Characterization of Phenolic Ionizations in Horse Heart Cytochrome *c*†

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ABSTRACT: The ionization constants and molar difference absorbance values of tyrosyl groups in horse heart ferricytochrome *c* were obtained and compared with those reported in the literature. A computer-assisted curve fitting procedure applied to experimental data and previous literature reports indicates two possible sets of parameter values. The first ionization constant pK'_1 is fixed at 10.1 ± 0.1 regardless of assigned molar difference absorbance while pK'_2 varies from 11.0 to 12.0 as a function of its assigned molar difference absorbance value. The remaining ionization constants pK'_3 and pK'_4 are each 12.35 ± 0.05 and appear to be independent of the assigned absorbance. Forward and reverse titrations are reversible between pH 10 and pH 13. Time-dependent changes were seen for ferrocytochrome *c* above pH 12.5 requiring pH jump experiments to extrapolate to zero time. Under conditions of a nitrogen atmosphere and in the presence of ascorbate, 0.97 group is ionized in water at pH 13.2. The analysis of this ionization in ferrocytochrome *c* is described by a pK' of 12.55 and a molar difference absorbance value of 14 420. The redox protonic tyrosyl difference curve was fitted with three ionization constants 9.9, 12.0, and 12.9 with the corresponding molar difference absorbance values of 9900, 10 900, and 10 800, respectively.

In an attempt to understand the complete hydrogen ion equilibria curves of horse heart ferri- and ferrocytochrome *c*, we undertook the spectrophotometric titration of tyrosyl residues in this mitochondrial heme protein. It has been reported that two, three, or four of the four tyrosines in ferricytochrome *c* ionize abnormally (Flatmark, 1964; Rupley, 1964; Stellwagen, 1964). The ionization of the tyrosyl residues in the apoprotein of cytochrome *c* has been evaluated (Stellwagen & Rysavy, 1972), and there is a report on the redox differences in the tyrosyl ionization of native cytochrome *c* (Stellwagen, 1964). Chemical reactivity and solvent perturbation have been used to probe further the native and microenvironment of normal and abnormal tyrosyl ionizations in cytochrome *c* (Stellwagen & McGown, 1960; Myer & Pal, 1972; Stellwagen & Van Rooyan, 1967; Skov et al., 1969; O'Hern et al., 1975). These studies have been used to identify and isolate redox-linked changes for the tyrosyl residues within cytochrome *c*. Since phenolic spectrophotometric titrations are needed to facilitate the analysis and interpretation of the more extensive hydrogen ion potentiometric measurements (Marini et al., 1981) of the protein and because of the variations reported in the literature, a reinvestigation of the ionization of tyrosyl residues in both redox species of horse heart cytochrome *c* seemed appropriate.

Materials and Methods

Cytochrome c Preparation. Horse heart cytochrome *c* Type VI was the generous gift of the Sigma Chemica Co. (St. Louis, MO; lot no. 72C-7490), which was treated with an excess of potassium hexaferricyanide or sodium ascorbate and sodium dithionite and dialyzed exhaustively against water at 4 °C and then passed through a mix-bed ion-exchange column (Rexyn I-300, Fisher Chemical Co.). Deionized stock solutions were millimolar in concentration and adjusted to neutrality with 1 N HCl and stored at 4 °C. A stock solution of phenol (0.05 M) was prepared daily and used for the potentiometric titrations. A 1:500 dilution was used for spectral titrations.

Spectral Titrations. Protein concentrations were determined from the absorbance at 550 nm for ferrocytochrome *c* and 528 nm for ferricytochrome *c* using the molar absorbance coefficients reported by Margoliash & Frohwirt (1959). Working solutions (10^{-5} M) were prepared daily and degassed with nitrogen. Three-milliliter samples were titrated slowly from pH 7 to pH 13.2 to allow for equilibrium to occur, and the titration was reversed. In some experiments continuous (nonequilibrium) titrations were conducted. The reversibility of ferricytochrome *c* titrations was further investigated by forward and reverse titrations from the isoionic pH ($pI \cong 10.3$). Solutions were titrated from the pI to pH 13 and back to pH 10 and from the pI to pH 7 and back to pH 10. All spectrophotometric titrations were performed with a Cary 14 recording spectrophotometer thermostated at 20 ± 1 °C. Careful attention was paid to the dynode setting, pen response, gain and scanning conditions, slit width settings, and chart speed. The slit width at 243 nm was 0.065 ± 0.005 mm, and a matched pair of 10 mm quartz cuvettes was used. Solutions containing guanidine hydrochloride required a slit width of 0.10 ± 0.01 mm at 243 nm. Visible spectra slit width requirements were less than 0.05 mm. All volume additions were made with either a micrometer attached to a calibrated syringe or a conventional mechanical micropipet. All readings were corrected for the volume changes. Saturated potassium hydroxide was used to obtain higher pH values to determine the maximum observable absorbance at 243 nm. Details of the procedure have been reported by Martin & Marini (1982). Base lines were obtained by using water and zeroed throughout the spectral range studied. For cytochrome *c* titrations the base line was set at 0.2 to allow negative deflections (Figure 1).

