Optical Rotatory Dispersion of Cytochrome c

III. EFFECT OF IONIC STRENGTH ON THE CONFORMATION OF HORSE HEART FERRICYTOCHROME c AND ITS FULLY ESTERIFIED DERIVATIVE*

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SUMMARY

Decrease in the ionic strength of a solution of horse heart ferricytochrome c at pH 1.5 results in changes in optical rotatory dispersion similar to those observed upon helix-random coil transition in model polypeptide systems. These changes are accompanied by modification of the dispersion pattern in the Soret region and parallel alterations in the absorption spectrum indicative of transition from a partially low spin system to a predominantly high spin one. A corresponding ionic strength effect is not observed with the unmodified protein at pH 7 but can be obtained at neutral pH with a derivative in which all the carboxyl groups are esterified. Such an esterified preparation, even under conditions where the dispersion pattern in the region from 200 to 250 mm remains essentially that of the unmodified protein, exhibits rotations which, at longer wave lengths, approximate more nearly those recorded for the nonesterified molecule in the presence of guanidine hydrochloride or urea. The possibility is considered that the propionate side chains of the porphyrin play an important role in the binding between the protein and its prosthetic group.

In an earlier study of the effect of pH on the optical rotatory dispersion of horse heart ferricytochrome c, it was noted that, at low pH, diminution of the ionic strength results in a decrease in the levorotation at the major negative extremum near 231 nm and the appearance of a new negative extremum near 210 nm. Although the optical activity at these wave lengths is in part attributable to transitions of the central coordination complex (3), it seems reasonable to assume, by analogy to relationships for model polypeptides (4), that this shift reflects primarily a helix-random coil conversion. Such a conversion upon change in the ionic strength could be a consequence of a number of factors, including: (a) alteration in the degree of shielding of the charged groups of the molecule; and (b) a change in the nature of the central coordination complex (5). The studies outlined here were undertaken in an effort to help resolve some of these factors.

EXPERIMENTAL PROCEDURE

Materials—Horse heart cytochrome c was obtained from Sigma (Type III). The fully esterified derivative was prepared by reaction with methanol-HCl at 25° for a period of 24 hours. The results of acidimetric titration and determination of the amount of methanol liberated upon saponification indicated that there remained essentially no free carboxyl groups.

Methods—Optical rotatory dispersion measurements were conducted with a Cary model 60 recording spectropolarimeter, as described previously (3). A 1-cm jacketed cell was used in determinations at temperatures other than 25°. In experiments with nonesterified cytochrome c, potassium ferricyanide at a molarity 0.15 that of the protein was added to each solution to be studied at 25° (2), and double this concentration was used in work at higher temperatures. Esterified cytochrome c, which is autoxidizable, was studied in the absence of ferricyanide. Protein concentrations were calculated on the basis of absorbance readings in the visible range and were kept between 1 and 4 × 10⁻³ m. Rotations are given as mean residue rotations.

RESULTS

When a solution of horse heart ferricytochrome c in 0.1 m potassium phosphate buffer, pH 6.8, is adjusted to pH 1.5 by the addition of HCl, marked changes occur in the optical rotatory dispersion in the visible, Soret, and near-ultraviolet ranges, but little or no alteration is observed in the rotation at the negative extremum near 231 mm. The main modification in this region is a slight broadening of the trough, accompanied by a shift in the extremum to about 233 mm (Fig. 1, Curve A and B). If, on the other hand, a solution of the protein in water is adjusted with HCl to pH 1.5, a significantly different result is obtained. As illustrated by Curve E, there is a further simplification of the pattern in the Soret region, and a major change is introduced at wave lengths below 250 mm. The trough near 231 mm gives way to a single peak at about 226 mm (Fig. 1, Curve E).

1 Position varies with conditions.
FIG. 1. Optical rotatory dispersion of horse heart ferricytochrome c at neutral and acid pH. Temperature, 25°. A, 0.1 M phosphate buffer, pH 6.8; B, 0.1 M phosphate-HCl, pH 1.5; C, 0.01 M phosphate-HCl + 0.1 M KCl, pH 1.5; D, 0.01 M phosphate-HCl, pH 1.5; E, HCl, pH 1.5.

FIG. 2. Optical rotatory dispersion of horse heart ferricytochrome c as a function of chloride concentration. A, pH 1.6; temperature, 25°. A, HCl; B, HCl + 0.02 M KCl; C, HCl + 0.04 M KCl; D, HCl + 0.07 M KCl; E, HCl + 0.09 M KCl; F, HCl + 0.14 M KCl; G, HCl + 0.20 M KCl; H, HCl + 0.35 M KCl. Inset, mean residue rotation at 210 mμ as a function of chloride concentration; midpoint, 0.1 M; n = 2.9.

