Circular Dichroism Studies of the Perturbations of Cytochrome c by Alcohols*

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SUMMARY

The circular dichroic (CD) behavior of ferri- and ferro-

cytochrome c in the presence of methanol, ethanol, and

1-propanol was investigated in an attempt to elucidate some

structure-function relationships of the protein.

The circular dichroic spectrum of ferricytochrome c in the

Soret region exhibited extrema at 417 and 402 nm, which

were susceptible to the presence of alcohols in solution. In

concentrations of 1-propanol exceeding 6 mole % the protein

became modified to a form that showed a positive CD band

at 406 nm with a large increase in ellipticity and the disap-

pearance of the 417-nm trough. Similar changes were also

observed in solvents containing greater than 13 mole % of

ethanol and greater than 21 mole % of methanol. These

changes in the Soret region occurred at the same alcohol

concentration as those in the aromatic region, thus possibly

implicating the aromatic residues in heme crevice conforma-

tion changes. Changes in the intrinsic region of ferricyto-

chrome c indicated the enhancement of α helix content at

elevated alcohol concentrations.

In contrast, ferrocytochrome c under anaerobic conditions

showed no changes in either absorption or CD spectra even in

50 volume per cent of 1-propanol. These spectra were,

however, converted immediately by higher alcohol concen-

trations to that resembling the modified ferricytochrome c.

The heme of this apparently "autoxidized" cytochrome c

was reactive to carbon monoxide and thus the iron was still

in the reduced state.

These results indicate that alcohols perturb effectively the

interactions between the heme and the protein moiety form-

ing the heme crevice. At increasing alcohol concentrations,

ferrocytochrome c which is less susceptible to modification

than ferricytochrome c is gradually deformed. Conse-

quently, the heme becomes exposed and reactive toward CO.

The similarity in the CD spectra of ferro- and ferri-

cytochrome c under these conditions is a reflection of inter-

actions between the distorted heme coordination sphere and

the altered heme environment.

Attempts have frequently been made to relate the structure

or conformational state of cytochrome c to its biological function

in the mitochondrion. At the forefront of such investigations

have been numerous studies on the elucidation of properties of

chemically modified cytochromes c. The modifications involve

amino acids considered to be involved in electron transfer to and

from the heme iron, and include tyrosine (1-5), tryptophan (6,

7), methionine (8, 9), and lysine (1). Other attempts to define

this relationship involve spectrophotometric observations of

cytochrome c in mitochondria (10) and fluorescent (11) and spin

labeling (12) of the protein molecule. The extensive studies of

Margoliash and Schejter (13) on relating the primary structures

of cytochromes, from many species, to their biological function

has also yielded much valuable information. The synthesis of a

cytochrome c analogue (14) has opened the door to a further ave-

nue of attack.

Our approach to this problem is to modify the conformation

of the protein with various agents, such as alcohols, and to at-

tempts to elucidate and relate the resultant conformation of the

cytochrome c to a variety of in vitro properties, determined under

the same conditions. In earlier papers we reported on the effect

of alcohols and ethers on the Soret absorbance of ferricytochrome

c (15, 16) and on the alcohol-induced autoxidation of ferrocyto-

chrome c (17). Further studies on the carbon monoxide bind-

ing2 and the effects of temperature on the 695-nm band of cyto-

chrome c, both under the influence of alcohols, have been com-

pleted.

ORD-CD techniques appeared to be potentially useful for

following alcohol-induced conformational changes. By these

techniques the effects of denaturants such as urea, pH, tempera-

ture, and ionic strength on cytochrome c structure have been

examined (e.g. 18-20). In this communication we present our

studies on the circular dichroism spectra of horse heart ferri- and

ferrocytochrome c in the presence of varying concentrations of

methanol, ethanol, and 1-propanol and discuss possible structural

changes which occur as a result of this treatment. A preliminary

report of this work has been presented (21).

EXPERIMENTAL PROCEDURE

Materials—Horse heart cytochrome c, grade III, was obtained

from the Sigma Chemical Co. The protein was brought to its

fully oxidized state by addition of a small quantity of potassium


2 L. S. Kaminsky and M. J. Byrne, in preparation.

* The abbreviations used are: CD, circular dichroism; ORD, optical rotatory dispersion.

