pressure perturbation lends itself well to the required signal averaging techniques. Perhaps less obvious is the ease with which very small perturbations can be applied. For GDH with ΔV = 25 mL/mol and ΔH = 5 kcal/mol, a 1.0-atm pressure jump is equivalent to a temperature jump of 0.03 °C. The pressure-volume work of 25 (mL atm)/mol would perturb the free energy by 0.6 cal/mol, and the experiment would approach the realm of fluctuation kinetics.

Despite the attractive features of techniques in the frequency domain (simplicity and economy of instrumentation and analysis), this approach does not prove sufficiently powerful for studying systems with small signals and slow relaxations. It may be more valuable in studying other kinds of interactions.

Added in Proof

Information recently obtained from Dr. K. A. Heremans (personal communication) suggests that ΔV^* is about +10 mL/mol. His estimate (to be published in detail elsewhere) derives from experiments specifically designed to measure this parameter (temperature jump at 1–600 atm) and must be regarded as being more precise than that obtained in this work. The mechanistic implication is that surface water molecules are displaced more or less independently.

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Ion Binding to Cytochrome *c* Studied by Nuclear Magnetic Quadrupole Relaxation†

Thomas Andersson,* Eva Thulin, and Sture Forsén*

ABSTRACT: The enhancement of the $^{35}Cl^-$ transverse relaxation rate on binding of chloride ions to oxidized and reduced cytochrome *c* has been studied under conditions of variable sodium chloride concentration, temperature, pH, sodium phosphate, iron hexacyanide, and sodium cyanide concentration. The results revealed the presence of a strong binding site(s) for chloride in both oxidized and reduced cyt *c*, with a higher affinity in ferrocytochrome *c*. Competition experiments suggest that these sites also bind iron hexacyanide and phosphate. Cyanide binding to the iron in ferricytochrome *c* at alkaline and neutral pH was shown to decrease the binding of chloride. The pH dependence of the $^{35}Cl^-$ relaxation rate has been fitted by using literature p*K* values for ionizable groups. No indications of Na^+ binding to oxidized and reduced cytochrome *c* have been observed by using $^{23}Na^+$ NMR. Our results suggest that chloride is bound near the exposed heme edge and that the surface structure or dynamics in this region are different in the two oxidation states.

Eucaryotic cytochrome *c* is a small protein, composed of 103–113 amino acid residues, having an iron porphyrin covalently attached to the protein. The biological role of cytochrome *c* is to receive electrons from cytochrome c_1 and in turn deliver them to cytochrome oxidase (Dickerson & Timkovich, 1975). The crystal structure of tuna cytochrome *c* has been determined for both oxidation states (Swanson et al., 1977; Takano et al., 1977; Mandel et al., 1977), and no

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significant differences have been observed. However, there is an extensive body of physical and chemical data indicating that the physical properties of cytochrome *c* differ in its oxidized and reduced states (Salemme, 1977). The origin of these differences may possibly be sought in the surface region of the protein molecule, in particular the arrangement of the amino acid side chains. At the present time, several studies indicate that the positively charged residues play an important role in the interaction between cytochrome *c* and the complexes in the respiratory chain (Salemme, 1977; Ng et al., 1977; Smith et al., 1977; Ferguson-Miller et al., 1978).

Any changes involving the surface charges upon reduction should be reflected in differences in the ion binding properties of the two forms. Ion binding to cytochrome *c* has been a widely investigated phenomenon. From electrophoretic studies, Margoliash and co-workers (Barlow & Margoliash, 1966; Margoliash et al., 1970) found that oxidized cytochrome *c* binds chloride and phosphate, while the reduced form only binds phosphate. Sodium was found only to bind to the reduced protein. Margalit and Schejter (Schejter and Margalit, 1970; Margalit et al., 1970; Margalit & Schejter, 1973a,b, 1974) have studied ion binding by redox potential measurements and gel filtration techniques. They came to the conclusion that phosphate and chloride ions bind exclusively to the oxidized protein with a binding constant of about 10^4 M^{-1}. No evidence of sodium binding to the protein was found. Chloride and phosphate binding to reduced cytochrome *c* has, however, been observed by Stellwagen & Shulman (1973a) by using proton NMR. These authors were able to estimate the binding constant (K_b = 400 M^{-1}) for phosphate to a site in the vicinity of His-26.

The results above are partially contradictory and suggest that ion binding to cytochrome *c* is worthy of further investigation. Nuclear magnetic quadrupolar relaxation spectroscopy is a sensitive and direct method for studying ion binding to macromolecules (Lindman & Forsén, 1976; Forsén & Lindman, 1978), and the work presented here is a $^{23}Na^+$ and a $^{35}Cl^-$ NMR investigation of binding of these ions to both oxidation states of horse heart cytochrome *c*.

