K14E cyto-550, show higher esr rates than wt in the absence of salt [7(2) $\times 10^7$ and 1.2(0.4) $\times 10^4$ M$^{-1}$ s$^{-1}$], like CDNP-K13 cytc (Concar et al., 1986). This is not unexpected since the ring of positive charges around the heme crevice is unfavorable for esr. Replacement of a positive charge by a neutral or a negative one decreases this effect. However, unlike CDNP-K13 cytc, K14Q and K14E cyto-550 show a lower salt dependence of their esr rates than wt. The slope of log $k_{es}$ versus the square root of the ionic strength is reduced by 30–40%. This suggests that K14 contributes considerably to the repulsive effect. It looks as if at infinite ionic strength the $k_{es}$ values may converge to the wt value. Further analysis of these rates using molecular dipole moment calculations [like, e.g., Dixon et al. (1989, 1990)] has to await more reliable structural data of *T. versutus* cyto-550.

### CONCLUSIONS

The conserved residue K14 has no role in maintaining the native conformation of cyto-550 at high pH, unlike K13 of cytc. NMR experiments show that wt cyto-550 exists in more than one conformation at low ionic strength. The protein conformations of K14Q and K14E cyto-550 appear much less sensitive to low salt concentrations than that of wt. Changes in the surface charge of the ferric cyto-550 influence the electron distribution in the heme, presumably via a structural rearrangement. Saturation transfer proved, in our hands, an unreliable method to determine the electron self-exchange rates; the $T_1^\ast$ method and the line-broadening method are preferred. The esr rate of wt cyto-550 shows a strong ionic strength dependence, which is ascribed to repulsive effects of the positive charges around the heme. This repulsion is diminished considerably when residue K14 is changed into a glutamine or glutamate residue.

### REFERENCES


Lys14 Mutants of Cytochrome c-550