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Hydrogen Ion Titration of Horse Heart Ferricytochrome *c*†

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ABSTRACT: Continuous hydrogen ion titration curves of deionized solutions of horse heart ferricytochrome *c* have been obtained at 25 °C at a constant ionic strength of 0.10 from pH 3.0 to 11.0. Titration of the oxidized protein in KCl required 28.4 equiv over that pH range, and a small hysteresis between the forward and reverse limbs was displayed. The Linderstrom-Lang approximation, which takes into account electrostatic interactions between charged groups on the protein surface, was used in a computer simulation program to analyze the forward and reverse limbs of the titration curve separately. The results indicated 1 α-, 12 β- and γ-, and 1 heme propionic carboxylic, 1 imidazole, 1 phenolic, and 18 ϵ-amino residues appear to titrate normally. Variations in the electrostatic interaction factor ω suggest conformational changes in the protein at the extremes of pH, although the relationship of the variations in ω to the magnitude of the conformational changes does not appear to be strictly quantitative for cytochrome *c*. These results show the acid–base behavior of cytochrome *c* to be complex in nature, and suggest that the Lindenstrom-Lang model may not be adequate for cytochrome *c*.

In mammalian species, cytochrome *c* is composed of 104 amino acid residues having a molecular weight of 12 400 and an iron porphyrin covalently attached via the porphyrin ring vinyl groups to two cysteine sulfhydryls of the protein. Cytochrome *c*, receiving electrons from cytochrome c_1 and in turn reducing cytochrome oxidase, is a necessary link in the mitochondrial electron transfer between NADH or succinate to molecular oxygen (Margoliash and Schejter, 1966). A reversible Michaelis complex between cytochrome *c* and cytochrome oxidase was postulated (Stotz et al., 1938) and some of the properties of the cytochrome *c*–cytochrome oxidase complex were recently reviewed by Nichols (1974). In order to elucidate even the most elementary points of the cytochrome *c*–cytochrome oxidase reaction mechanism and the roles of their interaction in mitochondrial electron transfer and energy coupling, further physical chemical characterization is required. To obtain a better understanding of the thermodynamic factors affecting cytochrome *c* interaction with cytochrome oxidase, we have undertaken the study of hydrogen ion titrations of both oxidized and reduced cytochrome *c* at constant ionic strength.

Acid–base titration curves for this protein have been reported by several authors including Theorell and Åkesson (1941a,b), Paul (1951), Paléus (1954), Bull and Breese (1966a), Greenwood and Wilson (1971), and Marti and Marini (1972). These curves were determined under a variety of experimental conditions including in many instances vari-

† From the Department of Biochemistry and Biophysics, Paul M. Althouse Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received July 22, 1975.* This paper represents the first in a series of studies dealing with the interactions between mammalian cytochrome *c* and cytochrome *c* oxidase. This work was done during the tenure of C. R. Hartzell as an Established Investigator of the American Heart Association and was supported in part with grants from the Central Fund for Research, The Pennsylvania State University and the U.S. Public Health Service, National Heart and Lung Institute (HL 17559). A preliminary report on a part of this work has been presented (Shaw and Hartzell, 1975).

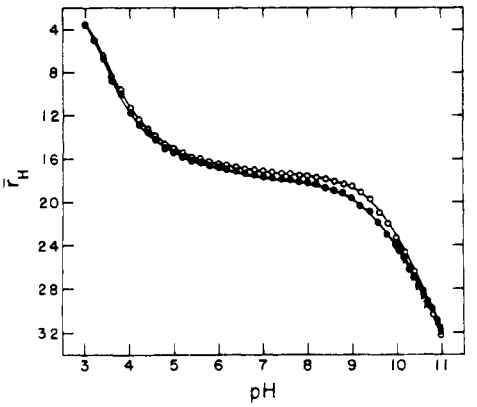

FIGURE 1: Titration curve for horse heart ferricytochrome *c* in 0.1 M KCl showing the dependence of $\bar{r}_H$, the average net number of hydrogen ions dissociated per protein molecule, on pH. The temperature was 25 °C and protein concentration was near 0.4 mM. The titration begins in the isoionic condition and X designates titration with 0.100 N NaOH upward from the isoionic point; (●) titration downward with 0.098 N HCl; and (○) the reverse titration with NaOH. The solid lines represent the theoretically generated curves obtained using the data of Table II and Figures 2a and 2b.

able ionic strengths and the presence of organic solvents and their measurements were often restricted to pH values up to and including the isoionic point. We report herein the hydrogen ion titration at 25 °C of horse heart ferricytochrome *c* from pH 3 to 11 in the presence of 0.10 M KCl.