Experimental Procedure

Materials. Horse heart cytochrome *c* was prepared by the method of Margoliash & Walasek (1967) and further purified on an ion-exchange resin to separate deamidated forms from the native protein. The absence of artifactual forms was checked by gel electrophoresis and carbon monoxide combination (Tsou, 1951). After elution from the ion-exchange column, the protein was dialyzed against triple distilled water for several days in the cold room, and finally deionized on a mixed bed ion-exchange resin. The cytochrome oxidase activity was assayed spectrophotometrically by using the method of Smith & Conrad (1956). By these procedures, we were able to obtain a pure and highly active protein.

Protein concentrations were determined on the basis of the extinction coefficient ϵ 29.5 cm^{-1} mM^{-1} at 550 nm for the reduced protein. A stock solution of the oxidized protein was kept at −20 °C and used within 1 month of preparation. Reduced cytochrome *c* was prepared by ascorbate reduction and the salt was removed by dialysis followed by deionization on a mixed bed resin.

Ferricytochrome *c* was carboxymethylated at room temperature by using a buffered solution, pH 7, containing NaCN and bromoacetate (Stellwagen, 1968). An amino acid analysis showed that cytochrome *c* had been modified at Met-65, Met-80, and His-33. The product also showed the characteristic absorbance spectrum of cytochrome *c* with both methionyl residues alkylated. Reduced carboxymethylated cytochrome *c* was prepared by adding an excess of sodium dithionite.

In the NMR experiments, no buffers were used; pH was adjusted by addition of dilute HCl or NaOH. In the $^{23}Na^+$ measurements D_2O was used instead of H_2O. All chemicals were of the finest grade available.

Relaxation Measurements. The nuclear magnetic resonance spectra of $^{35}Cl^-$ and $^{23}Na^+$ were obtained by using a modified Varian XL-100-15 spectrometer operating in the Fourier transform mode. For the $^{35}Cl^-$ measurements we used an external proton lock, and for the $^{23}Na^+$ recordings the magnetic field was internally locked, on the deuterium signal from the solvent. A few measurements were performed on a home-built Fourier transform spectrometer equipped with an Oxford Instruments 6-T wide-bore magnet. The probe temperature was held constant by a stream of dry thermostated nitrogen gas. The spectra were recorded at 28 °C unless otherwise stated. The linewidths, $\Delta\nu$, taken at half-height of the absorption signal, are related to the transverse relaxation rate, R_2, through $R_2 = \pi\Delta\nu$. The error in the measurements was estimated to be 3%.

Theory

For any nucleus with a spin quantum number (I) greater than 1/2, such as ^{35}Cl and ^{23}Na, the distribution of charge over the nucleus deviates from spherical symmetry, a deviation which can be described in terms of a nuclear electric quadrupole moment (eQ). If the electron distribution about the nucleus has less than cubic symmetry, then an electric field gradient at the nucleus will result which can couple to the nuclear quadrupole moment. Fluctuations in this field gradient coupled to the quadrupole moment can provide an efficient mechanism for nuclear magnetic relaxation, so efficient that, for most quadrupolar nuclei, it is the only relaxation mechanism that need be considered.

For a "free" solvated quadrupolar ion in aqueous solution, quadrupolar relaxation is relatively inefficient. For a quadrupolar ion bound in an asymmetric environment, for example, to a macromolecular binding site, the quadrupole relaxation is usually very efficient—a direct study of the bound ion is then extremely difficult. Studies of the binding of quadrupolar ions to macromolecules are, however, possible if there is a fast (or intermediate) chemical exchange between the bound and "free" ions (Forsén & Lindman, 1978).

If the chemical exchange between the protein sites and the bulk solution is much faster than the transverse relaxation rates at the protein sites, the excess transverse relaxation rate is given by

$$R_{2,e} = R_{2,o} - R_{2,f} = \sum_i p_i R_{2,i} \qquad (1)$$

where $R_{2,o}$ is the observed relaxation rate and $R_{2,f}$ is the relaxation rate in the absence of protein, and p_i and $R_{2,i}$ are the fraction of ions bound to and the relaxation rate at site i, respectively. For the case with no competing ions in the solution, the fraction of bound ions is given by

$$p_i = \frac{n_i c_{pr} K_{i,X}}{1 + K_{i,X}(X)} \qquad (2)$$

where n_i is the number of ions bound to site i with the binding constant $K_{i,X}$. The total protein concentration is denoted c_{pr}, and (X) refers to the concentration of unbound ions. In the general situation where other ions, L, compete with X for the same binding sites, the average fraction of the ion X bound

