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Studies on the Peroxidase Effect of Cytochrome c

V. Spectrophotometric Titration of a Reversible Conformational Change of Ferricytochrome c in Acid Aqueous Solution

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(1) A reversible conformational change of native, monomeric beef heart cytochrome *c* (ferric form) takes place in acid aqueous solution.

(2) The conformational change was studied by means of ultraviolet difference spectra; the main peaks were observed at 290.5 mμ ($-\Delta\varepsilon_{max}$($cm^{-1} \times mM^{-1}$) = 3.6 and at 230.5 mμ ($-\Delta\varepsilon_{max}$ ($cm^{-1} \times mM^{-1}$) = 24.0).

(3) Equilibrium data were obtained for the spectral transition at 290.5 mμ. Thus, the pH-dependence revealed an S-shaped titration curve with pK' = 2.80 (μ = 0.1; 23.2°C) for the single protonated group (probably carboxyl) involved in the spectral transition; the process was endothermic ($\Delta H'$ = −9.1 kcal $mole^{-1}$).

(4) The titration of one abnormal carboxyl group in ferricytochrome c agrees well with previous acid-base titrations.

(5) The spectral transition is paralleled by an activation of ferricytochrome c as a peroxidase, as well as by a change in its reactivity towards ionic ligands and its optical rotatory dispersion spectrum, indicating a common mechanism for these effects.

In some previous papers in this series the over-all reaction of the peroxidation of purpurogallin catalyzed by ferricytochrome *c** was studied.[1,2] The activation of cyt. c as a peroxidase in acid solution revealed some characteristic features which suggested a conformational** change of the polypeptide chain as a prerequisite for its catalytic activity.[2] Thus, the pK' values of the two catalytically active groups, and as a consequence the pH optimum, shifted towards the alkaline side when the reaction temperature was increased ($\Delta H_1'$ = −7.4 kcal $mole^{-1}$ and $\Delta H_2'$ = −10.4 kcal $mole^{-1}$). Further evidence in support of this view has recently been given by Urry.[3] By means of optical rotatory dispersion (ORD) measurements, a conformational change of ferri-cyt.

* The following abbreviation will be used: Cyt. c = cytochrome c.

** The term *conformation* is used synonymously with the sum of the secondary and tertiary structures of the protein molecule.

c was revealed at pH < 5. However, no attempt was made to determine either the pK' or the number of protonated groups involved during that transition. Since ultraviolet difference spectra provide a sensitive and simple means of detecting conformational changes in proteins,[4,5] this technique was applied in the present study.

MATERIALS AND METHODS

Cytochrome c. Beef heart cyt. c was obtained in the monomeric form by gel filtration on Sephadex G-75, and the characteristics of this preparation have been described in a previous paper.[6] The main subfraction (Cy I), accounting for about 90 % of the total material, was obtained by preparative disc electrophoresis on polyacrylamide gel.[7] Ferri-cyt. c was prepared as previously described.[8]

The concentration of the hemoprotein was assayed spectrophotometrically by using the specific extinction coefficient $E^{1\,\%}_{1\,cm}$ at 550 mμ red = 23.94 [6] and a molecular weight of 12 130 (calc. from the known amino acid composition [9] and by taking into account the contribution of the heme [10]).

Electrolyte solutions. HCl—NaCl solutions of different pH values, but with constant ionic strength ($\mu = 0.1$) and constant anion concentration ($[Cl^-] = 0.1$), were prepared. Deionized water and reagent grade chemicals were used.

pH measurements were made with glass electrodes (Radiometer, Denmark, Model 25 SE) standardized at pH 4.01 (25°C) with Beckman standard buffer. The temperature of the electrode vessels was controlled by circulating water from a constant temperature bath.

Difference spectra were obtained with a Beckman DK-2A recording spectrophotometer; however, for measurements of absorbancy differences at a single wave-length a Beckman DU spectrophotometer was used. The temperature of the cell compartments of both instruments was controlled by water circulation. The temperatures reported in the results were measured directly in the cuvettes at the time of thermal equilibrium. The measurements were usually made on protein concentrations of about 26 μM, and the data were calculated as $\Delta\varepsilon$ ($cm^{-1} \times mM^{-1}$).

RESULTS

The ferric form of the native, monomeric form of the hemoprotein (subfraction Cy I) was used in all experiments.