Experimental Procedure

Horse heart cytochrome *c* was purchased from Miles Research Laboratories. The protein was further purified, concentrated, and dialyzed by the methods described by Margoliash and Walasek (1967), and the purity of the protein sample was confirmed by their criteria.

The titration procedure employed was very similar to that described by Hartzell et al. (1967). Immediately prior to each titration, 2 ml of approximately 2 mM purified protein in deionized, distilled water was allowed to pass slowly through a column (0.5 × 5 cm) of Rexyn 300 (Fisher) mixed bed resin. The deionized sample was collected under water-saturated nitrogen in a 50-ml filter flask. In some of the titration experiments, the electrodialysis procedure of Katz and Ellinger (1963) was used as an alternative method of deionization. Identical titration results were obtained regardless of which deionization technique was applied. One milliliter of the deionized sample was transferred to a water-jacketed titration vessel which was continuously flushed with a stream of nitrogen saturated with the electrolyte solution. Four milliliters of 0.125 M KCl was added to the sample to bring the ionic strength to 0.1. The protein concentration was determined spectrophotometrically at 550 nm on a separate sample of the deionized solution using the reduced minus oxidized millimolar extinction coefficient of 21.1 mM^{-1} cm^{-1} reported by Van Gelder and Slater (1962) for horse heart cytochrome *c* in 0.055 M phosphate buffer (pH 7.6).

Continuous titrations were performed using an Orion Model 801 digital pH meter equipped with external calomel (Radiometer K101/K) and glass (Radiometer G202B) electrodes. During the course of the titration, constant stirring was maintained with a magnetic bar and the temperature of the protein solution and the calomel cell was regulated at 25.00 ± 0.05 °C. In each titration, the pH meter was standardized with potassium hydrogen phthalate (pH 4.01), sodium phosphate (pH 6.86), and sodium borate (pH 9.18) buffers (National Bureau of Standards). After each titration the meter was again standardized and if the agreement was not within 0.02 pH unit the entire titration was discarded. Micrometer syringe burets (Micro-Metric Instrument Company, Cleveland, Ohio) of 1- or 3-ml capacities were used to make additions of standardized solutions of HCl (0.098 N) or $HClO_4$ (0.101 N) and CO_2-free NaOH (0.100 N). Equilibrium was usually achieved instantaneously after each addition of acid or base except within the pH region around neutrality which required periods as long as 10 min to attain equilibrium. Each sample was first titrated from the isoionic point up to pH 11.0 with NaOH, then titrated to pH 3.0 with HCl (referred to as the acid limb), and finally back-titrated to pH 11.0 with NaOH (referred to as the basic limb). The observed net uptake of acid or base at a given pH was corrected for the unbound forms by blank titrations of 0.100 M KCl. Three independent titration experiments were performed and the results are reported as $\bar{r}_H$, the average number of hydrogen ions per protein molecule dissociated from the initially isoionic protein, vs. pH.

In order to compute simulations of the experimental titration curves using various reported titration parameters, a Fortran computer program was developed which permitted successive approximations to the experimental curve using the Linderstrom-Lang (1924) formalism. The key feature of this program is that the electrostatic interaction factor ω may be varied with pH. The calculations were performed using the IBM 370/168 computer located at the Computation Center of the Pennsylvania State University.