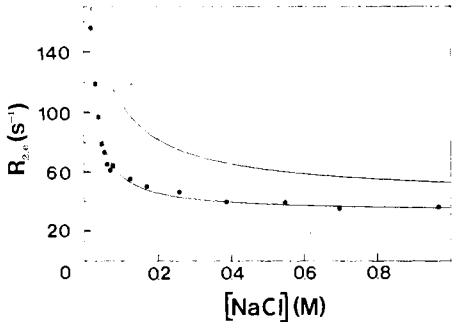

FIGURE 1: The $^{35}Cl^-$ excess transverse relaxation rate as a function of NaCl concentration in the presence of oxidized (○) and reduced (●) cytochrome *c* at pH 7.3 and 7.2, respectively. The data are normalized to a protein concentration of 3.0 mM. Lines are theoretical curves calculated by using the parameters given in Table I.

to *i* on the protein may be expressed as (Steinhardt & Reynolds, 1969)

$$p_i = \frac{n_i c_{pr} K_{i,x}}{1 + K_{i,X}(X) + \sum_L K_{i,L}(L)} \quad (3)$$

$K_{i,L}$ is the association constant for the competing ligand L, and (L) denotes the concentration of the free ions. For a moderately large protein, such as cytochrome *c*, and, for the nuclei and magnetic fields employed in this work, extreme narrowing conditions ($\omega\tau_c \ll 1$) apply (Lindman & Forsén, 1976) and the intrinsic transverse relaxation rate $R_{2,i}$ for a quadrupolar nucleus at site *i* is given by

$$R_{2,i} = \frac{2\pi^2}{5}\chi_i^2 \tau_{ci} \quad (4)$$

where χ_i is the nuclear quadrupolar coupling constant and τ_{ci} is the correlation time describing the reorientation of the electric field gradient at site *i*. τ_{ci} can, in principle, contain contributions from the overall protein rotational motion, chemical exchange, and the internal motion of the bound ion. Equation 1 shows that in the fast exchange limit there are two main parameters determining the relaxation rate, the fraction of bound ions and the intrinsic relaxation rate. The latter is determined by the quadrupole coupling constant and correlation time according to eq 4.

Results and Discussion

Dependence of the $^{35}Cl^-$ Relaxation Rate on NaCl Concentration. The dependence of the $^{35}Cl^-$ transverse relaxation rate on chloride concentration at pH near neutrality for the oxidized and reduced forms of cytochrome *c* is shown in Figure 1. The recordings were obtained by using protein concentrations between 1 and 4 mM, and the relaxation rates are corrected to 3.0 mM cytochrome *c* concentration. The data can be interpreted in terms of a two-site model, having one class of high affinity sites (index *i*) and one class of low affinity sites which gives a constant contribution ($\sum_n P_n R_{2,n}$) to the observed relaxation rate. By combining eq 1 and 2, and by making the approximation that the concentration of free chloride ions equals the total concentration, C_{Cl}, of these ions, the excess transverse relaxation rate may be expressed as

$$R_{2,e} = \frac{n_i C_{pr} K_{Cl} R_{2,i}}{1 + K_{Cl} C_{Cl}} + \sum_n P_n R_{2,n} \quad (5)$$

The experimental relaxation rates were fitted to eq 5 by using a least-squares fitting program. The results of this operation are given in Table I. Since the number of sites contained in each class is not known, the intrinsic relaxation rate is given as the product $n_i R_{2,i}$. Due to experimental limitations, it is not possible to determine large binding constants, but it can clearly be stated that chloride binds more tightly to the high affinity sites of reduced cytochrome *c*. The derived intrinsic relaxation rates are much smaller than expected on the basis of $^{35}Cl^-$ binding studies to other proteins (Lindman & Forsén, 1976). A possible explanation for this is that rapid local motions at the binding sites lead to a reduced effective quadrupolar coupling constant.

Table I: Results of the Fitting of the Binding Parameters of Chloride to Oxidized and Reduced Cytochrome *c*

	high-affinity class		low affinity class
	K_{Cl} (M^{-1})	$n_S R_{2,S}$ (s^{-1})	$\Sigma_W p_W R_{2,W}$ (s^{-1})
Fe^{3+}-cyt *c*	17	2.7×10^3	44
Fe^{2+}-cyt *c*	$\geq 10^2$	9×10^2	33

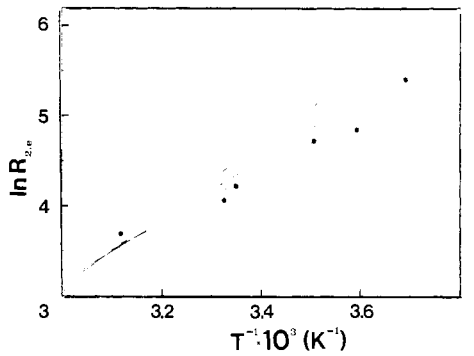

FIGURE 2: The $^{35}Cl^-$ excess transverse relaxation rate of a 0.10 M NaCl solution as a function of the inverse absolute temperature in the presence of oxidized cytochrome *c* at pH 7.3 (○) and pH 9.2 (□), and at pH 7.2 for reduced cytochrome *c* (●). The relaxation rates are normalized to 3.0 mM protein concentration. The lines are drawn by using a least-squares fitting program.