Time-dependent changes were seen for ferrocytochrome *c* above pH 12.5, and therefore "pH jump" type experiments were used to obtain absorbances at 243 and 550 nm. A predetermined amount of 1 N potassium hydroxide was added to a 3-mL sample and rapidly mixed (approximately 3–5 s). A continuous time tracing permitted linear extrapolation to zero time. In this way, the amount of reduced species at 550 nm could be correlated with the amount of ionized phenolic residues at 243 nm. In addition to the nitrogen purge of ferrocytochrome *c* solutions, sodium ascorbate (10^{-5} M) was sometimes added. The contribution of ascorbate to the absorption at 243 nm was <2% and delayed the oxidation of

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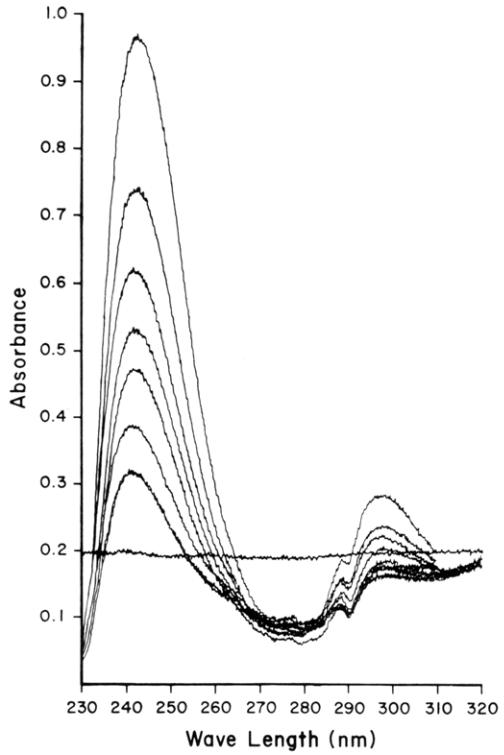

FIGURE 1: pH difference spectral titration of ferricytochrome *c* in water at 20 °C. The reference solution was maintained at neutral pH and volume corrections were made. The scan rate was 10/2 Å/s with a chart speed of 2 in./min. The cytochrome *c* concentration was $\sim 1.34 \times 10^{-5}$ M, and the pH (lower to upper curve) was 9.5, 10.0, 10.5, 11.0, 11.5, 12.0, and 12.5.

ferrocytochrome *c*, which permitted a more precise extrapolation to zero time. Due to the rapid conversion of ferro- to ferricytochrome *c* in 4.8 M guanidine hydrochloride, a 2.4 M guanidine hydrochloride solution was used.

Potentiometric pH Titrations. The pH of protein solutions was determined by using a Radiometer pHMC4 meter with a glass membrane electrode (type 202B) with a Radiometer open junction calomel electrode (type K100). The standardization of the pH scale has previously been reported (Marini & Wunsch, 1968). Using 1 N KOH as a titrant and titrating in 0.25 pH unit increments, we determined the amount of base needed for a given pH for a 3-mL sample. The reproducibility of these titrations were within 1–2% for any pH value. For pH values above 13.2, saturated KOH was used.

Data Analysis. Six reported titration curves were selected from the literature and digitized as previously described (Martin & Marini, 1982). This procedure yields the coordinates of the curve in a form that can be analyzed for ionization constants (pK') and the optimal difference absorbance per tyrosyl residue. The curves selected for analysis were those of Flatmark (1964), Rupley (1964), Stellwagen (1964), Stellwagen & McGown (1970), Myer & Pal (1972), and O'Hern et al. (1975).

Results

The pH difference absorbance spectra of ferricytochrome *c* in water at 20 °C are shown in Figure 1 and are representative of seven separate experiments obtained at equilibrium. Maximum absorption occurs at 242 ± 1 nm; although this peak apears slightly skewed, the variation of ΔA_M is small and the distance between the ascending and descending segments of the peak is constant at the half-height rise. This symmetry suggests that a single chromophore is responsible for the observed absorption, as is seen for the ΔA_M difference

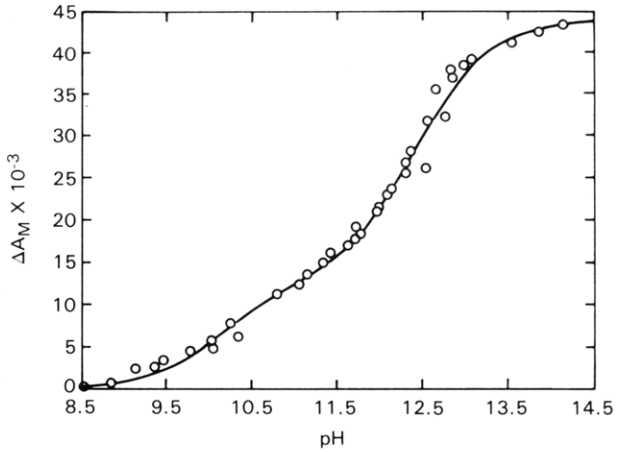

FIGURE 2: Change in absorbance at 243 nm as a function of pH for horse heart ferricytochrome *c* under equilibrium conditions. The open circles are experimental points while the smooth curve is the calculated curve. At pH 10.1, the molar absorbance is 12 720 ± 910, while at pH 12.4, it is 31 6000 ± 980. See the text for details.

spectra of *N*-acetyltyrosine amide (Martin & Marini, 1982). A blue shift of the ΔA_M from 232 to 243 nm for cytochrome *c* is assumed to be due to the protein–chromophore electronic interaction. The second maximum of the protein at 298 nm is also shifted upscale from the 293-nm maximum of *N*-acetyltyrosine amide, but it has a much smaller ΔA_M than the 243-nm peak and was not further studied. The small shoulder at 288 nm is believed to be related to the tryptophan absorption.