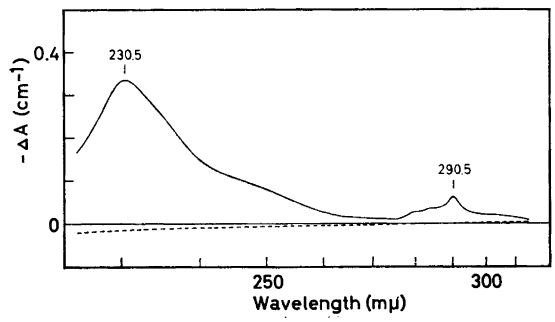

Fig. 1. Ultraviolet difference spectrum of ferricytochrome c at pH 1.03 *vs.* pH 6.9; $\mu = 0.1$; 23.2°C; cyt. c concentration, 14 μM. The dotted line indicates the base line.

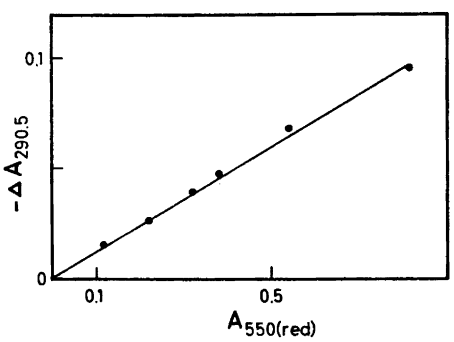

Fig. 2. Absorbancy difference between ferricytochrome c at pH 1.03 and at pH 6.9 as a function of the cyt. c concentration.

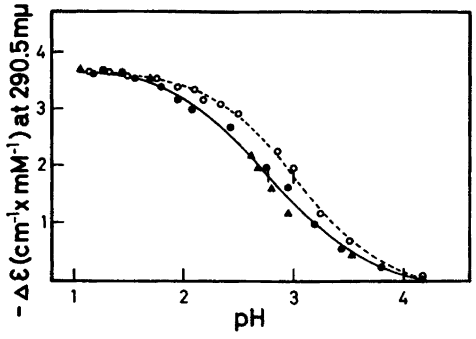

Fig. 3. Difference in absorbancy at 290.5 mμ for native, monomeric ferricytochrome c at the pH indicated on the abscissa, *vs.* ferricytochrome c at pH 6.9. μ = 0.1 and $[Cl^-]$ = 0.1 M. Step-wise titration from pH 6.9 to pH 1.03 at 23.2°C (●) and at 33.2°C (○); ▲, continuous backtitration from pH 1.03 at 23.2°C. The curves drawn through the experimental points follow the equation describing the ionization of a univalent weak acid with pK' = 2.80 at 23.2°C and pK' = 3.02 at 33.2°C. For experimental details see text.

The ultraviolet difference spectrum of ferri-cyt. c at pH 1.03 *vs.* pH 7.0, shown in Fig. 1, revealed two main peaks, *i.e.* at 230.5 mμ ($-\Delta\varepsilon$ ($cm^{-1} \times mM^{-1}$) = 24.0 and 290.5 mμ ($-\Delta\varepsilon$ ($cm^{-1} \times mM^{-1}$) = 3.6). At λ = 290.5 mμ, Beer's law was obeyed, within the limits of experimental error, over a concentration range of 4 to 28 μM (Fig. 2).

a) Time independence. No change in the difference spectrum was observed over a 1-hour period at any pH studied.

b) Effect of pH. A plot of the absorbancy difference at 290.5 mμ as a function of pH (Fig. 3) yields an S-shaped titration curve with pK' = 2.80 (23.2°C) for the spectral transition (for calculation of pK' see Fig. 4). The maximum absorbancy difference was found to be $-\Delta\varepsilon_{max}$ ($cm^{-1} \times mM^{-1}$) = 3.6 at pH = 1. Unfortunately, at pH > 3.5 the value of $-\Delta\varepsilon$ is quite small and the precision of the measurements is rather poor.

c) Reversibility. The reversibility was tested in the following way: Cyt. c solutions of appropriate concentration were allowed to stand at pH 1.03 (0.1 N HCl) for 10 min at 23.2°C, and the back-titration was then started by the addition of 1 N NaOH; exactly the same volume of buffer was added to the sample cuvette (pH 7.0) each time. 5 min were allowed for temperature equilibration after each addition of alkali (buffer) and the back-titration was completed within 1 h. After each addition of HCl (buffer) corrections were made for the dilutions in calculating the value of $\Delta\varepsilon_{290.5}$ ($cm^{-1} \times mM^{-1}$). Fig. 2 shows that the back-titration follows the forward titration, indicating that the spectral transition is completely reversible.