The origin of the differences in the observed $^{35}Cl^-$ excess relaxation rates between the oxidized and reduced forms of cytochrome *c* is at the present time not clear. Since the chloride chemical exchange rate is fast in comparison with the chloride relaxation rate at the protein binding site (cf. following section), this rules out differences in chemical exchange rates. Upon oxidation, the heme charge increases by one unit but simple calculations show that this will not have any significant effect on the quadrupole relaxation of chloride ions bound near the heme crevice. Neither is any significant paramagnetic contribution to the $^{35}Cl^-$ transverse relaxation rate expected on the basis of substituting reasonable parameters into the Solomon–Bloembergen equations (Dwek, 1973).

Dependence of the $^{35}Cl^-$ Relaxation Rate on Temperature. If the exchange of chloride ions is rapid enough to influence the $^{35}Cl^-$ signal from the bulk solution, the excess relaxation rate can be interpreted in terms of an average of the intrinsic relaxation rates of the different sites, weighted according to their population. It can be shown (Lindman & Forsén, 1976) that, in the fast exchange limit, a plot of ln $R_{2,e}$ vs. T^{-1} is linear with a positive slope. Figure 2 shows the results obtained for 0.10 M NaCl solutions at pH 7.3 and 9.2 for oxidized cytochrome *c*, and at pH 7.2 for reduced cytochrome *c*. Protein concentrations between 1 and 2.5 mM were used, but the data are normalized to 3.0 mM. The results indicate chloride ion exchange to be fast, confirming the validity of eq 1.

Dependence of the $^{35}Cl^-$ Relaxation Rate on pH. The effect of pH on the $^{35}Cl^-$ relaxation rate for both oxidation states of cytochrome *c* is shown in Figure 3. The results were

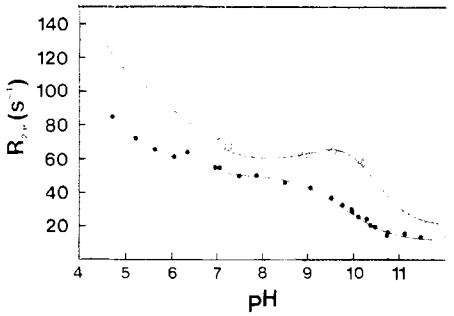

FIGURE 3: The pH dependence of the $^{35}Cl^-$ excess transverse relaxation rate for a 0.30 M NaCl solution containing oxidized (○) and reduced (●) cytochrome *c*. The data are normalized to a protein concentration of 3.0 mM. The solid lines are theoretical curves drawn by using the parameters appearing in Table II.

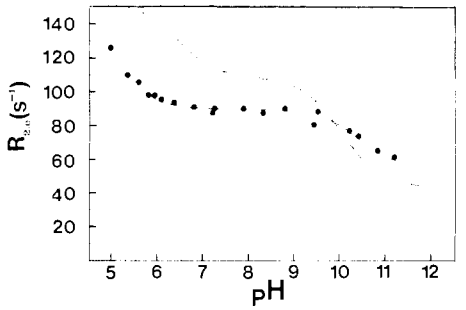

FIGURE 4: The pH dependence of the $^{35}Cl^-$ excess transverse relaxation rate for a 0.3 M NaCl solution containing oxidized (○) and reduced (●) carboxymethylated cytochrome *c*. The data are corrected to 3.0 mM protein concentration. Lines are theoretical curves drawn by using the parameters given in Table II.

obtained in 0.3 M NaCl solutions containing 2.2 mM ferricytochrome *c* and 3.4 mM ferrocytochrome *c*, respectively. The pH was adjusted by adding small amounts of dilute NaOH and HCl. The data are normalized to a protein concentration of 3.0 mM.

Again we can clearly observe a difference between the oxidized and reduced forms. The most conspicuous feature in Figure 3 is the "hump" in the $^{35}Cl^-$ excess relaxation rate for the oxidized form at a pH around 9.5. It is known from spectroscopic studies that oxidized cytochrome *c* exists in five distinct conformational states. The physiologically active state, usually denoted state III, is stable between pH 2.5 and pH 9.5 where it undergoes a transition to state IV (Dickerson & Timkovich, 1975). The disappearance of the methionine methyl signal in the proton NMR spectrum during this alkaline isomerization indicates that methionine-80 is displaced as the sixth heme ligand (Redfield & Gupta, 1971). The heme iron remains, however, in a low spin state, indicating that another strong field ligand, possibly Lys-79 but cf. Pettigrew et al. (1976), coordinates to the heme iron. If cytochrome *c* is carboxymethylated at Met-80, a lysine, probably the same as in state IV, is already coordinated to the heme iron at neutral pH. Figure 4 shows the pH dependence of oxidized and reduced carboxymethylated cytochrome *c*. It is seen that the oxidized protein does not show any increase in the $^{35}Cl^-$ relaxation rate in the pH range 9–10. The "hump" in Figure 3 must obviously be related to the conformational transition between states III and IV, resulting in either an increased binding to Fe^{3+}-cyt *c*,[1] an increased correlation time, and/or an increased quadrupole coupling constant of a binding site. The temperature dependence of Fe^{3+}-cyt *c* at pH 9.2 rules out

[1] Abbreviations used: Fe^{3+}-cyt *c*, ferricytochrome *c*; Fe^{2+}-cyt *c*, ferrocytochrome *c*.