FIG. 3. Absorption spectra of horse heart ferricytochrome c as a function of chloride concentration. pH 1.5; temperature, 25°; 2-cm absorption cell. A, HCl; B, HCl + 0.03 M KCl; C, HCl + 0.06 M KCl; D, HCl + 0.09 M KCl; E, HCl + 0.13 M KCl; F, HCl + 0.19 M KCl. Inset, absorbance at 528 mμ as a function of chloride concentration; midpoint, 0.1 M; n = 2.7.

At neutral pH, the absorption spectrum remains, to the lowest salt concentrations attainable, fully that characteristic of the low spin species. The analysis of a helix-random coil transition...
under these conditions would thus be a simpler proposition than it is at pH 1.5. However, a solution of the protein in water, adjusted to pH 6.8 with HCl, exhibits the same optical rotatory dispersion in the region between 200 and 250 nm, as does a solution in 0.1 M phosphate buffer of pH 6.8; i.e., diminution of the ionic strength at neutral pH does not result in a helix-random coil transition. This lack of random coil formation could be a reflection of a number of factors, including: (a) the smaller net charge of the protein at neutral pH; (b) the possible presence at neutral pH, but absence at low pH, of structurally significant bonds involving carboxylate groups of the molecule; and (c) the difference in nature of the central coordination complex at the two values of pH. To obtain further information, comparative measurements were conducted with a preparation in which the carboxyl groups were all in the form of the methyl ester.

As shown in Fig. 4, such fully esterified ferricytochrome c has an absorption spectrum which at neutral pH differs appreciably from the spectrum of the unmodified molecule and resembles more nearly that of the nonesterified preparation in the presence of 8 M urea. It would appear that blockage of the carboxyl groups is attended by a significant alteration in the protein-prosthetic group interaction, and this is borne out fully by the dispersion pattern recorded over the range from 250 to 450 nm (Fig. 5). Here again, the data for the esterified derivative (Curve B) bear a closer similarity to those for the parent molecule in 8 M urea (Curve A) than to those for the unmodified system. On the other hand, there is an important difference between the esterified and urea-containing systems at wave lengths below 250 nm. Whereas the nonesterified molecule in the presence of urea yields a pattern with a trough near 210 nm, the esterified derivative in 0.1 M phosphate buffer, pH 6.8, continues to yield a curve with a trough near 231 nm and exhibits about the same rotation at the negative extremum as is observed with the unmodified protein under these conditions. Clearly, modification of the carboxyl groups permits a loosening of the structure in the vicinity of the prosthetic group, with little or no accompanying helix-random coil transition.

If the esterified preparation is subjected to a reduction of the ionic strength, then a shift in the dispersion pattern indicative of a lowering of the helix content can be observed even at pH 6.8, and this change is largely unattended by further alteration in the form of the curve in the Soret region (Fig. 5, Curves B and C). It thus is possible to study the helix-random coil transition under conditions where there is minimal overlap with change in the protein-heme binding.

At a constant ionic strength low enough to bring about a partial conversion to the random coil form at pH 6.8, decrease of the pH leads to additional diminution of the trough near 231 nm and enhancement of that near 210 nm (Fig. 5, inset). This shift of course cannot, in this instance, be attributed to a disruption of carboxylate bonding or to a major increase in the net charge of the molecule. On the other hand, it is paralleled by further

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5 The data for solutions containing urea (Figs. 4 and 5) are at variance with the view that this agent is without effect on horse heart ferricytochrome c (cf. Reference 6).
Since at pH 6.8 the unmodified protein is insensitive to lowering of the ionic strength at 23°C, dispersion patterns were determined also for a solution of neutral pH and low ionic strength at a series of higher temperatures (Fig. 7). With increasing temperature, there is a diminution of the levoration at the extremum near 231 mμ, but, as upon decrease of the pH, concomitant alterations occur in the Soret and other regions of the wave length range studied. As shown for three wave lengths in Fig. 8, the change occurs in two steps. No midpoint can be assigned to the second of these, except perhaps in the case of the data for 232 mμ, where it seems to fall near 88°. The first transition is centered at approximately 53° and, as shown in the inset, exhibits the same slope at all three wave lengths represented. There is no indication of a change in helix content unattended by change in the Soret region.

**DISCUSSION**

Reactions involving the central coordination complex of cytochrome c have long been recognized to result in important changes in the conformation of the molecule, and it is evident that information on the detailed nature of these changes is essential to an understanding of the relationship between structure and function. Optical rotatory dispersion studies can help to provide such information and have recently begun to be applied intensively in this connection (1–3, 7–9). The data thus far obtained show, in part, that reduction of the heme group, complex formation with extrinsic ligands, and change in pH all bring about extensive modification of the dispersion pattern at wave lengths above 250 mμ. On the other hand, comparatively little change has been noted at lower wave lengths. A small, and perhaps functionally significant, increase in helicity may occur upon reduction of the prosthetic group, but, in general, it appears that the helix content of horse heart cytochrome c is not one of the more variable features of the molecule. Marked changes in the optical rotatory dispersion below 250 mμ, similar to those associated with helix-random coil transition in model polypeptides (4), have been observed only upon the addition of agents such as guanidine hydrochloride and urea, upon diminution of the ionic strength at low pH, and upon study of the esterified derivative at low ionic strength.