A titration of the absorbance changes at 243 nm for ferricytochrome *c* as a function of pH is shown in Figure 2 using a cytochrome *c* solution at pH 7 as a reference. The complete ionization of the phenolic hydroxyl groups of the protein gave a value ΔA_M at 243 nm of 46 300, which corresponds to an average molar difference absorbance of 11 600 M^{-1} cm^{-1} per tyrosine. Flatmark (1964) reports 10 530 and Stellwagen (1964) found 11 000. This value (ΔA_M = 11 600) was initially used to calculate the ionization constants of the tyrosine residues of both redox forms.

Experimental tyrosyl ionization curves for horse heart ferricytochrome *c* can be analyzed with the use of a computer-assisted, iterative, curve-fitting procedure (MLAB), which adjusts the parameter values of a function to minimize the sum of the squared errors of the curve generated from the calculated parameter values and the experimental data (Knott & Schrager, 1972). The function is

$$\bar{r} = \sum_{i=1} N_i/(1 + 10^{pK'_i - pH})$$

where $\bar{r}$ is the sum of all the groups ionized (or titrated) with the ionization constants pK'_1 to pK'_4 and N_1 to N_4, the molar absorbance of the individual tyrosyl group. Initially N was assumed to be the same for each tyrosine residue and the ionization constants pK'_1 to pK'_4 were determined on this basis. Alternatively, the ionization constants may be assigned, thereby permitting an evaluation of absorbance. A more useful procedure is the simultaneous fitting of all parameter values. To that end, a systematic evaluation of pK's and tyrosyl absorbance was made, and these data are summarized in Table I. The experimental curve can be described by one ionization constant with pK' = 10.13 ± 0.10 and a molar absorbance of 12 720 ± 910; the remaining portion of the curve could be fitted for three ionizations with a pK' of 12.36 ± 0.05 and a total absorbance of 31 600 ± 980 (11 100, 10 900, and 9600, respectively). This solution is not unique (see Table I). The ionization constants pK'_1 to pK'_4 are listed in order of in-

Table I: Evaluation of Tyrosine Ionizations and Millimolar Absorbance Coefficients in Horse Heart Ferricytochrome *c* Using Constraints

constraints[a]	pK'_1	ΔA_{mM_1}	pK'_2	ΔA_{mM_2}	pK'_3	ΔA_{mM_3}	pK'_4	ΔA_{mM_4}
A	10.01	11.00	11.87	11.00	12.52	11.00	12.56	11.00
B	10.10	12.20	12.35	10.43	12.35	10.43	12.35	10.43
C	10.13	12.36	12.76	9.60	12.36	10.90	12.36	10.90
D	10.13	12.69	12.36	9.64	12.37	10.43	12.37	11.10
E	10.35	11.12	10.35	11.12	10.35	11.12	10.35	11.12

[a] A = evaluation of pK' values with ΔA_{mM} extinction held constant for all four tyrosyl groups. B = evaluation of pK' values with ΔA_{mM} extinction held constant for three tyrosyl groups. C = evaluation of pK' values with ΔA_{mM} extinction held constant for two tyrosyl groups. D = evaluation of pK' values with ΔA_{mM} extinction different for each tyrosyl group. E = best-fit pK' and ΔA_{mM} for ferricytochrome *c* in 4.8 M guanidine hydrochloride.

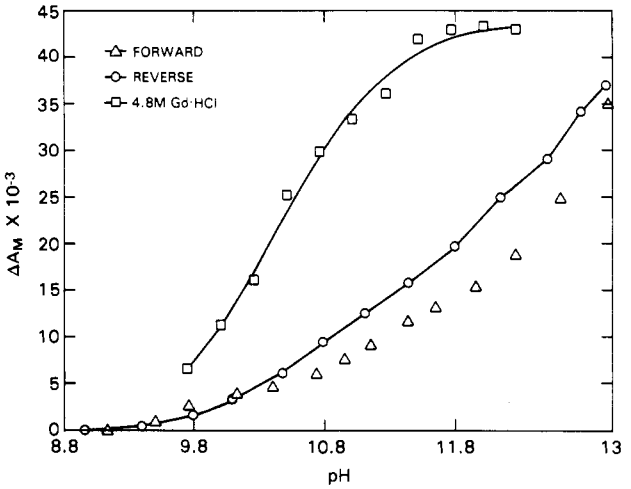

FIGURE 3: Continuous forward (Δ) and reverse (O) spectrophotometric titrations of horse heart ferricytochrome *c* at 243 nm as a function of pH in water at 20 °C. Forward (□) titration of horse heart ferricytochrome *c* in 4.8 M guanidine. The mean on each experimental point is the same as in Figure 2.

creasing magnitude and do not indicate residue assignment. The first ionization constant $pK'_1 \sim 10.1$ appears to be reasonably constant by any analysis while pK'_2 shows the greatest variation. Closer analysis, however, suggests that variation of pK'_2 is dependent upon the assigned value of its absorbance. Thus, when the assignment of individual tyrosyl absorbance is varied, two cases are possible: (1) one normal ($pK \sim 10$) and three abnormal tyrosyl ionizations ($pK > 12$); (2) one norrmal ($pK \sim 10$), one abnormal with $11 < pK < 12$, and two abnormal ($pK > 12$) ionizations. Any set of parameter values will adequately describe the experimental curve (Figure 2).