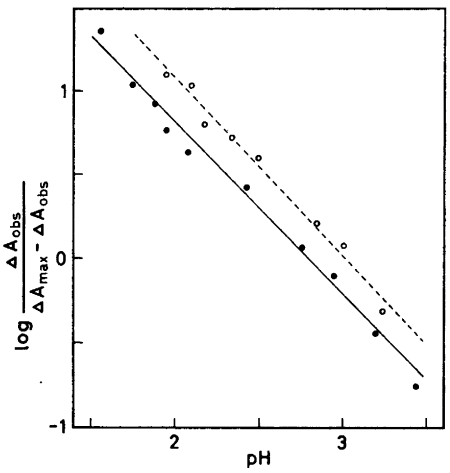

Fig. 4. Graphical determination of the number (n) of H^+-ions which combine with one molecule ferricytochrome c in the spectrophotometric transition. The equations of the lines fitting the experimental values were,

at 23.2°C: $\log \frac{\Delta A_{obs}}{\Delta A_{max}-\Delta A_{obs}} = 2.85-1.02\ pH$

at 33.2°C: $\log \frac{\Delta A_{obs}}{\Delta A_{max}-\Delta A_{obs}} = 3.19-1.06\ pH$

Data obtained from Fig. 3.

d) Effect of temperature. In the titration experiment reported above the value $-\Delta\varepsilon_{max}$ ($cm^{-1} \times mM^{-1}$) = 3.6 was found at pH = 1 and 23.2°C. This extinction coefficient was found to be independent of the temperature at least up to 46°C.

By increasing the temperature the whole titration curve is displaced towards the alkaline side (Fig. 3); thus, the pH of the inflection point increased 0.22 pH units by increasing the temperature 10°C (*i.e.* from 23.2 to 33.2°C). This corresponds to a value of $\Delta H' = -9.1$ kcal. $mole^{-1}$.

c) Stoichiometry. From the almost symmetrical titration curves in Fig. 2 it is possible to obtain an accurate estimate of the number (n) of protonated groups involved (*i.e.* the number of H^+-ions combining with one molecule of ferri-cyt. c) in the spectral transition. Thus, by plotting

$$\log \frac{\Delta A_{obs}}{\Delta A_{max}-\Delta A_{obs}}$$

vs. pH, straight lines of unit slope were obtained at 23.2°C (n = 1.02) and 33.2°C (n = 1.06) (Fig. 4). This shows that the transition has the character of a univalent dissociation.

DISCUSSION

The acid difference spectrum of ferri-cyt. c is very similar to that found in several other proteins, and there can be no doubt that it reflects a conformational change of the native hemoprotein molecule. Generally, in acid difference spectra of proteins a prominent peak in the region about 230 mμ has been attributed mainly to changes in the conformation of the peptide backbone,[11,12] whereas peaks in the region between 270 and 300 mμ have been attributed to changes in the environment of the aromatic chromophores of the protein molecule.[4,5,13-18] Since beef heart cyt. c contains two "buried" tyrosine

residues,[19] it is reasonable to attribute the difference peak at 290.5 mμ to the accessibility of one or both of these residues, which results from a conformational change. λ_{max} at 290.5 mμ is, however, slightly higher than expected for tyrosyl groups alone,[5,13] which may indicate that the single tryptophan residue [9] also contributes to this peak.

The pH-dependence of the spectral transition revealed an S-shaped titration curve with $pK' = 2.80$ (at 23.2°C) for the single protonated group ($n_{H^+} = 1$) involved in the transition. It is difficult to interpret the data otherwise than as the titration of a carboxyl group, the pK' of which is slightly lower than that of a "free" carboxyl group. This conclusion is in good agreement with, and gives an explanation of, the difference in acid-base titration curves of ferro- and ferri-cyt. c. Thus, Theorell and Åkeson [20] found that the two titration curves differed by 1 equiv. from pH 8 to about pH 3. At more acid pH values the difference increased to about 2 equiv., indicating the titration of one carboxyl group more in the ferric than in the ferrous form. This difference is consistent with the recent observation that ferri-cyt. c, but not ferro-cyt. c, undergoes a conformational change in acid solution.[3] Finally, the reversibility of the spectral transition agrees well with earlier observations that cyt. c, as determined by its catalytic activity in the succinate oxidase system,[21] is perfectly stable from pH 1.6 to 12.3.