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ferricytochrome to the cell, just prior to running the spectra. Ferricytochrome c was prepared by reduction of cytochrome c with excess dithionite followed by the removal of the unreacted dithionite and its oxidation products on a column of Bio-Gel P-2. The alcohols were Baker Analyzed Reagents and were used without further purification. Water was double glass-distilled.

**Methods**—Alcoholic solutions of ferricytochrome c were prepared as described in an earlier paper (17). Spectra of ferrocytochrome c were determined anaerobically. The buffer-alcohol mixture was bubbled with oxygen-free, water-saturated argon in a Thünenberg cuvette. Ferricytochrome c and enough solid dithionite to reduce any portion of the cytochrome c, which may have become oxidized, was placed in the side arm and was mixed with the solvent after 30 min of argon bubbling. Absorbance spectra of the solutions were determined in the 550-nm region with a Cary 14 spectrophotometer before and after determination of the circular dichroism spectra. An experiment was repeated if any change in absorbance at 550 nm was noted. Concentrations of cytochrome c are reported in the figure legends and were determined with molar extinction coefficients as reported by Schejter (22).

Circular dichroism spectra were obtained in a Cary Spectropolarimeter model 60, with CD attachment 6002, at 27°. The instrument was calibrated with twice recrystallized d-10-camphorsulfonic acid. The slit width was programmed to give a constant light intensity over the region 625 to 210 nm. Rotations are reported in deg cm² per mole of cytochrome c.

Spectra of carbon monoxide ferrocytochrome c in alcoholic solution were determined in Thünenberg cuvettes with side arms replaced with serum caps. The solvent was bubbled with argon for 30 min and freshly reduced cytochrome c was injected into the solution through the serum cap followed shortly by oxygen-free carbon monoxide for a further 5 min.

**RESULTS**

Ferricytochrome c—Addition of low concentrations of methanol, ethanol, or 1-propanol to solutions of ferricytochrome c at pH 5 in 0.1 M sodium acetate produced only minor changes in the CD spectrum. At higher concentrations of alcohol, however, marked changes in the spectra were observed. The extrema in the Soret region at 417(-)nm and 402(+)nm rapidly altered with increasing alcohol concentration above a certain level, which was dependent on the alcohol used. The trough at 417 nm decreased and ultimately disappeared and the peak at 402 nm increased dramatically in ellipticity and shifted to 405 nm. An inflection on the 402-nm band at approximately 395 nm also disappeared at higher alcohol concentrations as did the other major trough in the Soret region at 370 nm.

The double peak in the ultraviolet region at 250 and 262 nm slowly increased in ellipticity and ultimately merged into a single band at 258 nm. The weak negative peak in this region at 285 nm became masked at high alcohol concentrations and was observed as a positive inflection on the 258-nm band.

In the visible metal-ligand transition region the three positive peaks at 492, 530, and 550 nm increased slightly in ellipticity with initial increases in alcohol concentration as did the trough at 577 nm, but at higher alcohol concentrations a general decrease in the ellipticities in the range 550 to 480 nm was observed and the spectrum in this region became featureless. Representative families of spectra are shown in Figs. 1 and 2 which illustrate the effect of 1-propanol on ferricytochrome c. The major changes observed in these spectra are induced by the solvents over a narrow range of their concentrations. This is exemplified in Fig. 3 where the ellipticities at wave lengths associated with the major peaks and troughs are plotted against the mole % of propanol. Although the various bands are from widely differing areas of the spectrum they all undergo the transition at essentially the same 1-propanol concentration.

The effectiveness of the three alcohols investigated in inducing changes in the conformation of ferricytochrome c is a function of their hydrocarbon chain length. This is shown in Fig. 4 where almost 23 mole % of methanol is required to induce one-half the maximum change in ellipticity in the Soret region while the corresponding figures are only 13 mole % for ethanol and 6 mole % for 1-propanol. A similar dependence of effectiveness of induction of conformational changes on hydrocarbon chain lengths was also noted for peaks and troughs at all the wave lengths investigated.