The continuous (nonequilibrium conditions) forward titration from pH 7 to pH 13 and reverse spectral titration curves of ferricytochrome *c* in water are shown in Figure 3. These titrations are not coincident; the reverse titration is shifted downscale. The difference occurs primarily between pH 10 and pH 13. However, the isoionic point is used as a reference point; the forward and reverse titration from pH 10 to pH 13 is essentially reversible (Figure 4B). Although a more difficult region to access experimentally the titration from pH 10 to pH 7 is not reversible (Figure 4B). This irreversibility from pH 7 to pH 10 may represent a slow conformational change that also causes an irreversibility (hysteresis) in the potentiometric titration of ferricytochrome *c* (Shaw & Hartzell, 1976; Marini et al., 1980). The effect of 4.8 M guanidine hydrochloride on the ferricytochrome *c* titration at 243 nm causes all of the phenolic groups of tyrosine to ionize with nearly normal ionization constants (see Table I). In 4.8 M guanidine hydrochloride, tyrosine titrates with a ΔA_M of 11 120 and a pK' of 10.35.

Time-dependent changes are also found in the 243- and 500-nm bands for ferrocytochrome *c* at pH >12.5. Resolution

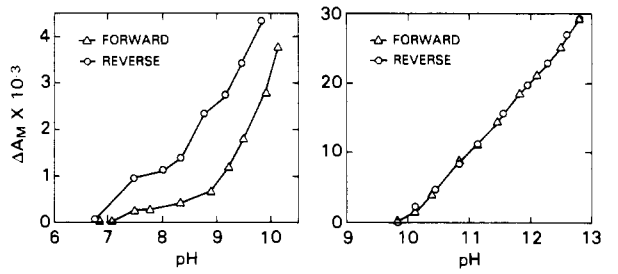

FIGURE 4: Forward (Δ) and reverse (O) spectrophotometric titration of horse heart ferricytochrome *c* with pH 10 as a reference point.

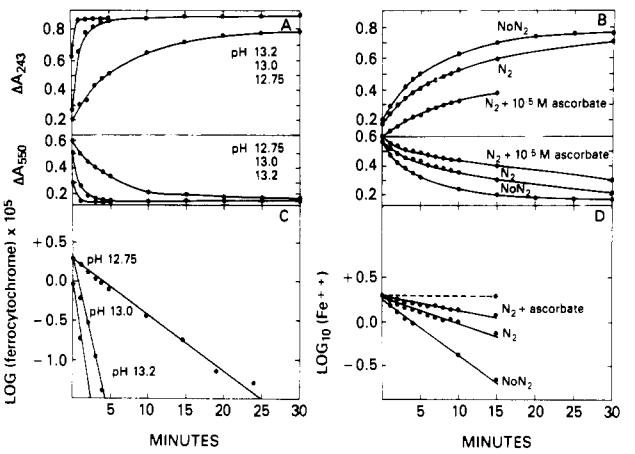

FIGURE 5: (A) Increase in absorption at 243 nm for ferrocytochrome *c* and the decrease in absorption at 550 nm for ferrocytochrome *c* (2.3×10^{-5} M) at different pH conditions as a function of time. The reference cuvette contains ferrocytochrome *c* at pH 7. (B) Increase in absorption at 243 nm and the decrease in absorption at 550 nm for a solution of ferrocytochrome *c* (2.39×10^{-5} M) at pH 12.75 under varying conditions (N_2 and/or ascorbate) as a function of time. The reference cuvette contained the same concentration of ferrocytochrome *c* at pH 7 and the pH of the sample cuvette was 12.75. (C) A log plot of the change of absorbance at 550 nm for ferrocytochrome *c* as a function of time with varying pH. (D) A log plot of the change in absorbance at 550 nm for ferrocytochrome *c* as a function of time at a constant pH of 12.75 under varying conditions of nitrogen and ascorbate.

of time-dependent changes above pH 12.5 requires the presence of nitrogen and the addition of 10^{-5} M ascorbate. These changes in the absorbance are undoubtedly related to the frequently observed phenomenon of autoxidation of ferrocytochrome *c* at alkaline pH (Tsou, 1951). Extrapolation to zero time under these conditions will correct for autoxidation at any given pH and give an estimate of the tyrosine ionization of the reduced form. The time course of this reaction with and without nitrogen and with nitrogen plus 10^{-5} M ascorbate is summarized in Figure 5. At pH 12.75 the half-life of the conversion in water is 8.6 min; the use of nitrogen and 10^{-5} M ascorbate decreases the autoxidation ($t_{1/2}$ = 39.5 min). Above pH 13.2 the conversion to the oxidized form is complete before spectral scanning can occur. Under these conditions of nitrogen and ascorbate, the number of ionized tyrosyl groups

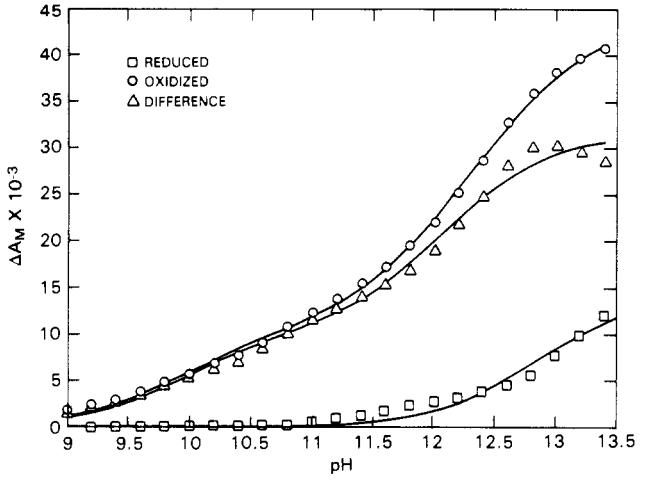

FIGURE 6: Spectral titration of horse heart ferri- (○) and ferrocytochrome *c* (□) and their difference curve (△).