Table II: Results of the Fitting of the pH Dependence of the Relaxation Rates for Native and Carboxymethylated Cytochrome *c*

	$pK_{a,i}$	$R_{2,i}$ (s^{-1})	assignment
Fe^{3+}-cyt *c*	10.4	70	Lys residue(s)
	9.5	−31	alkaline isomerization
	6.5	38	His-33
	4.6	60	carboxylate residues
Fe^{2+}-cyt *c*	10.2	18	Lys residue(s)
	9.4	20	Lys-79?
	6.4	14	His-33
	4.6	45	carboxylate residues
Fe^{3+}CM-cyt *c*	10.2	65	Lys residue(s)
	6.4	45	?
Fe^{2+}CM-cyt *c*	10.4	35	Lys residue(s)
	4.4	220	carboxylate residues

the possibility of chemical exchange effects. It is not straightforward to separate these different factors. A large number of positive surface charges around the heme edge are due to lysyl groups that have considerable flexibility. A theoretical study of the relaxation of quadrupolar ions attached to flexible binding sites (Bull, 1978) has shown that the observed apparent correlation time and quadrupole coupling constant depend in a complex way on the mode of internal motion. Therefore, it can not be ruled out that the alkaline isomerization is accompanied by a conformational change in the backbone that influences the freedom of motion of these positive side chains in such a way as to cause increased relaxation rates of interacting halide ions. On the other hand, the alkaline isomerization may be accompanied by a change in the protein fold in such a way that a new anion binding site(s) is created or the affinity of a weak binding site is considerably increased. The recently observed effect of different ions on the transition III → IV provides evidence favoring the latter explanation. In the presence of increasing concentration of ClO_4^-, an ion that interacts strongly with cytochrome *c*, the p*K* value is shifted toward lower pH values, as would be expected if state IV has a higher affinity for ClO_4^- than state III (J. Ångström, K.-E., Falk, and T. Andersson, unpublished results).

The binding of ions to protein molecules is primarily due to electrostatic interactions between the ion and charged residues on the protein. It is sometimes possible to fit the pH dependence of the relaxation rate by using known p*K* values of the ionizable groups on the macromolecule (Halle & Lindman, 1978). Shaw & Hartzell (1976) have determined these p*K* values for ferricytochrome *c* using hydrogen ion titrations, and they are in good agreement with p*K* values determined by other techniques. For reduced cytochrome *c*, to the authors' knowledge, no similar investigation of the ionizable groups has been carried out. Proton NMR shows that hisitidine-33 has nearly the same p*K* value in the two oxidation states (Cohen et al., 1974; Cohen & Hayes, 1974). The experimental relaxation rates were fitted to the equation

$$R_{2,e} = \sum_i \frac{R_{2,i}}{1 + 10^{(pH - pK_{a,i})}} \qquad (6)$$

where $R_{2,i}$ is the contribution to the relaxation rate from chloride ions exchanging with site *i*. The p*K* value of this site, denoted $pK_{a,i}$, was taken from the literature. For reduced cytochrome *c*, we needed to make only very small corrections to these p*K* values in order to obtain a good fit. The "hump" in the relaxation rate was introduced by using a negative $R_{2,i}$ corresponding to the p*K* of the alkaline isomerization. The

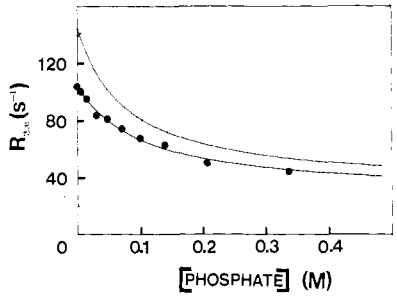

FIGURE 5: The $^{35}Cl^-$ excess transverse relaxation rate of a 0.073 M NaCl solution, pH 7.0, containing 3.1 mM oxidized (○) and reduced (●) cytochrome *c*, as a function of the total concentration of added phosphate. Lines are theoretical curves drawn by using the calculated relative binding constants: $K'/K = 0.8$ for oxidized and $K'/K = 1.7$ for reduced cytochrome *c*.

results of the fitting procedures are shown as the solid lines in Figures 3 and 4 with the corresponding parameters appearing in Table II. The data indicate that His-33 is involved in chloride binding in both oxidation states. It is also seen that the decrease in the relaxation rate above pH 8 for the carboxymethylated cytochrome can be fitted by using only one pK value, whereas the native reduced cytochrome *c* requires at least two pK values. This can be explained by the fact that a lysyl residue (probably Lys-79) in the carboxymethylated protein is coordinated to the heme iron and is no longer able to participate in the binding of chloride.

