The Enthalpy Change Accompanying the Oxidation of Ferrocytochrome *c* in the pH Range 6–11 at 25°

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ABSTRACT: The enthalpy changes in the oxidation of ferrocytochrome *c* by ferricyanide ion, and in the reduction of ferricytochrome *c* by ascorbate ion, have been measured by flow calorimetry in the pH range 6–11 at 25°. These data, together with potentiometric equilibrium data give for reaction 1 (see text) the thermodynamic parameters $\Delta G' = -5.97$ kcal mole^{-1}, $\Delta H = -14.1$ kcal mole^{-1}, and $\Delta S' = -27.3$ cal deg^{-1} mole^{-1} at pH 7.00 (standard state for H^+: unit activity at pH 7.00).

The sigmoidal variation of ΔH with pH for this reaction is probably due to a conformational change in ferricytochrome *c* which takes place with uptake of a proton. Interpretation of the data on this basis gives for the conformational transition $\Delta G' = -3.1$ kcal mole^{-1}, $\Delta H = -16.0$ kcal mole^{-1}, and $\Delta S' = -43$ cal deg^{-1} mole^{-1} (standard state for H^+: unit activity at pH 7.00). The pH of half-completion of the conformational transition is approximately 9.3, which is the same as the pH of half-completion of various other changes in ferricytochrome *c* reported by other workers.

Numerous observations of changes in the physicochemical properties of cytochrome *c* accompanying change in the oxidation state of the iron atom have been interpreted by various authors to indicate that ferricytochrome *c* and ferrocytochrome *c* differ in conformation, the oxidized protein having a more open and flexible conformation than the reduced protein (Margoliash and Schejter, 1966). Among recent reports in this connection may be mentioned studies of the optical rotatory dispersion (Ulmer, 1965; Urry and Doty, 1965; Myer and Harbury, 1965; Mirsky and George, 1966) and the circular dichroism (Myer, 1968a,b) of cytochrome *c*. It appears from these studies that the conformational changes probably do not involve a large part of the molecule, since the changes in Cotton effects are largely restricted to those due to asymmetry of the heme transitions, with only minor changes in the intrinsic Cotton effects which reflect asymmetry in the conformations of peptide bonds. This conclusion is further supported by observations on the rate of deuterium–hydrogen exchange in cytochrome *c* (Ulmer and Kägi, 1968; Kägi and Ulmer, 1968).

In addition to the conformational changes resulting from its reduction, ferricytochrome *c* has been shown to undergo a reversible "isomerization" at alkaline pH to form a species with altered electron transfer properties (Greenwood and Palmer, 1965; Urry, 1965; Myer and Harbury, 1965; Brandt *et al.*, 1966). Ferrocytochrome *c* appears to remain essentially in its native conformation under these conditions.

As part of a program of investigating the thermodynamics of various protein reactions, it was of interest to determine by direct calorimetry the enthalpy change in the reduction of ferricytochrome *c* over a wide range of pH. If it is indeed true that ferrocytochrome *c* remains in its native conformation as the pH is raised while the oxidized protein undergoes a conformational change, the resulting data should reflect, in addition to enthalpies of ionization, the enthalpy change accompanying the transition of ferricytochrome *c*. In this paper we report calorimetrically determined enthalpy values for the oxidation of ferrocytochrome *c* by ferricyanide ion, and for the reduction of ferricytochrome *c* by ascorbate ion, in the pH range 6–11. While these measurements were in progress, George *et al.* (1968) reported $\Delta H = -14.5 \pm 1.5$ kcal mole^{-1} for the reaction at pH 7.0

$$Fe^{III}\text{-cyt } c + 0.5H_2 \rightleftharpoons Fe^{II}\text{-cyt } c + H^+ \qquad (1)$$

where Fe^{III}-cyt *c* and Fe^{II}-cyt *c* represent ferricytochrome *c* and ferrocytochrome *c*, respectively. This value is in good agreement with the value $\Delta H = -14.1 \pm 0.2$ kcal mole^{-1} derived from our work.