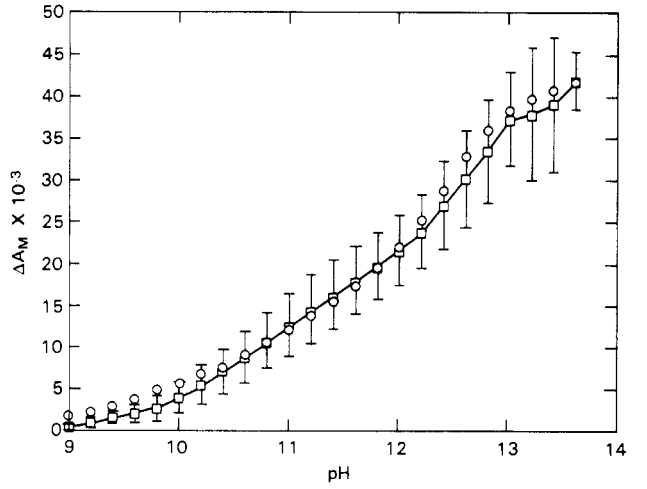

FIGURE 7: Average tyrosine ionizations for horse heart ferricytochrome *c* taken from Table I for individual curves. (○) were experientally obtained while (□) are from the literature.

in ferrocytochrome *c* at pH 13.2 at zero time in water is 0.97 group after correction for the presence of the oxidized species. Analysis results in a best fit of $pK' = 12.5$ and a tyrosyl absorbance of 14400. In 2.4 M guanidine hydrochloride at pH 11 <0.8 group is titrated. At guanidine hydrochloride concentrations >2.4 M the conversion of ferro- to ferricytochrome *c* is too rapid to be detected by this procedure. Spectral titrations of ferrocytochrome *c* at 243 nm are shown in Figure 6. The redox protonic tyrosyl difference is obtained by subtracting the reduced titration from the oxidized titration and is also shown. This difference curve can be described with three ionization constants 9.9, 12.0, and 12.9 with the corresponding molar absorbance coefficients of 9900, 10900, and 10800, respectively.

Discussion

Spectrophotometric detection of phenolic hydroxyl ionizations in proteins has been investigated extensively following the original report by Cramer & Neuberger (1943). For cytochrome *c*, a number of such curves have been reported and are summarized in Figure 7 with the ionic constants reported in Table II. There are many difficulties inherent in such a compilation involving variations in the reported molar absorbance for tyrosine, ionic strength, protein concentration and preparation, species differences, buffers, temperature, reference solution, pH scale standardization, and time of titration. Despite these admitted difficulties, the averaged data found

Table II: Reported Values for the Tyrosine Ionization Constants in Ferricytochrome *c*[m]

preparation	pK_1	pK_2	pK_3	pK_4	pK_5
native, beef[a]	10.8	13.0	13.0	13.71	
native, horse[b]	10.13	11.18	12.67	13.36	
native, horse[c]	10.26	12.33	12.33	12.33	
native, horse[d]	10.07	11.64	12.62	12.64	
native, horse[e]	10.11	10.83	12.37	12.38	
native, horse[f]	10.73	12.35	13.22	13.22	
average curve[g]	10.27	11.55	12.77	12.79	
native, tuna[d]	10.5	10.5	10.5	12.4	12.4
8 M urea, beef[a]	10.8	10.8	10.8	10.8	
8 M urea, horse[c]	11.0	11.0	11.0	11.0	
9.4 M urea, tuna[d]	10.9	10.9	10.9	10.9	10.9
tryptic digest, horse[c]	11.0	11.0	11.0	11.0	
apocytochrome *c*, horse[h]	10.2	10.2	10.2	10.2	
N-bromosuccinamide, horse[i]	10.2	10.2	10.6	10.6	
iodinated, horse[j]	7.0	9.0	12.2	12.2	
iodinated, tuna[j]	7.0	7.0	7.0	10.0	12.0
nitrated, horse[k]	6.4	6.4	12.7	12.7	
enzymatic iodination, horse[l]	7.0		12.2	12.2	