Competition between Phosphate and Chloride. Several studies have pointed out that phosphate binds to cytochrome *c*. Margoliash and co-workers (1970) reported that phosphate binds to both oxidation states at neutral pH. On the other hand, Margalit & Schejter (1973b) state that phosphate binds exclusively to the oxidized form of cytochrome *c*.

Figure 5 shows the excess $^{35}Cl^-$ relaxation rate of 0.073 M NaCl solutions, pH 7.0, containing oxidized and reduced cytochrome *c*, as a function of the total concentration of added phosphate. The measurements in Figure 5 were performed on a 6-T spectrometer. The sample temperature was somewhat lower than for the other experiments (23 °C compared with 28 °C), resulting in a small relative signal broadening. Our results are most easily interpreted in terms of phosphate and chloride competition for common sites in both oxidized and reduced cytochrome *c*. The experimental relaxation rates were fitted to the equation

$$R_{2,e} = \frac{n_i C_{pr} K_i R_{2,b}}{1 + K'_i(PO_4) + K_i(Cl^-)} + \sum_{n \neq 1} P_n R_{2,n} \qquad (7)$$

where n_i is the number of ions bound at site i, K_i and K'_i are the intrinsic association constants for chloride (given in Table I) and phosphate, respectively, $R_{2,b}$ is the relaxation rate for the bound chloride ion, and C_{pr} the total protein concentration. The concentrations of free phosphate and chloride ions are denoted (PO_4) and (Cl^-) and they can, under the experimental conditions used, be approximated by the total concentrations of these ions. The second term in eq 7 describes the contribution to the observed $^{35}Cl^-$ relaxation rate from sites which are not affected upon addition of phosphate. The results of the fitting procedures are given as the solid lines in Figure 5, and the obtained relative binding constants K'_i/K_i are shown in the figure legend. The magnitude of the decrease in the relaxation rates would seem to indicate that phosphate competes for the high affinity site(s) on both oxidized and reduced cytochrome *c*. The values of the relative binding constants, K'_i/K_i, show that phosphate binds with approximately the same affinity as chloride to the sites observed.

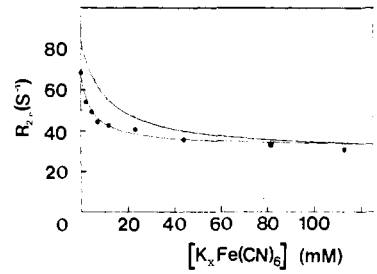

FIGURE 6: The $^{35}Cl^-$ excess transverse relaxation rate of a 0.054 M NaCl solution, containing Fe^{3+}-cyt *c* (pH 7.3) and Fe^{2+}-cyt c (pH 7.4), as a function of the total concentration of added iron hexacyanide. To the oxidized protein (○) ferricyanide ($X = 3$) was added, and to the reduced cyt *c* (●) ferrocyanide ($X = 4$) was added. The data are normalized to a protein concentration of 3.0 mM.

Competition between Iron Hexacyanide and Chloride Ions. Iron hexacyanide binding to cytochrome *c* is a well-studied phenomenon. NMR measurements by Stellwagen & Shulman (1973) showed that a complex is formed between the two species. Equilibrium dialysis experiments have shown that ferricytochrome *c* contains two iron hexacyanide binding sites, and that chloride ion interacts with both of these binding sites (Stellwagen & Cass, 1975). One of these binding sites is proposed to be located near the exposed heme edge, and ^{13}C NMR of guanidinated cyt *c* suggests the lower portion of the exposed heme edge (Lys-79) is not directly involved in the iron hexacyanide binding (Stellwagen et al., 1977). Due to experimental limitations, Stellwagen & Cass were not able to make a definite statement about the binding parameters for reduced cyt *c*. Figure 6 shows the $^{35}Cl^-$ relaxation rate in a 0.054 M NaCl solution, containing oxidized or reduced cytochrome *c*, as a function of the total concentration of added iron hexacyanide. To avoid changing the oxidation state of cyt *c*, we added ferrihexacyanide to Fe^{3+}-cyt *c* and ferrohexacyanide to Fe^{2+}-cyt *c*. The data were obtained by using 3.0 mM oxidized and 2.5 mM reduced cyt *c*, but the data are normalized to a protein concentration of 3.0 mM. The data in Figure 6 suggest that chloride competes with the iron hexacyanides for (a) common site(s) in both oxidation states. The magnitude of the decrease of the relaxation rate implies that the high affinity chloride binding sites are involved in the hexacyanide binding. A rough analysis of the binding curves shows that ferrocyanide competes at least twice as efficiently with chloride for ferrocytochrome *c* as does ferricyanide in the case of ferricytochrome *c*. Investigations by Margalit & Schejter have pointed out that addition of negatively charged ions such as chloride and phosphate affects the redox potential of cytochrome *c* (Margalit & Schejter, 1970, 1973a,b). The redox potential has been measured indirectly by observing the equilibrium:

$$Fe^{2+}\text{-cyt } c + Fe(CN)_6^{3-} \underset{}{\overset{K_{obsd}}{\rightleftharpoons}} Fe^{3+}\text{-cyt } c + Fe(CN)_6^{4-} \qquad (8)$$