Experimental Section

Horse heart cytochrome *c* (type III), from Sigma Chemical Co., was completely oxidized by ferricyanide ion and then purified on columns of Amberlite CG-50 (Margoliash, 1954). Analysis of the product (Margoliash *et al.*, 1959) showed that only the monomer was present. Ferrocytochrome *c* was prepared by reducing the oxidized protein with excess ascorbate ion and then chromatographing anaerobically on columns of Amberlite CG-50. The material obtained this way was 70–90% in the reduced form.

Cytochrome c Concentration. A number of extinction coefficients at 550 mμ for ferrocytochrome *c* have been reported. The uncertainty in these values is large enough so that a redetermination was made in this study. The iron concentration of purified cytochrome *c* was determined with

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a Perkin-Elmer Model 303 atomic absorption spectrophotometer which was calibrated with standard ferricyanide ion solutions. The optical densities at 550 mμ for ferricyanide ion oxidized and ascorbate ion reduced cytochrome *c* and at 520 mμ for the reduced form were determined on a Cary 14 spectrophotometer. The ϵ_{550} values for oxidized and reduced cytochrome *c* were found to be 8.5×10^3 and 29.5×10^3 M^{-1} cm^{-1}, respectively, and ϵ_{520} for reduced cytochrome *c* was found to be 17.0×10^3 M^{-1} cm^{-1}. The ϵ_{550} values given here agree well with those reported by Massey (1959) and Van Gelder and Slater (1962).

The total cytochrome *c* concentration used in all of the studies reported in this paper was determined at 25°, pH 7.0 in 0.10 M phosphate buffer by measuring the optical density at 550 mμ for the ascorbate ion reduced sample. This procedure eliminates errors arising from pH or temperature variation of the extinction at 550 mμ. The fraction of reduced cytochrome *c* in a sample was calculated under the above conditions from optical density measurements and extinction coefficients of the sample at 550 mμ before and after reduction with ascorbate ion.

Buffers. When cytochrome *c* undergoes oxidation (reduction) above pH 7.0 protons are released (absorbed). Consequently, the calorimetric measurements reported in this study were made in 0.05 M buffer solutions so that the state of the protons was defined and appropriate thermal corrections could be made where necessary. The buffers were prepared in the sodium ion form from reagent grade chemicals and were used in the indicated pH ranges: phosphate, pH 6–9; borate, pH 9.0; and carbonate, 9.75–10.9. These buffers were chosen not only for their buffering ability but also for their relatively small heats of ionization in order to minimize the corrections for buffer ionization reactions. The enthalpy of ionization for these buffers was determined under the same conditions as existed in the cytochrome *c* oxidations or reductions, by reaction with standardized HCl in the flow calorimeter (see below), the HCl being present in limiting amount. The following values were obtained for the ionization reactions: phosphate (second ionization), +1.13 kcal $mole^{-1}$; borate, +2.80 kcal $mole^{-1}$; and carbonate (second ionization), +4.60 kcal $mole^{-1}$. The accuracy of these values is considered to be 1–2%.

Proton Change Accompanying Valence Change. The proton change per mole of cytochrome *c* undergoing a valence change was measured with a Radiometer automatic titrator TT1, for both the oxidation by ferricyanide ion and the reduction by ascorbate ion.

Calorimetric Measurements. The calorimeter used in this work was a flow modification (Lyons and Sturtevant, 1969) of the Beckman Model 190 microcalorimeter. Except for one set of measurements at 15°, all calorimetric measurements were performed at 25°.