[a] Tyrosyl $E'_{243} = 10.53$ mM^{-1} cm^{-1}; $\mu = 0.3$ (glycine KCl–KOH); temperature = 25 °C; cytochrome *c* $E_{550} = 30.53$ mM^{-1} cm^{-1}; ~10 μm (Flatmark, 1964). [b] Tyrosyl $E'_{243} = 9.70$ mM^{-1} cm^{-1}; 0.15 M NaH_2PO_4–0.15 M $NaHCO_3$ with 10 M NaOH; temperature = 30.0 °C; cytochrome *c* concentration ~ 5 μM (Rupley, 1964). [c] Tyrosyl $E'_{243} = 11.0$ mM^{-1}; 0.20 M KCl; temperature not stated; Cytochrome *c* concentration = 1.08×10^{-5} M (Stellwagen, 1964). [d] Tyrosyl $E'_{243} = 11.0$ mM^{-1} cm^{-1}; 0.20 M KCl at room temperature; Cytochrome *c* concentration = 0.01–0.02 mM (Stellwagen & McGowan, 1970). [e] Tyrosyl $E'_{243} = 11.0$ mM^{-1} cm^{-1}; ionic strength, temperature, and cytochrome *c* concentration not given (Myer & Pal, 1972). [f] Tyrosyl $E'_{243} = 11.0$ mM^{-1} cm^{-1}; ionic strength, temperature, and cytochrome *c* concentration not given; $E'_{528} = 10.4$ mM^{-1} cm^{-1} (O'Hern et al., 1975). [g] The above six (*a*–*f*) reported titration curves were processed by the DIGITIZE procedure using the PROPHET system, an NIH-supported national computer resource. Information Handling Program, Division of Research Resources, National Institutes of Health, Bethesda, MD 20205. [h] Tyrosyl $E'_{295} = 2.33$ M^{-1} cm^{-1}; ionic strength, temperature, and cytochrome *c* concentration not given (Stellwagen & McGowan, 1970). [i] There is an increase of $\Delta E'_{243}$ for the modified protein of 65 mM^{-1} cm^{-1} over the native preparation of 45 mM^{-1} cm^{-1} (Myer & Pal, 1972). [j] The $\sim E'_{243}$'s for tyrosine, monoiodotyrosine, and diodotyrosine are 11.0, 8.0, and 4.0 cm^{-1} mM^{-1}, respectively; in horse only tyrosyl residues at positions 67 and 74 in the amino acid sequence are modified while in tuna only position 97 in unmodified. The ionization constants for these iodinated derivatives are estimated from the data given (McGowan & Stellwagen, 1970). [k] E'_{243} for nitrotyrosine is 4 mM^{-1} cm^{-1} and residues 48 and 67 have been reported to be modified; the ionization constants are estimates (Skov et al., 1969). [l] Enzymatic iodination with lactoperoxidase led to the modification of a single tyrosyl residue at position 74 without loss of the 695-nm band or biological activity; ionization constants are estimated (Ferguson-Miller et al., 1976). [m] Note that the older term extinction (E') is used in the compliation of this table rather than absorbance.

in this study are not appreciably different from the average of six separate reported curves. There are major differences, however, in the values for the ionic parameters that result from the analytical methods used to evaluate the spectral titrations. When the absorbance coefficients are allowed to vary (constraints D of Table I), the values for the ionic constants are $pK'_1 = 10.1$ for a single group and three groups with $pK' = 12.35$. These values are comparable with those reported by Stellwagen (1964). However, McGowan & Stellwagen (1970) report pK' values of 10.1, 11.2, 12.5, and 12.8 based on the same data as well as that of Rupley (1964). These values are those found for a constant molar absorbance (constraints A, Table I). Differences in the values for $pK's$ can be explained in several ways. Originally it was postulated that two, three, or all four tyrosyl groups ionized abnormally high (Flatmark, 1964; Rupley, 1964; Stellwagen, 1964). From the spectrophotometric titration of iodinated horse heart ferricytochrome *c* (McGowan & Stellwagen, 1970), the estimated ionization

constants are 7.0, 9.0, 12.2, and 12.2. These authors proposed that tyrosyl residues 67 and 74 have native pK' values of 10.1 ± 0.1 and 11.2 ± 0.3 and these two tyrosyl residues are involved in a conformational transition accompanying reduction of the heme iron. This would imply that the pK' values for Tyr-48 and Tyr-97 are both ≥12.7. The averaged titration curve (Figure 7) can be described with pK'_1 = 10.27, pK'_2 = 11.55, and two groups with pK' = 12.78 (line g, Table II). As we have indicated, the value for the second ionization is dependent on the assigned molar absorbance of tyrosine.

The differences reported may also be due to the hysteresis observed in the acid and base titrations (Figure 3). Shaw & Hartzell (1976) have assigned ionization constants of 10.1, 12.2, and 13.1 for the potentiometric titration of horse heart ferricytochrome *c* from pH 11 to pH 3. For the reverse titration, they assign pK' = 10.4, 12.2, and 13.1. The change in the first ionization occurs near the isionic point. With ferricytochrome *c* at pH 10 as a reference, the titration to pH ~13 and return is reversible (Figure 4B) but the titration to pH 7 is not reversible (Figure 4A). Thus, a previous history of the protein with respect to pH is required to interpret the spectral titrations. The exposure to high pH may explain the differences reported for the ionization of Tyr-67 of 10.4 (Czerlinsky & Dor, 1971) and 11.0 (Cupta & Koenig, 1971). For reduced cytochrome *c*, Stellwagen (1964) reports that the ionization of tyrosyl groups in ferrocytochrome *c* lags behind the same ionizations in ferricytochrome *c*. Even though Stellwagen reports a lag, all four tyrosyl groups in ferrocytochrome *c* are titrated at pH 13.0 with the experimental curves at this pH being coincident with the ferricytochrome *c* titration. We find only a single ionization in ferrocytochrome *c* with a pK' of 12.6 (Figure 6). Although no ionization constant can be assigned to the remaining three tyrosyl residues in ferrocytochrome *c*, these residues must titrate with corresponding higher pK's (>13) than in ferricytochrome *c*.