By increasing the temperature, the pK of the titrable carboxyl group is shifted towards the alkaline side approaching more and more that of a normal carboxyl group; the intrinsic pK for a side chain carboxyl of a glutamyl or an aspartyl residue in a protein is usually about 4.6.[22] Thus, if the $\Delta H'$ value obtained in the present study is independent of the temperature range studied, a pK' value of 4.5 is to be expected at 100°C. This assumption agrees with the ORD studies of Urry [3] which revealed that ferri-cyt. c is nearly completely unfolded at this high temperature.

The pK' value obtained for the spectral transition (2.80 at 23.2°C) is lower than those found for the catalytically active groups in the peroxidase reaction ($pK_1' = 3.13$ and $pK_2' = 3.78$ at 25°C)[2] and this indicates that different groups are involved in these two reactions. However, their $\Delta H'$ values are very similar indicating that this thermodynamic parameter primarily reflects the unfolding process in either case. It is also worth mentioning here that the pH dependence of the spectral transition fairly well parallels the change in reactivity of ferri-cyt. c towards ionic ligands such as CN^- and N_3^-, [23] as well as the change in ORD spectrum.[3] This parallelism indicates a common mechanism for all these effects *i.e.* a change in the conformation of the native hemoprotein.

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REFERENCES

1. Flatmark, T. *Acta Chem. Scand.* **18** (1964) 2269.
2. Flatmark, T. *Acta Chem. Scand.* **19** (1965) 2059.
3. Urry, D. W. *Proc. Natl. Acad. Sci. U.S.* **54** (1965) 640.

4. Laskowski, M., Jr., Widom, J. M., McFadden, M. L. and Scheraga, H. A. *Biochim. Biophys. Acta* **19** (1956) 581.
5. Scheraga, H. A. *Biochim. Biophys. Acta* **23** (1957) 196.
6. Flatmark, T. *Acta Chem. Scand.* **18** (1964) 1517.
7. Flatmark, T. *Acta Chem. Scand.* **18** (1964) 1656.
8. Flatmark, T. *Acta Chem. Scand.* **20** (1966) 1476.
9. Yasunobu, K. T., Nakashima, T., Higa, H., Matsubara, H. and Benson, A. *Biochim. Biophys. Acta* **78** (1963) 791.
10. Paléus, S. and Paul, K. G. In *The Emzymes*, 2nd Ed., Academic, New York 1963, Vol. 8, p. 97.
11. Glazer, A. N. and Smith, E. L. *J. Biol. Chem.* **235** (1960) PC 43.
12. Wetlaufer, D. B. *Advan. Protein Chem.* **17** (1962) 303, p. 373.
13. Scheraga, H. A. *J. Am. Chem. Soc.* **82** (1960) 3847.
14. Hermans, J. and Scheraga, H. A. *J. Am. Chem. Soc.* **83** (1961) 3283.
15. Hermans, J. and Scheraga, H. A. *J. Am. Chem. Soc.* **83** (1961) 3293.
16. Broomfield, C. A., Riehm, J. P. and Scheraga, H. A. *Biochemistry* **4** (1965) 751.
17. Riehm, J. P., Broomfield, C. A. and Scheraga, H. A. *Biochemistry* **4** (1965) 760.
18. Riehm, J. P. and Scheraga, H. A. *Biochemistry* **4** (1965) 772.
19. Flatmark, T. *Acta Chem. Scand.* **18** (1964) 1796.
20. Theorell, H. and Åkeson, Å. *J. Am. Chem. Soc.* **63** (1941) 1818.
21. Paul, K. G. *Acta Chem. Scand.* **2** (1948) 430.
22. Steinhardt, J. and Beychok, S. In Neurath, H. (Ed.), *The Proteins*, Academic, New York 1964, Vol. 2, p. 171.
23. George, P., Glauser, S. C. and Schejter, A. In Desnuelle, P. (Ed.), *Molecular Basis for Enzyme Action and Inhibition, Proc. Fifth Congr. Biochem.*, Moscow 1961, Pergamon, Oxford 1963, Vol. 4, p. 192.

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