One set of calorimetric measurements was made by mixing a ferrocytochrome *c* solution (3×10^{-5}–7×10^{-4} M) with a ferricyanide ion solution (5×10^{-3} M), both in the same 0.05 M buffer and at the same pH. The pH of the solution was measured before and after reaction and changed by less than 0.05 pH unit. Separate heat of dilution measurements were made and appropriate corrections applied for the dilution of ferricyanide ion with appropriate 0.05 M buffer solutions. The heat of dilution of ferrocytochrome *c* was found to be negligible.

At pH 7.0, the equilibrium constant for

$$Fe^{II}\text{-cyt } c + Fe^{III} \rightleftarrows Fe^{III}\text{-cyt } c + Fe^{II} \quad (2)$$

where Fe^{III} and Fe^{II} represent ferricyanide ion and ferrocyanide ion, respectively, is about 420 and increases with increasing pH (Brandt *et al.*, 1966). Nevertheless, to be doubly sure of having complete reaction, a large excess of ferricyanide ion was always employed. The Fe^{III}:Fe^{II}-cyt *c* ratio was varied between 25 and 100 with no detectable variation in the enthalpy change. The concentration of ferrocytochrome *c* was determined spectrophotometrically at 550 mμ as described above by removing a 0.250-ml sample from the delivery syringe of the calorimeter at the conclusion of the calorimetric measurement. This procedure avoided errors due to air oxidation of ferrocytochrome *c* in the alkaline pH region resulting from filling the syringes. In those cases where the ferrocytochrome *c* was at a pH other than 7, it was allowed to stand for several minutes in the pH 7 buffer before standardization to allow complete equilibration at this pH.

Another set of calorimetric measurements was made for

$$Fe^{III}\text{-cyt } c + 0.5\text{ascorbate ion} \rightleftarrows Fe^{II}\text{-cyt } c + 0.5\text{dehydroascorbic acid} + 0.5H^+ \quad (3)$$

Heats of dilution were found to be negligible for both the ascorbate ion and ferricytochrome *c*. The ferricytochrome *c* concentration was determined at 550 mμ as described above.

Results

The calorimetric results are summarized in Table I. The calorimetric measurements could not be extended above pH 10.9 because a slow exothermic drift developed above this pH, perhaps due to irreversible protein denaturation.

It is convenient to use available data to refer the enthalpy values in Table I to the reaction of the standard hydrogen

$$H^+ + e^- \rightleftarrows 0.5H_2$$

electrode, for which the standard enthalpy is set equal to zero at all values of the pH. The thermochemical calculations involved are illustrated below for the data at pH 7.00. From Table I

$$Fe^{III}\text{-cyt } c + Fe^{II} \rightleftarrows Fe^{II}\text{-cyt } c + Fe^{III} \qquad \Delta H_4 = +12.6 \text{ kcal mole}^{-1} \quad (4)$$

According to Hanania *et al.* (1967)

$$Fe^{III} + 0.5H_2 \rightleftarrows Fe^{II} + H^+ \qquad \Delta H_5 = -26.7 \text{ kcal mole}^{-1} \quad (5)$$

Adding these equations gives eq 1 with $\Delta H_1 = -14.1$ kcal $mole^{-1}$. Since the value of ΔH for reaction 5 can be taken to be independent of pH above pH 6, we have in general that $\Delta H_1 = \Delta H_4 - 26.7$. For the reductions by ascorbate the following procedure applies. From Table I, at pH 7.00

$$Fe^{III}\text{-cyt } c + 0.5\text{ascorbate ion} \rightleftarrows Fe^{II}\text{-cyt } c + 0.5\text{dehydroascorbic acid} + 0.5H^+ \quad \Delta H_6 = -4.9 \text{ kcal mole}^{-1} \quad (6)$$

TABLE I: Enthalpy Changes in the Oxidation of Ferrocytochrome *c* by Ferricyanide Ion, the Reduction of Ferricytochrome *c* by Ascorbate Ion, and the Reduction of Ferricyanide Ion by Ascorbate Ion at 25°.