**Fig. 1. Circular dichroism spectra of horse heart ferricytochrome c in 1-propanol (volume percentage of 1-propanol is shown where 10% is equivalent to 2.4 mole %, 20% to 5.2 mole %, 22.5% to 6 mole %, 25% to 6.8 mole %, and 50% to 17 mole %); 0.1 M sodium acetate buffer, pH 5; ferricytochrome c concentration, 10.3 μM; path length 10 mm; temperature 27°; trace of potassium ferriyanide present.**
The CD spectrum of ferricytochrome c in the intrinsic region, from 240 nm to approximately 205 nm, exhibited solvent-induced changes. It is important to point out that these changes were not in accord with the remainder of the spectrum. The trough at 222 nm did not undergo a major modification in ellipticity until higher alcohol concentrations than those inducing effects in other regions of the spectrum. The changes observed in this 220 nm region were also not a function of the alcohol used as perturbant. The trough at 208 nm was more susceptible to the influence of alcohols and a large increase in ellipticity combined with a shift to lower wavelength, was observed at higher alcohol concentrations (Fig. 5).

Ferricytochrome c solutions in concentrations up to 200 μM in 12 mole % of 1-propanol (volume percentage of 1-propanol is shown where 30% is equivalent to 5.2 mole %, 22.5% to 6 mole %, and 50% to 17 mole %); ferricytochrome c concentration, 11.1 μM; path length, 1 mm. Other conditions as in Figs. 1 and 2.

Ferrocytochrome c—In contrast to the results for ferricytochrome c, the alcohols had no apparent effect on the CD spectrum of ferrocytochrome c even up to 27 mole % of methanol. Small variations were observed throughout the range 450 to 240 nm.
Fig. 6. Molar extinction coefficients of ferricytochrome c for the Soret band in aqueous (■) and 12.3 mole % of 1-propanol (●) as a function of cytochrome c concentration. Concentrations varied from 2.7 to 1315 μM and path lengths from 50 to 0.1 mm. Other conditions as in Fig. 1.

Log [Cytochrome c]

0 1 2 3

10 12 14

$e_{Soret} \times 10^{-4}$

Fig. 7. Circular dichroism spectra of horse heart ferrocytochrome c in 1-propanol (volume percentage of 1-propanol is shown). Sodium acetate buffer (0.1 M), pH 5; ferrocytochrome c concentration, 18.8 μM; path length, 10 mm; cytochrome c reduced with sodium dithionite and maintained under anaerobic conditions, temperature 27°.

Fig. 8. Circular dichroism spectra of horse heart ferrocytochrome c in 1-propanol. Cytochrome c concentration 40.5 μM. Other conditions as in Fig. 7.

Fig. 9. Absorbance spectra of ferrocytochrome c in different 1-propanol concentrations. A, 17 mole %; B, 8.5 mole %; C, 22.5 mole %. —, anaerobic conditions; ——, anaerobic conditions with carbon monoxide bubbling for 5 min; ——, carbon monoxide compound with subsequent oxygen bubbling for 5 min. Other conditions as in Fig. 7.

but the scatter of the results is indicative of experimental error rather than a systematic effect. In the visible range between 425 and 450 nm variations were slightly larger and the trough at 547 in particular, increased very slightly with increasing alcohol concentration. In Figs. 7 and 8 the effect of 1-propanol on the CD spectrum of ferrocytochrome c is demonstrated which exemplifies the apparent ineffectiveness of the alcohols in contrast to Fig. 3.

A very dramatic conformational change in ferrocytochrome c was however, indicated by both absorbance and CD spectra at high alcohol concentrations under anaerobic conditions. The absorbance band at 550 nm slowly declined to ultimately yield a spectrum resembling that of ferricytochrome c (Fig. 9), while the CD spectrum of this sample was almost identical with that...
The interactions of alcohols with proteins have been extensively investigated and the resultant effects well documented (15, 23). It is generally accepted that organic molecules such as alcohols act in solution by the formation of stable hydrophobic bonds with the hydrophobic residues of the protein chain. The net result of this is that amino acid residues which in the native state (in aqueous solution) are constrained in the interior of the molecule can now, in alcoholic solution, be stabilized at the surface of the protein.

In view of the obvious importance of the environment around the heme for the function of cytochrome c and the known hydrophobicity of this environment (24), it might be expected that alcohols would radically affect this function by exposing the buried heme to the solvent. Such an opening of the protein crevice might simply involve a loosening of the hydrophobic residues from the heme or in addition a displacement of the heme iron ligands.