Results of the tyrosyl ionization for each redox species of cytochrome *c* can be further analyzed comparing the tyrosyl (protonic) redox difference (Figure 6). The difference curve obtained by subtracting the reduced titration from the oxidized titration suggests that at least two processes are involved. The tyrosyl protonic redox difference consists of two components that overlap. The first component is proposed to consist of the shift of one ionizable group (pK' = 12.6) in ferrocytochrome *c* to a pK' ≥ 10.0 in ferricytochrome *c*. The remainder of the tyrosyl difference curve constitutes the second component and is assumed to be the shift of the three nontitrable tyrosyl groups in ferrocytochrome *c* to the pK'_2, pK'_3, and pK'_4 of ferricytochrome *c*.

X-ray crystallographic studies have revealed three important aspects of cytochrome *c* structure: the cytochrome fold, the absence of crystal differences between the oxidized and reduced protein, and extensive hydrogen bonding (Ferguson-Miller et al., 1976). Although the specific residues involved in hydrogen-bond formation are controversial, Tyr-48 and Trp-59 are bound to the inner heme propionic side chain while Thr-49 is bound to the other heme propionic in both redox states. Try-67 is bonded to Thr-78 in both redox states while Tyr-74 is at the surface and Tyr-94 is external: Tyr-74 and Tyr-97 are unbonded (Ferguson-Miller et al., 1976).

The phenolic group may be further characterized as weakly or strongly hydrogen bonded with or without a hydrophobic microenvironment. Siamiorza et al. (1975) have interpreted the 850- and 830-cm^{-1} bands from Raman spectra as a sensitive measure of hydrogen-bond formation for the tyrosyl residue, which is insensitive to the hydrophobic environment and the conformation of the amino acid backbone. We attempted to determine the 850, 830 cm^{-1} doublet for ferricytochrome *c* using an argon ion laser but no Raman resonance was observed between 750 and 850 cm^{-1}. This is consistent with a previous report of Strekas & Spiro (1972). The absence of the 850, 830 cm^{-1} doublet may be attributed to increased fluorescence of heme compounds increasing the background noise and quenching may mask the doublet. If this is the case, fast-reaction Raman spectroscopy at the nanosecond level will be required for the detection of this doublet (Woodruff & Farquharson, 1978).

The reported number of tyrosyl residues in eukaryotic cytochromes *c* varies from 2 to 6. Wheat cytochrome *c* contains six tyrosyl side chains, baker's yeast and tuna contain five, and most mammalian species such as chimpanzee, rhesus monkey, man, pig, cow, horse, donkey, dog, and rabbit contain four tyrosyl groups; *Neurospora crassa*, *Euglena gracilis*, and *Crithidia oncopelti* contain three groups while *Tetrahymena pyriformis* contains two tyrosyl groups (Borden & Margoliash, 1974). These results would suggest that only position 48 is invariant while positions 67, 74, and 94 show conservative substitutions.

Horse and beef heart cytochrome *c* contain tyrosyl residues at positions 48, 67, 74, and 97; positions 48, 67, and 74 are invariant with position 97 showing only conservative changes. Tuna cytochrome *c* is the same as horse and beef cytochrome *c* except that the fifth tyrosyl residue is present at position 46, which represents a conservative substitution for a phenylalanine residue (Borden & Margoliash, 1975).

With the data at hand it is still not possible to unequivocally assign values to each tyrosyl ionization in horse heart ferricytochrome *c*. However, several assumptions can be made that permit a tentative assignment. The ionization of native tuna cytochrome *c* tyrosyl groups is almost identical with that of native horse heart cytochrome *c* except for the additional Tyr-46 ionization. As a first approximation, tuna Tyr-46 may be assigned the ionization constant of 10.5. If this is true, it may be further inferred that tuna Tyr-46 is accessible to both the titrant and solvent and as such horse Tyr-48 might be in a same or similar microenvironment. Horse Tyr-48 would then have the same or similar pK' of 10.1. To the previous assignment of horse Tyr-74 pK' 11.2 (McGowan & Stellwagen, 1970) and 11.0 (Gupta & Koenig, 1971) can be added Tyr-67 and Tyr-94 as 12.7. Such an assignment leaves unexplained the chemical reactivity differences between tuna and horse cytochrome *c* toward iodination and nitration. Also, the X-ray crystallographic studies do not completely support this assignment.

The ionization constants of horse heart ferricytochrome *c* have been obtained and compared with those reported in the literature. The lowest pK value has been tentatively assigned to Tyr-48 while Tyr-74 has been proposed to be strongly hydrogen bonded. The ionization constants of Tyr-67 and Tyr-94 represent their hydrophobic environments. The redox-linked, protonic-dependent changes involve the ionization constants of horse heart cytochrome *c* for Tyr-48 in particular and Tyr-67, -74, and -94 in general and are consistent with previously proposed compact, closed/loose, open structures for cytochrome *c* (Ferguson-Miller et al., 1976). Conformation of these assignments will have to await the spectrophotometric titration of other eukaryotic cytochromes *c*.

Registry No. Tyrosine, 60-18-4; cytochrome *c*, 9007-43-6.