pH	No. of Expt	$-\Delta H$ (kcal mole^{-1})	Av Dev (kcal mole^{-1})	$-\Delta H_1$ (kcal mole^{-1})
		Oxidation of Ferrocytochrome *c*		
6.00	2	12.8	±0.1	13.9
6.50	2	12.6	0.1	14.1
7.00	7	12.6	0.1	14.1
7.50	3	11.6	0.2	15.1
8.00	3	10.8	0.2	15.9
8.45	3	8.92	0.21	17.8
8.90	3	7.80	0.18	18.9
9.00	3	8.87	0.28	17.8
9.75	3	1.88	0.40	24.8
10.00	2	−0.70	0.20	27.4
		Reduction of Ferricytochrome *c*		
7.00	3	4.93	±0.10	14.2
8.00	3	6.85	0.22	16.1
10.00	3	17.9	0.2	27.2
10.90	5	21.4	0.1	30.7
		Reduction of Ferricyanide Ion		
7.00	5	17.4	±0.3	

Also from Table I

$$0.5H^+ + Fe^{II} + 0.5\text{dehydroascorbic acid} \rightleftharpoons Fe^{III} + 0.5\text{ascorbate ion} \quad \Delta H_7 = +17.4 \text{ kcal mole}^{-1} \quad (7)$$

As above

$$Fe^{III} + 0.5H_2 \rightleftharpoons Fe^{II} + H^+ \quad \Delta H_5 = -26.7 \text{ kcal mole} \quad (5)$$

Adding these three equations gives eq 1 with $\Delta H_1 = -14.2$ kcal mole^{-1}. These independent values of ΔH for reaction 1 are seen to be in excellent agreement. We cannot as easily assume ΔH_7 to be independent of pH as we did ΔH_5, since ascorbate ion has a pK at 11.57 (Khan, 1962) and dehydroascorbic acid has one at about 9.0 (Borsook *et al.*, 1937). In the absence of information on both of the corresponding heats of ionization we shall, nevertheless, make this assumption. It is interesting that values of $\Delta H_1 = \Delta H_6 - 9.3$ calculated on this basis agree very well with those derived from the ferrocytochrome *c* oxidations.

Calorimetric measurements of reaction 2 at 15° yielded $\Delta H_2 = -13.2$ kcal mole^{-1}, so that $\Delta C_p = +60$ cal deg^{-1} mole^{-1} for this process. If ΔC_p for reaction 5 is assumed to be zero, ΔC_p for reaction (1) is -60 cal deg^{-1} mole^{-1}.

The standard reduction potential at neutral pH, E', for the beef or horse cytochrome *c* couple has been reported by several authors. Recent values determined by potentiometric titration are 0.254 V at 30° (Rodkey and Ball, 1950), 0.255 V at 25° (Henderson and Rawlinson, 1956), and 0.255 V at 30° (Frohwirt, 1961). Our calorimetric ΔH gives a reliable value for the temperature coefficient, in the vicinity of 25°, of $-0.356/T$ V deg^{-1} (T = absolute temperature). Using this temperature coefficient, we obtain for the mean value at 25°, $E' = 0.259$ V. Thus, for reaction 1 at pH 7.00 the standard change in Gibbs free energy is $\Delta G' = -5.97$ kcal mole^{-1} and in entropy is $\Delta S' = -27.3$ cal deg^{-1} mole^{-1} (taking the standard potential of the hydrogen electrode to be zero at pH 7.00). It is important to note that these values cannot be converted to other values of pH in the usual manner since reaction 1 as written does not include the proton uptake which accompanies reduction of ferricytochrome *c* at low or high values of the pH.