Although the protein-prosthetic group interaction characteristic of the parent molecule at neutral pH can thus readily be altered without appreciable change in the helix content, there is no indication that the reverse can be accomplished. Changes in the dispersion pattern below 250 mμ have thus far been obtained only under conditions resulting also in major modification of the curve at longer wave lengths. It clearly would be useful, in the further study of the underlying relationships, to have procedures permitting introduction of the changes above and below 250 mμ in an sequential manner as possible, and the ionic strength effects dealt with here illustrate one approach to such stepwise change. The nonesterified protein in 0.1 M phosphate-HCl of pH 1.5 and the esterified derivative in 0.1 M phosphate buffer of pH 6.8 exhibit curves having much the same simplified form.

It should be noted that the changes in the range from 200 to 250 mμ differ markedly from those seen upon helix-random coil transition at 23°. The magnitude of the change in rotation at 231 mμ exceeds that obtained upon reduction in pH at low ionic strength, or upon the addition of guanidine hydrochloride or urea (cf. Fig. 5). Yet the curve in this vicinity retains the form of a trough, and there is no indication of the development of a new trough near 210 mμ.

**ALTERATION OF THE CENTRAL COORDINATION COMPLEX**

Altering the central coordination complex, as indicated by a change in spectrum from the low spin type to the high spin type, and by the emergence of an over-all dispersion pattern similar to that exhibited under comparable conditions by the unmodified protein (Fig. 1, Curve D).

If the optical rotatory dispersion of the esterified protein is determined at pH 6.8 as a function of chloride concentration, the group of curves shown in Fig. 6 is obtained. The midpoint of the transition occurs at a 10-fold lower concentration than that which marks the midpoint for the nonesterified system at pH 1.5. This, too, would be consistent with a relationship between the ease of random coil formation and the state of binding of the heme and protein parts of the molecule. At pH 1.5, the chloride dependence for the esterified derivative becomes essentially the same as that for the unmodified preparation, both in terms of the midpoint of the transition and in terms of the greater n value observed under these conditions.
above 250 μm as do those obtained at pH 6.8 in the presence of 8 M urea or 4 M guanidine hydrochloride; but, below 250 μm, the pattern remains similar to that for the parent molecule at pH 6.8 in the absence of denaturing agents. Only upon lowering of the ionic strength are changes indicative of a helix-random coil transition observed.

The helix-random coil transition obtained with the nonesterified protein at pH 1.5 is still, however, accompanied by other major changes. There is a parallel alteration of the absorption spectrum, from a form characteristic of the system in a partially low spin state to one typical of the high spin state (5), and a further simplification occurs in the dispersion pattern at the longer wave lengths, principally in the Soret region. The reduction in helix content observed under these conditions might thus be attributable to decrease in the shielding of charged groups, to the change in coordination indicated by the alterations in spectrum and longer wave length optical rotatory dispersion, or to both of these factors.

A much simpler situation prevails in the case of the helix-random coil transition obtained at pH 6.8 with the fully esterified derivative. Here there is no further appreciable simplification of the dispersion pattern at the longer wave lengths and no change in the absorption spectrum indicative of a change in spin state. Decrease in the helicity upon diminution of the ionic strength thus clearly does not require the additional disruption of the central coordination complex encountered in work at low pH and can conveniently be studied in its absence.

On the other hand, the midpoint of transition for the esterified derivative at pH 6.8 occurs at a chloride concentration approximately 10-fold lower than that which characterizes the transition of the esterified and nonesterified systems at pH 1.5. The change in coordination brought about at the low pH thus does significantly facilitate decrease in the helix content. This, of course, is consistent with the view that the bonding between the protein and the prosthetic group has an important influence on the folding pattern of the molecule and, through that, on the susceptibility to helix-random coil transition.

The fact that elimination of the negatively charged groups results in major modification of the protein-prosthetic group interaction suggests that one or more of the carboxylate-containing side chains have an essential function in maintenance of the native structure. The present data do not permit distinction in this regard between the side chains of the aspartic and glutamic acid residues and the propionate side chains of the metalloporphyrin. The latter are, however, in an obviously favorable position to influence the protein-heme interaction in a very direct manner and would seem deserving of special attention. Efforts are under way to prepare a derivative in which only the porphyrin side chains are modified.

REFERENCES
Optical Rotatory Dispersion of Cytochrome c: III. EFFECT OF IONIC STRENGTH ON THE CONFORMATION OF HORSE HEART FERRICYTOCHROME c AND ITS FULLY ESTERIFIED DERIVATIVE
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