Results

The titration curve for the oxidized protein in 0.1 M KCl in the pH region from 3.0 to 11.0 is shown in Figure 1. This figure represents the average of three independent experiments. In this figure, the X's indicate the initial titration with NaOH starting from the isoionic pH value of 10.04 up to pH 11.0, and the reverse-titration is denoted by filled circles. Finally, the back-titration from pH 3.0 up to pH 11.0 is indicated by the open circles. The titration from pH 10.0 to 11.0 with NaOH is found to be completely superimposable on the reverse titration with acid, as is the back-titration with NaOH from pH 3.0 to 6.0. However, the curve shows a small but persistent hysteresis between the base and acid limbs[1] extending from pH 5.8 to 10.0, and the magnitude of the difference never exceeds 1.1 equiv. There were no visible signs of protein precipitation in any pH region throughout the entire course of the titration.

Although it is impossible to specify exactly the number of carboxylic, histidine, and amino residues from the titration curves alone, it is generally possible to divide the titration curve into three portions and to count separately the groups which titrate in the acid, neutral, and alkaline regions. Then, the groups titrated in the acid part of the curve may be tentatively designated as carboxylic groups while the middle region of the curve may be attributed to histidine imidazole ionizations (there is no free α-amino group in horse heart cytochrome *c*) and the alkaline region may be assigned to side-chain amino and phenolic groups (Tanford, 1962). The results of such an analysis are listed in the bottom two rows of Table I. The values designated HCl titration correspond to the number of groups titrated from pH 3.0 to a given point on the acid limb of the

[1] The terminologies "acid limb" and "base limb" are used in an operational sense, referring to the fact that the protein is being titrated with acid or base, respectively. These terms have nothing to do with the acidity or basicity of the solutions.

Table I: Numbers of Expected and Observed Titratable Groups for Horse Heart Ferricytochrome *c*.

	Equiv Titrated from pH 3.0 to 5.5	Equiv Titrated from pH 5.5 to 8.5	Equiv Titrated from pH 8.5 to 11.0	Total Equiv Titrated
No. expected from corresponding mixture of small molecules[a]	13.2	5.2	19.7	38.1
No. expected in absence of electrostatic interactions[b]				
HCl titration	13.7	2.8	16.6	33.1
NaOH back-titration	13.0	2.2	16.4	31.6
No. observed in 0.1 M KCl[c]				
HCl titration	12.8 ± 0.14	2.5 ± 0.15	12.8 ± 0.84	28.1 ± 1.10
NaOH back-titration	12.5 ± 0.09	2.0 ± 0.00	14.3 ± 0.96	28.8 ± 0.99

[a] Calculated from the Henderson–Hasselbach equation using amino acid analysis data (Margoliash et al., 1961) and data on dissociation constants of small molecules (Tanford, 1961, 1962; Nozaki and Tanford, 1967). The pK values used were 3.75 for the α-carboxylic, 4.6 for β- and γ-carboxylic, 4.87 for propionic carboxylic, 7.0 for histidine imidazolium, 9.6 for phenolic, 10.2 for ϵ-amino, and 12.5 for guanidino groups. [b] Calculated from the Henderson–Hasselbach equation assuming no electrostatic interactions between charged groups, and using the numbers of groups and pK' values from Table II. [c] Reported as mean ± standard error of the mean, $N = 3$.

Table II: Number of Groups and Intrinsic pK Values Used to Simulate Titration Curve of Horse Heart Ferricytochrome *c* in 0.1 M KCl.

Acid Limb (pH 11.0 to 3.0)		Base Limb (pH 3.0 to 11.0)		
No. of Groups	Intrinsic pK	No. of Groups	Intrinsic pK	Residue Assignment
1	2.7	1	2.1	His-18
1	2.9	1	2.9	His-26
12	3.6	1	3.6	α-Carboxylic
1	4.4	12	4.4	β- and γ-carboxylic
1	4.8	1	4.6	Propionic carboxylic
1	6.5	1	6.2	His-33
1	9.5	1	9.4	Lys-79
1	10.1	1	10.4	Phenolic
18	10.4	1	10.5	ϵ-Amino
1	12.2	1	12.4	Phenolic
2	12.6	2	12.6	Arginines
1	13.1	1	13.1	Phenolic
1	5.4	1	9.4	Propionic carboxylic

curve, while the values under NaOH back-titration are calculated using the $\bar{r}_H$ value from the base limb of the titration curve. Also included in Table I are entries for the number of groups expected to be titrated in each pH region calculated using the Henderson–Hasselbach expression. The first set of entries includes the numbers expected from the titration of a mixture of noninteracting small molecules corresponding to the amino acid composition determined by Margoliash et al. (1961). The second set of entries corresponding to a similar Henderson–Hasselbach analysis using the pK' values and numbers of groups generated from the computer simulation (see Table II) of the experimental titration curve (Figure 1) agrees much more closely with the observed numbers.