The gradual effect of the homologous series of alcohols studied here (Fig. 4) in modifying the CD spectrum of ferrocytochrome c supports the view that hydrophobic interactions are involved. The sharp changes observed in the different spectral regions (excluding the intrinsic region) all occur at very similar alcohol concentrations and this is probably an indication that the conformational and structural moieties represented by the CD bands are undergoing interrelated alterations. In particular, aromatic residues are possibly involved in conformational changes about the heme and tyrosines 48 and 67 and tryptophan 59 are well placed candidates for this role (24).

The concentrations of alcohols required to induce one-half the maximum change in molar ellipticities agree well with those required to produce similar effects on the Soret absorbance of ferrocytochrome c (15, 16). A major difference which arises from a comparison of the two studies is that in the case of the Soret absorbance a maximum value is reached at a particular alcohol concentration after which further increases in alcohol produce large reductions in the absorbance (see Fig. 4, Reference 15) while the molar ellipticities remain fairly constant after reaching a maximum (Figs. 3, 4). The marked and sharp change in the CD spectra as the alcohol concentrations are increased, is probably indicative of the displacement of a heme iron ligand (see below). Subsequent studies which have shown the disappearance of the 605 nm absorbance band of cytochrome c at alcohol concentrations very similar to those producing marked changes in the CD spectra appear to substantiate this explanation. Furthermore this agreement would further implicate methionine 80 as an involved ligand (25, 26). It is interesting to compare these studies with the results of Ulmer (27) on the ORD spectra of cytochrome c-phospholipid complexes in iso-octane. Although iso-octane provides a more hydrophobic environment than the alcohols used in the present investigation, Ulmer could detect no difference between the ORD spectrum of the complex in iso-octane and that of ferrocytochrome c in water. It is thus tempting to conclude that the phospholipid stabilizes the native conformation of the protein under hydrophobic conditions and this aspect is being investigated further.

The apparent lack of change in the CD of ferrocytochrome c at alcohol levels which produce such marked changes in ferrocytochrome c is further proof of the greater stability of the reduced form. However, as shown by its reactivities to carbon monoxide, ferrocytochrome c in as low as 5 mole % of 1-propanol is already susceptible to carbon monoxide binding, and has also become autoxidizable. It would appear, therefore, that the compactness of the molecule is already loosened in such low 1-propanol concentration but this change must be relatively minor in order to produce no significant alteration in the asymmetry of the heme environment.

The major changes observed in the CD spectra of ferrocytochrome c at high alcohol concentrations were suggestive of aut-
oxidation of the protein. The ability of the cytochrome c to react with carbon monoxide, and yield the typical carbon monoxide cytochrome c absorbance and CD spectra, however, is convincing evidence that the iron was in the reduced form although exposed to the solvent. The overall conformation of the protein must thus resemble that of the solvent-modified oxidized form, having a less compact structure and with the liganding pattern of this oxidized form.

The occurrence of changes in the 222-nm trough which were out of step with those arising from the aromatic and heme absorption regions (by being induced at higher alcohol concentrations) indicates that the earlier effects are localized in the heme crevice. The increased ellipticity of the 222-nm band at high alcohol concentrations could possibly denote the increased \( \alpha \) helix content of the protein, which is in accord with the findings of other studies which indicate the enhancement of \( \alpha \) helix contents of low helix proteins by hydrophobic reagents (e.g. 28). However, the possibility of the participation of heme transitions in this region (29) cannot be excluded.

The gross effects of urea and elevated temperatures on the CD spectrum of ferricytochrome c (19) in the Soret region resemble the alcohol-induced effects of this study. In the intrinsic region, however, very marked differences are noted. In the case of urea and thermal denaturation the 222-nm trough is ultimately almost completely removed while alcohols enhance this ellipticity. Thus, although it is now generally accepted that urea denatures globular proteins by stabilizing their hydrophobic residues at the surface of the protein, and appears to produce an opening of the heme crevice in like manner to the alcohols, its ultimate action on cytochrome c is at variance with that of the alcohols. It is possible that the hydrogen-bonding capability of urea prevents the stabilization of intramolecular hydrogen bonds of the protein by the formation of segments of \( \alpha \) helix as is the case in alcohols.

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