Our results suggest an interpretation of this phenomenon. Since chloride ions are shown to compete with iron hexacyanide, we propose that the changes of the redox potential upon addition of chloride result from a simple competitive effect rather than from structural or dynamic changes induced by chloride.

Effect of Cyanide on the $^{35}Cl^-$ Relaxation Rate. Cyanide ions are known to displace methionine-80 as the sixth iron ligand in ferricytochrome *c* (Wütrich, 1971). Figure 7 shows the effect of the addition of cyanide on the $^{35}Cl^-$ relaxation rate for cytochrome *c* solutions at neutral and alkaline pH. The upper curve in Figure 7 illustrates the result of addition of KCN to a 0.25 M NaCl solution, pH 7.2, containing 6.0

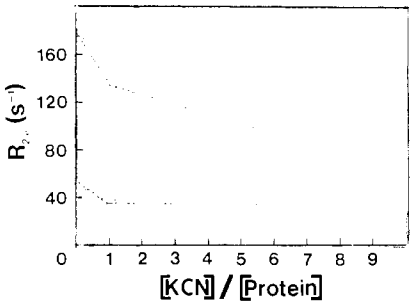

FIGURE 7: The $^{35}Cl^-$ excess transverse relaxation rate of a 0.25 M NaCl solution, containing 6.0 mM oxidized cytochrome *c* at pH 7.2 (○), and a 0.30 M NaCl solution containing 2.9 mM oxidized cyt *c* at pH 9.9 (□), as a function of the cyanide/protein ratio.

mM oxidized cytochrome *c* and shows that cyanide has a marked effect on the chloride binding. In the phosphate and iron hexacyanide competition experiments, we interpreted the decrease in the $^{35}Cl^-$ relaxation rate as arising from direct competition with the chloride ions. This is a reasonable assumption, since there is no evidence of structural changes upon binding these ions to the protein molecule. Cyanide is known to replace methionine-80 as the sixth heme ligand, with a resulting conformational change (Wütrich, 1971). The data in Figure 7 show a kink near a cyanide/protein ratio = 1, and it is reasonable to assign the first part of this curve to this conformational change. In the second part of the upper curve (i.e., cyanide/protein ratio > 1), the relaxation rate decreases, presumably due to competition with other chloride binding sites. In order to find out what causes the "hump" in the pH dependence of the $^{35}Cl^-$ relaxation rate in the presence of Fe^{3+}-cyt *c*, we added KCN to a 0.3 M NaCl solution, pH 9.9, containing 2.9 mM oxidized cytochrome *c*. The result of this experiment is shown in the lower curve in Figure 7. It is worth noting that in this pH region it took more than 1 h after each addition of cyanide before equilibrium was reached, in agreement with the findings of George & Tsou (1952).

The results presented above suggest that oxidized cytochrome *c* has (a) chloride binding site(s) at or very near the exposed heme edge. When the pH is raised, the charge arrangement near the heme is changed, resulting in either an increased chloride binding or possibly a change in the $^{35}Cl^-$ quadrupolar coupling constant.

Sodium Binding to Cytochrome c. The binding of Na^+ was studied by using 5 and 12 mM NaCl solutions containing varying amounts of reduced and oxidized protein. Within the error of the measurements, no enhancement of the $^{23}Na^+$ relaxation rate was observed. From these results, we thus conclude that sodium ions do not bind to oxidized or reduced cytochrome *c*. The only alternative interpretation is that there are sodium ions exchanging too slowly to influence the observed signal. It can be estimated that this would require a k_{off} of about 10^2 s^{-1}, which is not a very likely situation. In this instance, our results are at variance with those of Margoliash and co-workers (1970).

Conclusions

The $^{35}Cl^-$ NMR data clearly show binding to cytochrome *c* in both oxidized and reduced form. Although a detailed interpretation of the data is not possible at this stage, the results clearly reveal a difference in the anion binding properties at the oxidized and reduced form of cytochrome *c*. No evidence of Na^+ binding to the oxidized or reduced protein was obtained.

Iron hexacyanide and phosphate are shown to compete with chloride for the strong chloride binding sites. The effect of anions on iron hexacyanide binding, thus, seems to be site specific. These results suggest an interpretation of the changes in redox potential upon addition of chloride and phosphate as arising from a simple competitive effect.