The results of the simulation are shown in Figure 1 in which the solid lines represent the theoretically generated titration curves which most closely fit the experimental curve. The pK' values used to obtain the theoretical curves are listed in Table II. The electrostatic interaction factor varied with pH and the values used in the simulation are presented in the form of plots

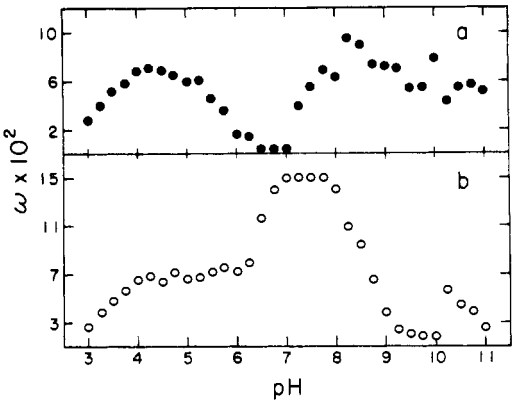

FIGURE 2: The dependence of the electrostatic interaction factor ω on pH. The upper curve (a) shows the dependence for the acid limb of the titration curve, while the lower plot (b) shows this dependence for the base limb. The symbols used are the same as those in Figure 1.

of this parameter as a function of pH for the acid and base limbs of the titration curve in Figures 2a and 2b, respectively.

For both the acid and base limbs of the titration curve several classes of groups were required to simulate the experimental data adequately. Thirteen classes were required for simulation of the basic limb while an additional class consisting of one group with a pK' value of 5.4 was required in the simulation of the acid limb.

The electrostatic interaction factor ω varies with pH for both limbs of the curve but over the most of the titration at a given pH, ω is larger for the base limb than for the corresponding point on the acid limb. Also, ω achieves a maximum between pH 7 and 8 on the basic limb which has no comparable counterpart on the acid limb of the titration.

The classical expression (Linderstrom-Lang, 1924) for the analysis of protein titration curves taking into account electrostatic interactions between charged groups is:

$$\mathrm{pH} - \log \frac{\bar{r}_i}{n_i - \bar{r}_i} = \mathrm{p}K_i' - 0.868\omega\bar{Z} \qquad (1)$$

in which $\bar{r}_i$ is the average number of protons per molecule dissociated from groups of the ith class, n_i is the total number of groups in the class, $\bar{Z}$ is the average charge on the protein, pK_i' is the intrinsic pK of the groups of the class, and ω is the electrostatic interaction factor. The usual procedure, in accordance with Tanford (1962), is to plot the left side of eq 1

against $\bar{Z}$; values of ω are taken from the slope of the straight line portion so obtained and values of pK' from the value of the left-hand sum at $\bar{Z} = 0$. Corrections for ionization of groups outside the ith class are then calculated from assumed or experimentally determined values for ω and the pK values of interfering groups. Such a procedure will show whether ω can be considered a constant or if it must be regarded as a variable. Initial calculations following this procedure indicated that ω varied with pH. Thus, we have elected to use a curve-fitting technique to simulate the experimental titration curves.