Discussion

A number of studies have been made of the temperature-induced conformational changes occurring in ferricytochrome *c* (Urry, 1965; Myer and Harbury, 1965; Schejter and George, 1964; Myer, 1968a,b). The most detailed of these studies is the one by Schejter and George (1964) who determined the apparent thermodynamic quantities for the process responsible for the temperature variation of the intensity of the 695-mμ band in the spectrum of ferricytochrome *c*. These authors estimate that at 26° only 5% of the ferricytochrome *c* is in the "hot" form. The fact that the difference between our ΔH values for reaction 2 at 15 and 25° is essentially negligible substantiates the results of Schejter and George and further indicates that at 25° only the low temperature form of ferricytochrome *c* is present in significant concentration. These results allow us to restrict the discussion which follows to the properties of and modifications occurring in the low temperature or "cold" form of ferricytochrome *c*.

It was pointed out earlier that optical and hydrogen–deuterium-exchange studies indicate that the conformational change accompanying reduction of ferricytochrome *c* is not very extensive. The small magnitude of ΔC_p for reaction 1

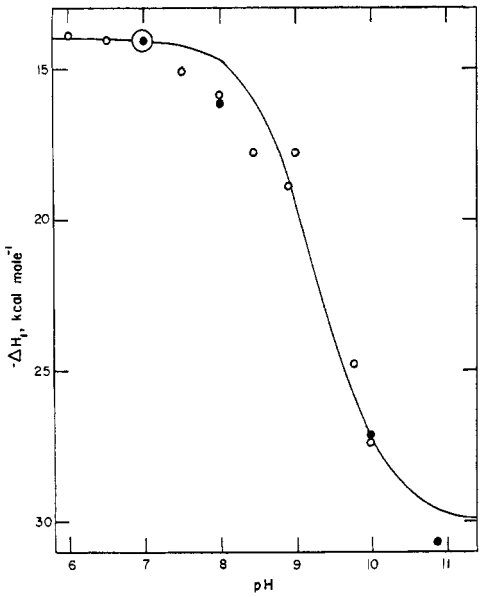

FIGURE 1: The variation with pH of the enthalpy change in the reaction 1 at 25°. (○) Values calculated from enthalpies of oxidation by ferricyanide; (●) values calculated from enthalpies of reduction by ascorbate ion. The curve is calculated assuming control by a single onizing group with $pK = 9.3$, with ΔH (pH 7) = -14.0 kcal mole^{-1} and ΔH (pH > 11) = -30.0 kcal mole^{-1}.

further supports this conclusion. It was been found, as reviewed by Tanford (1968, p 244 ff), that in the thermal unfolding of proteins, changes in apparent heat capacity of the solute amounting to +0.1 to +0.2 cal deg^{-1} g^{-1} are observed, whereas in the present case the value is only -0.005 cal deg^{-1} g^{-1}. These large increases in heat capacity are attributed to exposure of hydrophobic groups to the solvent with accompanying orientation of water molecules around them (Kauzmann, 1959).

The favorable free energy for reaction 1 at pH 7.00, $\Delta G' = -5.97$ kcal mole^{-1}, is the result of a favorable enthalpy change overcoming an unfavorable entropy change, $\Delta S' = -27.3$ cal deg^{-1} mole^{-1}. A decrease in entropy is consistent with the view that the reduced form of the protein has a more rigid, compact conformation than the oxidized form.

The variation with pH of the enthalpy change in reaction 1 shown in Figure 1 suggests that a pH-dependent equilibrium exists involving one or, less likely, both of the cytochrome *c* species. Since the calorimetric measurements cannot delineate the source of this variation, we assume, from the conclusions of previous studies (Greenwood and Palmer, 1965; Urry, 1965; Myer and Harbury, 1965; Brandt *et al.*, 1966), that ferricytochrome *c* is the species undergoing modification. The reversibility of this modification is demonstrated in Figure 1 where it is seen that the ΔH for reaction 1 is independent of whether it is calculated from ascorbate ion reduction of ferricytochrome *c* or ferricyanide ion oxidation of ferrocytochrome *c*.