The effect of KCN for oxidized cytochrome *c* at neutral pH implies that the slight conformational change, induced by cyanide binding to the heme iron, reduces the binding of chloride. The "hump" in the relaxation rate accompanying the alkaline isomerization of ferricytochrome *c* implies either that the relaxation effects of an existing site are increased or that a new site is formed.

The competition experiments and pH dependence of the $^{35}Cl^-$ relaxation rate may be taken to indicate that chloride is bound at the "front side" of the molecule and that the ion binding properties of chloride in this area are different for oxidized and reduced cytochrome *c*. Thus, it is reasonable to suggest that the surface structure or dynamics of the "front side" of the molecule is different in the two oxidation states with possible implications for the redox mechanism of cytochrome *c*.

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Resonance Raman Spectra and Optical Properties of Oxidized Cytochrome Oxidase†

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ABSTRACT: Raman spectra of oxidized cytochrome oxidase and its inhibitor complexes with cyanide and formate have been recorded by using 441.6-nm HeCd laser excitation. Photoreduction effects were avoided by flowing the protein samples through the scattering volume. As an aid in the interpretation of the protein data, Raman spectra of low- and high-spin ferric heme *a* complexes dissolved in water or in non-hydrogen-bonding organic solvents were recorded. The model compound data demonstrate that heme *a* vibrational bands in the 1540–1660-cm^{-1} region are sensitive to iron spin state and indicate that the Raman spectrum of oxidized cytochrome oxidase obtained with 441.6-nm excitation is due primarily to vibrations of low-spin cytochrome a^{3+}. The spectra of the inhibitor complexes of the enzyme are consistent with this interpretation. The selective enhancement of cytochrome a^{3+} vibrational modes under these conditions is rationalized by using simple considerations of the Raman excitation profile and the optical spectra of cytochromes *a* and a_3 deduced by W. H. Vanneste [(1966) *Biochemistry 5*, 838–848]. In contrast to the Raman spectrum of reduced cytochrome a_3, those of oxidized and reduced cytochrome *a* do not show a well-defined heme *a* formyl vibration in the 1670-cm^{-1} region. The model compound data indicate that either hydrogen bonding or lack of conjugation of the formyl π electrons with the porphyrin π system can account for this observation. The implications which this may have for heme–heme interaction in the protein are discussed.

Cytochrome oxidase, the terminal oxidase in mitochondrial respiration, contains two heme *a* bound iron atoms and two copper atoms. In the protein, the hemes *a*, as well as the copper atoms, are functionally and magnetically distinct as reflected in the cytochrome aa_3 nomenclature (Palmer et al., 1976; Erecinska & Wilson, 1978). Cytochrome *a* comprises one of the hemes *a* and its protein surroundings and is a site of cytochrome *c* oxidation. Its iron is low spin in both the resting and fully reduced enzyme with histidine residues as the probable occupants of the fifth and sixth coordination positions (Babcock et al., 1979). Oxygen reduction occurs at cytochrome a_3; the heme *a* iron involved in this reaction is high spin and, in its ferric state, strongly antiferromagnetically coupled to one of the copper atoms (Falk et al., 1977; Tweedle et al., 1978). The second copper is magnetically isolated and, in its cupric state, shows an EPR[1] spectrum with an unusually small hyperfine splitting. The function and ligands of this copper are uncertain, although it may play a part, along with cytochrome *a*, in cytochrome *c* oxidation (Powers et al., 1979; Chan et al., 1979).

Electron paramagnetic resonance, magnetic circular dichroism, and magnetic susceptibility measurements have provided much of the information on cytochrome oxidase at the molecular level. These methods supply detailed information on the paramagnetic ferric, ferrous, and cupric species in the enzyme but are not very sensitive to the geometry and bonding of the unusual peripheral substituents of the heme *a* porphyrin ring (Babcock et al., 1979). The importance of these substituents, a formyl at ring position 8 and a hydroxyfarnesylethyl group at position 2, has been suggested by the reconstitution experiments of Hill & Wharton (1978). For *Pseudomonas aeruginosa* cytochrome oxidase, they observed that reconstitution with either heme *a* or heme d_1 in the enzyme active site restored function; reconstitution with protoheme did not produce oxidase activity.

Resonance Raman spectroscopy yields porphyrin structural information (Spiro, 1974; Warshel, 1977) which is complementary to that obtained by the magnetic techniques described above. For example, the vibrations of porphyrin ring substituents can be observed directly (Salmeen et al., 1973; Lutz, 1977), insight into the planarity of the porphyrin ring can be obtained (Spaulding et al., 1975; Spiro, 1974), and porphyrin

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[1] Abbreviations used: EPR, electron paramagnetic resonance; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MCD, magnetic circular dichroism; Me_2SO, dimethyl sulfoxide.

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