Discussion

The titration curve we have obtained for horse heart ferricytochrome c in KCl is in substantial agreement with previously reported experiments (Paul, 1951; Paléus, 1954; Bull and Breese, 1966a; Greenwood and Wilson, 1971; Marti and Marini, 1972), and with that reported by Theorell and Åkesson (1941b) for bovine cytochrome c if the difference in lysine content in the equine and bovine proteins is taken into account. The back-titration curve (open circles, Figure 1) is in particularly close agreement with their data in the pH region from 5.5 to 8.5, within which exactly 2.0 equiv is titrated. Greenwood and Wilson (1971) have reported that about 25 groups are titrated from pH 2.5 to 10. While the numbers of groups titrated and overall shape of their titration curve are very similar to ours, the hysteresis observed in our titrations was not revealed in their experiments. Discontinuous titrations of horse heart cytochrome c were performed by Bull and Breese (1966a) at an ionic strength of 0.2 in KCl from pH 10 to 2, along with back-titrations to pH 10. Although the pH which they report for an isoionic solution is somewhat low, indicating the possibility of incomplete deionization prior to titration, when the ordinate of their titration curve is shifted to correct for this discrepancy, a comparison with the data in Figure 1 shows that their curve is nearly identical with ours over the common pH ranges. The hysteresis revealed by our continuous titrations is shown to be present at the same magnitude in their discontinuous titrations. These authors report a value of 24 for ΣN^+, the number of hydrogen ions required to go from the point of zero net charge to the acid end point, with 20 of these equivalents titrated between pH 3 and 10. Marti and Marini (1972) report a slightly higher value of 25.8 for the titration in 0.15 M KCl from pH 2 to 10. Our data indicate that from pH 3 to 10, 20.5 equiv is titrated (Figure 1).

The total number of ionizable side chains in horse heart cytochrome c determined from amino acid and x-ray diffraction analyses is 43 (Margoliash et al., 1961; Dickerson et al., 1971). The number of groups ionizing between pH 3 and 11 is less than expected from the protein composition, but considering the known structure and chemistry of the molecule this observation is not unreasonable Theorell and Åkesson, 1941b; Rupley, 1964; Stellwagen and Cass, 1974; Dickerson et al., 1971; Babul and Stellwagen, 1972; Gupta and Koenig, 1971; Cohen et al., 1974; Harbury, 1966; Stellwagen, 1966). It should also be noted that a considerable portion of the discrepancy between the number of groups predicted to titrate from amino acid analysis and known dissociation constants of small molecules and the observed numbers (see Table I) is removed when the calculation is repeated using the numbers of groups and intrinsic pK (pK') values from the simulation in Table II. Thus it appears that much of the discrepancy is due to the pK values used.

The solid lines shown in Figure 1 represent the best fit obtained to the experimental curves and were achieved using the pK' values of Table II and the electrostatic interaction factor calculated at each pH taken (Figures 2a and 2b). Clearly, the agreement between the experimental and simulated curves is excellent.

Table III represents a summary of the various apparent pK values observed for equine and bovine heart ferricytochrome c. We believe that the data shown here include the ionizations that are most important in the equilibration of ferricytochrome c with hydrogen ions. In all likelihood, the minor fluctuations in the values reported for the same ionization are the result of slight variations in the experimental conditions used. The apparent pK values presented in Table III have been arranged in groups which probably represent the same ionization. Most of these apparent pK values have corresponding intrinsic pK values listed in Table II.

The results in Table II can be interpreted using the assignments presented in Table III. One α- (pK' = 3.6), 12 β- and γ- (pK' = 4.4), and 1 heme propionic carboxylic (pK' = 4.6–4.8), 1 histidine imidazolium (pK' = 6.2–6.5), 1 phenolic (pK' = 10.1–10.4), 18 ϵ-amino groups (pK' = 10.4–10.5), and 2 guanidyl groups (pK' = 12.6) appear to titrate normally. In addition, the intrinsic pK values of 2.1–2.7, 2.9, 9.4–9.5, 10.8–10.9, 12.2–12.4, and 13.1 are assigned to the ionizations of His-18, His-26, Lys-79, and three tyrosine phenolic residues, respectively. The only remaining ionizable group from charge group analysis unaccounted for is that of the buried heme propionic carboxylic group. The last entries in Table II include pK' values of 5.4 for the acid limb and 9.4 for the base limb and this ionization has been tentatively assigned to the ionization of the buried propionic carboxylic group. Obviously, the involvement of factors other than electrostatic interactions is suggested. We believe this group is conformationally masked and thus prevented from ionizing during the basic limb until after a conformational change occurs in the alkaline pH region. Such a conformational change might be coincident with the deprotonation and ligand exchange reaction of Lys-79 (Gupta and Koenig, 1971; Stellwagen and Cass, 1974). Then, reprotonation of the propionic carboxylic group would occur around pH 5.4. The pK' of 5.4 is somewhat higher than the pK of 4.87 for propionic acid, consistent with its hydrophobic location (Dickerson et al., 1971). This interpretation is consistent with the variations in the electrostatic work factor shown in Figures 2a and 2b. Throughout much of the titration, the electrostatic interaction factor for the acidic limb corresponds to a larger protein radius than that of the basic limb (except above pH 9). This suggests reversible swelling of the protein during titration. The hysteresis between the two limbs of the curve can be accounted for by these differences in pK' and ω.