References

Borden, D., & Margoliash, E. (1976) *Handbook of Biochemistry and Molecular Biology Proteins* (Fasman, G. D.,

Ed.) Vol. III, pp 268-279, CRC Press, Cleveland, OH.
Cramer, J. L., & Neuberger, A. (1943) *Biochem. J. 37*, 302.
Czerlinski, G. H., & Dar, K. (1971) *Biochim. Biophys. Acta 234*, 57.
Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1976) in *The Porphyrins* (Dolphin, D., Ed.) p 37, Academic Press, New York.
Flatmark, T. (1964) *Acta Chem. Scand. 18*, 1976.
Gupta, R. K., & Koenig, S. H. (1971) *Biochem. Biophys. Res. Commun. 45*, 1134.
Hermans, J. (1962) *Biochemistry 1*, 193.
Knott, G., & Schrager, L. (1972) *ACM-SIGG RAF Q. 6*, 138.
Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570.
Marini, M. A., & Wunsch, C. (1963) *Biochemistry 2*, 1454.
Marini, M. A., Martin, C. J., Berger, R. L., & Forlani, L. (1972) *Biopolymers 13*, 891.
Marini, M. A., Marti, G. W., Berger, R. L., & Martin, C. J. (1980) *Biopolymers 19*, 885.
Marini, M. A., Martin, C. J., Berger, R. L., & Foriani, L. (1981) *Biopolymers 20*, 2253.
Martin, C. J., & Marini, M. A. (1982) *Biopolymers 21*, 1657.
McGowan, E. B., & Stellwagen, E. (1970) *Biochemistry 9*, 3047.
Myer, Y. P., & Pal, P. K. (1972) *Biochemistry 11*, 4195.
O'Hern, D. J., Pal, P. K., & Myer, U. P. (1975) *Biochemistry 14*, 382.
Rupley, J. A. (1964) *Biochemistry 3*, 1648.
Shaw, R. W., & Hartzell, C. R. (1976) *Biochemistry 15*, 1909.
Siamioiza, M. J., Lord, R. C., Chen, M. C., Takamatsu, T., Haradi, I., Matsuura, H., & Shimanoucki, T. (1975) *Biochemistry 14*, 4870.
Skov, K., Hofmann, T., & William, G. R. (1969) *Can. J. Biochem. 47*, 750.
Stellwagen, E. (1964) *Biochemistry 3*, 919.
Stellwagen, E., & Van Rooyan (1967) *J. Biol. Chem. 242*, 4801.
Stellwagen, E., & McGowan, E. B. (1970) *Biochemistry 9*, 3764.
Stellwagen, E., & Babul, G. (1972) *J. Biol. Chem. 247*, 8047.
Stellwagen, E., & Rysavy, R. (1972) *J. Biol. Chem. 247*, 8074.
Strekas, T. C., & Spiro, T. G. (1972) *Biochim. Biophys. Acta 278*, 188.
Tsou, C. L. (1951) *Biochem. J. 49*, 362.
Uyeda, M., & Peisach, J. (1981) *Biochemistry 20*, 2028.
Woodruff, W. H., & Farquharson, S. (1978) *Anal. Chem. 50*, 1389.

Unfolding–Refolding Transition of a Hinge Bending Enzyme: Horse Muscle Phosphoglycerate Kinase Induced by Guanidine Hydrochloride†

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ABSTRACT: The unfolding–refolding transition of horse muscle phosphoglycerate kinase induced by guanidine hydrochloride was studied under equilibrium conditions using four different signals: fluorescence intensity at 336 nm, UV difference absorbance at 286 and 292 nm, ellipticity at 220 nm, and enzyme activity. From the following arguments, we found that the process deviates from a two-state model and intermediates are significantly populated even at equilibrum: (1) the noncoincidence of the transition curves and (2) the asymmetry of the transition curve obtained from CD measurements. From these different data and the thermodynamic analysis, it was suggested that the two domains of the horse muscle phosphoglycerate kinase refold independently of one another with different equilibrium constants, the most favorable constant referring to the folding of the C-terminal domain which contains all tryptophans.

Many proteins are folded into several, two or more globular units called domains. Among them, nucleotide binding enzymes have a bilobed structure, each domain being separated by a cleft. Substrate binding induces a conformational change. For hexokinase (Bennett & Steitz 1980a,b) and for phosphoglycerate kinase from yeast (Pickover et al., 1979) and from horse muscle (Banks et al., 1979), an open structure was described for the free enzyme and a closed structure was described or suggested in the presence of specific ligands. The conformational change, which leads to a modification in the relative orientation of the two domains, possibly occurs through a "hinge bending motion" of these domains.

Phosphoglycerate kinase (PGK; EC 2.7.2.3) is the first enzyme of the glycolytic pathway that generates ATP through the high-energy phosphoryl transfer reaction:

1,3-diphospho-D-glycerate + ADP $\overset{Mg^{2+}}{\rightleftharpoons}$ 3-phospho-D-glycerate + ATP

The complete amino acid sequence of the horse muscle enzyme is known; the three-dimensional structure has been determined by high-resolution X-ray analysis (Banks et al., 1979). The two domains of the molecule have almost the same size and correspond to the C-terminal and N-terminal parts of the molecule. The nucleotide substrates bind to the C-terminal domain, and the phosphoglycerate substrates bind to the N-terminal domain.

While there is only a slight and local change in the structure with ATP-Mg and ADP-Mg, 3-phosphoglycerate (3PG) binding induces a conformational change that encompasses the whole enzyme. Moreover, the location of binding sites for

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0006-2960/84/0423-6654$01.50/0