The data in Figure 1 provide some insight into the nature of the pH-induced modification in ferricytochrome *c*. Greenwood and Palmer (1965) and Brandt *et al.* (1966) concluded that at high pH ferricytochrome *c* exists partly in a form designated below by Fe^{III}-cyt *c'*, which must be converted to the low pH form, Fe^{III}-cyt *c*, before it can be reduced to ferrocytochrome *c*

$$Fe^{III}\text{-cyt } c' + nH^+ \rightleftharpoons Fe^{III}\text{-cyt } c \quad (8)$$

$$Fe^{III}\text{-cyt } c + 0.5H_2 \rightleftharpoons Fe^{II}\text{-cyt } c + H^+ \quad (9)$$

We have observed that there is no release or absorption of hydrogen ions when the oxidation state of cytochrome *c* is changed in the region of neutral pH, in agreement with the findings of Theorell and Åkesson (1941). These authors found that between pH 7 and 10 the difference in proton binding between the oxidized and reduced forms of cytochrome *c* changes by one proton per molecule. The reduction potentials for cytochrome *c* determined by Rodkey and Ball (1950) change with pH in the alkaline region in the manner to be expected if one proton per molecule is taken up when ferricytochrome *c* is reduced. Theorell and Åkesson (1941) found that the change with pH of the absorbance of ferricytochrome *c* at 650 mμ follows a simple titration curve with $n = 1$ and $pK' = 9.35$ (at an unspecified temperature). These observations all suggest that the value of *n* in reaction 8 is 1. Our measurements of the changes in protonation on changing the oxidation state of cytochrome *c* are also consistent with the value $n = 1$.

It is not possible to derive an independent estimate of *n* from our calorimetric data since a reliable value for the limit of ΔH_1 at high pH, ΔH_∞, cannot be obtained. This being the case we shall assume in what follows that $n = 1$. The curve in Figure 1 is drawn for $n = 1$, $pK = 9.3$, and $\Delta H_\infty = -30$ kcal mole^{-1}; since the experimental data show an average deviation from this curve of ±0.7 kcal mole^{-1}, we consider that the curve represents an adequate fit to the experimental data.

It is reasonable to equate the difference ΔH_1 (pH < 11) $- \Delta H_1$ (pH 7) = -16.0 ± 2.0 kcal mole^{-1} to ΔH_8, the enthalpy change in reaction 8. This quantity is much too large to be due solely to the association of one proton per molecule, so that it is necessary to conclude that the decrease in heat content is due at least in part to the conformational transition in ferricytochrome *c* at alkaline pH which has been postulated by others. The standard free energy and entropy of reaction 8, calculated from our data, are $\Delta G'_8 = -3.1 \pm 0.3$ kcal mole^{-1} (pH 7.00) and $\Delta S_8' = -43 \pm 6$ cal deg^{-1} mole^{-1} (pH 7.00). Since the addition of protons to amino or phenolic hydroxyl groups is accompanied by an increase in entropy (Edsall and Wyman, 1958) the entropy decrease in reaction 8 is another indication that a conformational transition is probably involved.

The apparent thermodynamic quantities at 25° reported by Schejter and George (1964) for the thermally induced process leading to the loss of the 695-mμ band in the spectrum of ferricytochrome *c* are remarkably similar to those given above for reaction 8: $\Delta G' = -1.7$ kcal mole^{-1}; $\Delta H = -14.6$ kcal mole^{-1}; $\Delta S' = -43$ cal deg^{-1} mole^{-1}. It seems rather likely that these two processes are indeed essentially identical.

The pH of half-completion of the ΔH variation shown in Figure 1 is approximately 9.3. A number of previous studies, including those mentioned above (see Margoliash and Schejter, 1966, for a summary), have given indication of a heme-linked ionization occurring in ferricytochrome *c* with a pK' of 9.3 which controls the pH dependence of various properties

of the protein. One can reasonably assume that all of these phenomena are manifestations of a single conformational change, involving also a change in protonation, the thermodynamic parameters for which have been estimated in this work.

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