The wide variations in the electrostatic interaction factor with pH indicate that cytochrome c does not fit the Linderstrom-Lang (1924) model exactly. Although changes in the conformation of ferricytochrome c in the presence of chloride begin to occur at about pH 4 in the acid region and at pH 9 at the alkaline end of the titration (with concomitant ligand exchange in the sixth position), total unfolding of the protein probably does not occur between pH 3.0 and 11.0 (Theorell and Åkesson, 1941a; Boeri et al., 1953; Bull and Breese, 1966a; Schechter and Saludjian, 1967; Gupta and Koenig, 1971; Rupley, 1964; Stellwagen and Cass, 1974; Babul and Stellwagen, 1972; Lemberg and Barret, 1973; Cohen et al., 1974). Thus, the variations in the electrostatic interaction factor shown in Figures 2a and 2b cannot be regarded as strictly quantitative predictions of the cytochrome c conformation, but we believe that the qualitative trends established are reasonable. In addition to swelling somewhat during the course of the titration, other factors cause cytochrome c to deviate from the

Table III: Previously Reported Apparent pK Values for Mammalian Heart Ferricytochrome *c*.

Apparent pK	No. of Groups	Source[a]	Method of Determination[b]	Author's Original Assignment[c]	Reference
0.4	1	B	ST	His-18	Theorell and Åkesson (1941a)
1.7	1	E	PT	His-18	Bull and Breese (1966b)
2.1	2	B	ST	His-18	Boeri et al. (1953)
2.3	1	E	PT	His-18	Bull and Breese (1966b)
2.5	1	B	ST	His-18	Theorell and Åkesson (1941a)
2.5	1	E	ST	His-18	Babul and Stellwagen (1972)
2.5	1	E	ST	His-18	Cohen et al. (1974)
2.8	1	B	ST	Abnormal carboxyl	Flatmark (1966)
3.2	1	E	ST		Greenwood and Wilson (1971)
<3.2	1	E	NMR of C-2 proton	His-26	Cohen et al. (1974)
3–4	12	E	PT	Carboxyl	Bull and Breese (1966b)
4.5	1	E	PT	Carboxyl	Bull and Breese (1966b)
5.0	1	E	KCF	His-18	George et al. (1967)
5.1	1	E	PT		Bull and Breese (1966b)
5–6	1	E	pH jump, ST		Czerlinski and Bracokova (1973) Zabinski et al. (1973)
5.4	2	B	PT	Porphyrin propionates	Paléus (1954)
6.1	1	E	PT		Bull and Breese (1966b)
6.4	1	E	NMR of C-2 proton	His-33	Cohen et al. (1974)
6.4	1	E	NMR of C-2 proton	His-33	Dobson et al. (1975)
6.8	1	B	PT	His	Paléus (1954)
6.9	1	B	$E^{0\prime}$ vs. pH	His	Paul (1947)
7.8	1	B	$E^{0\prime}$ vs. pH		Rodkey and Ball (1950)
8.4	1	E	PT	Amino	Bull and Breese (1966b)
8.6	1	E	$E^{0\prime}$ vs. pH		Margalit and Schejter (1973a)
8.8	1	B	KCF		George and Tsou (1952)
8.9	1	E	ST		Greenwood and Wilson (1971)
9.0	1	E	NMR	Lys-79	Gupta and Koenig (1971)
9.1	1	E	pH ROK	His-18 imino proton	Czerlinski and Dar (1971)
9.2	1	E	ST	Lys-79	Stellwagen and Cass (1974)
9.3	1	B	ST	His-18 imino proton	Theorell and Åkesson (1941a)
9.3	1	E	pH ROK	Lys-79	Lambeth et al. (1973)
9.8	1	B	PT	His-18 imino proton	Theorell and Åkesson (1941b)
11	1	E	ΔA, stopped-flow	Lys-79	Davis et al. (1974)
10.0	1	E	ST	Tyr	Rupley (1964)
10.4	1	E	pH ROK	Tyr-67	Czerlinski and Dar (1971)
11.0	1	E	ST	Tyr	Rupley (1964)
11.0	1	E	NMR	Tyr-67	Gupta and Koenig (1971)
12.4	1	E	ST	Tyr	Rupley (1964)
12.8	1	B	ST	Coordination of hydroxyl group	Theorell and Åkesson (1941a)
13.1	1	E	ST	Tyr	Rupley (1964)

[a] Abbreviations used are: B, bovine; E, equine. [b] Abbreviations used are: ST, spectrophotometric titration; PT, potentiometric pH titration; KCF, kinetics of complex formation; pH ROK, pH dependence of rapid oxidation kinetics; ΔA, difference spectroscopy. [c] Unless otherwise indicated, assignments of ionizations to histidine (His) residues are intended to represent dissociation of the imidazolium proton.

Linderstrom-Lang model. These include the failure of chemically identical groups to have the same intrinsic pK, the indication that cytochrome *c* is not truly spherical, and the fact that the charged groups are not uniformly distributed over the protein surface (Dickerson et al., 1971).

To calculate a theoretical value for the electrostatic interaction factor, we have made the usual assumption that $a = b + 2.5$ Å in the Debye–Hückel expression (Cohn and Edsall, 1943) and have used the Stokes radius of 17 Å reported by Tanford et al. (1974) for the hydrated protein radius. This leads to a theoretical value for ω of 0.11 at an ionic strength of 0.1. Since cytochrome *c* has approximately seven positive charges at neutral pH which must be removed from the surface upon titration up to the isoionic point, it seemed possible that the protein might actually shrink somewhat during this part of the titration. Thus a value of 0.15 was chosen for the upper limit of ω in the computer simulations, while a lower limit of 0.004 was used.

To our knowledge, no quantitative data are available on the exact numbers of chloride ions bound to cytochrome *c* as a function of pH although it is known that one to two chloride ions are bound at neutral pH (Barlow and Margoliash, 1966; Margoliash et al., 1970; Margalit and Schejter, 1973a,b; Dickerson et al., 1971). Therefore, it has been impossible to correct accurately the $\bar{Z}$ values obtained from Figure 1 for the extent of chloride binding to the protein.

Whether the interaction between cytochrome *c* and cytochrome *c* oxidase involves the protein, lipid, or both of these components of the oxidase is uncertain (Nichols, 1974). During the course of study on this problem we became aware of the incompleteness of the hydrogen ion titration data for the oxidized and reduced forms of cytochrome *c*. A complete Linderstrom-Lang analysis for both forms of this protein has never been reported. We also learned of the complications in the

thermodynamics and kinetics of the cytochrome *c*-cytochrome oxidase system introduced by the presence of various anions and cations. Therefore, we have initiated a study of these problems on the assumption that the complex between cytochrome *c* and cytochrome *c* oxidase is largely an electrostatic protein-protein interaction (Sekuzu et al., 1960; Kuboyama et al., 1962; Nichols, 1964; Nichols et al., 1969; Hartzell and Shaw, 1973; Nichols, 1974). Knowledge of the intrinsic p*K* values, electrostatic interaction factor, and net surface charge for the horse heart cytochrome *c* molecule should contribute to a better understanding of the cytochrome *c*-cytochrome *c* oxidase complex.

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