Optical Rotatory Dispersion and Spectral Properties of Yeast Isocytochromes *c**

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ABSTRACT: The optical rotatory dispersion spectra of yeast iso-1- and iso-2-cytochromes *c* were examined in the wavelength range 215–680 mμ, and changes seen in the optical rotatory dispersion spectra of the ferricytochromes *c* between pH 7 and 9 were correlated with the disappearance of the 695-mμ optical absorption band. A similar transition, related to a conformation change round the heme, occurs in horse heart ferricytochrome *c* over the pH range 7–9.5. The optical rotatory dispersion spectra of both oxidized and reduced yeast cytochromes *c* were very similar to that of horse heart cytochrome *c*, although the depth of the 232-mμ trough was less. Iso-1-ferrocytochrome *c* is not as stable as horse heart or iso-2-cytochromes *c* at pH 10, and a change in the optical rotatory dispersion is seen.

It is concluded that histidine residue 26, present in both horse heart and yeast iso-1-cytochrome *c*, but absent in iso-2-cytochrome *c*, does not contribute the sixth ligand to heme iron in cytochrome *c*.

There have been several studies on the optical rotatory dispersion of cytochrome *c* from different species (Ulmer, 1965, 1966a,b; Urry, 1965; Myer and Harbury, 1965; Mirsky and George, 1966). The most complete data are for horse heart cytochrome *c* and its derivatives. Since optical rotatory dispersion has been used to investigate structural features of cytochrome *c*, we thought it would be interesting to compare the optical rotatory dispersion of two variants of baker's yeast cytochrome *c*. Baker's yeast cytochromes *c* differ from mammalian cytochromes in several important respects, including amino acid sequence, and yet they function at least as efficiently in mammalian electron-transport and phosphorylating systems. They show no splitting of the optical absorption band at low temperatures, and are relatively unstable to heat, acid, and alkali, particularly at low ionic strength. They have five extra amino acids in place of the acetyl group found at the N-terminal end of the protein in horse heart cytochrome *c*. They also have a single cysteine near the C-terminal end which makes them susceptible to dimerization (Margoliash and Schejter, 1966). The complete ordered amino acid sequence of the iso-1-cytochrome *c* is known (Narita *et al.*, 1963), and the exact sequence of the iso-2-cytochrome *c* is known up to position 26. The two cytochromes differ by at least 13 amino acid residues. In the iso-2-cytochrome the histidine residue normally found at position 26 is replaced by an asparagine (Stewart *et al.*, 1966). Formerly, this histidine residue was thought to contribute the sixth ligand to the heme iron and to be invariant in all species. However, experiments on carboxymethylation of methionine 80 (Tsai and Williams, 1965a,b; Ando *et al.*, 1966) and trinitrophenylation of lysine residues 72 and 73 (Okunuki *et al.*, 1965) have implicated the invariant sequence of amino acids from positions 70–80 in heme coordination, and methionine 80 and tyrosine 74 have been suggested as possible ligands, although it has also been suggested that there may be only one strong-field ligand, namely histidine 18, bound to the heme (Fanger *et al.*, 1967; Heller and Smith, 1966). *Pseudomonas* cytochrome *c* contains only one histidine residue and is quite different in amino acid sequence from mammalian-type cytochrome *c*, and yet modification of methionine 61 in the amino acid sequence causes changes similar to those seen in mammalian-type cytochrome *c* (Fanger *et al.*, 1967). Previous optical rotatory dispersion experiments suggest that the invariant tryptophan residue in position 59 is affected by heme–protein interaction (Ulmer, 1966a).

Our results show that the shape of the optical rotatory dispersion of the yeast isocytochromes *c* is similar to that of other cytochromes so far examined in the spectral region 270–680 mμ, although the numerical values of the various Cotton effects differ considerably from horse heart cytochrome *c*. The change in the optical rotatory dispersion of the yeast ferricytochromes *c* which occurs between pH 7.8 and 8.8 can be correlated with the disappearance of the 695-mμ optical absorption band. Yeast iso-1-ferrocytochrome *c* is less stable at alkaline pH than horse heart ferrocytochrome *c*.

Materials and Methods

Baker's yeast iso-1- and iso-2-cytochromes *c* were a gift from Dr. E. Margoliash. They were further purified

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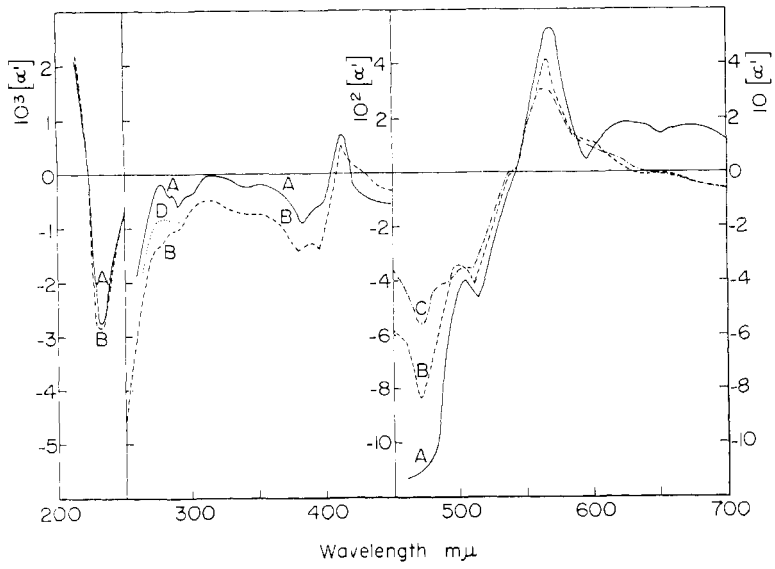

FIGURE 1: Optical rotatory dispersion of yeast iso-1-ferricytochrome *c*. (A) pH 7, (B) 8.8, (C) 9.5, and (D) 7.8. Solutions in phosphate buffer (≈0.1 M)–0.8 M NaCl, 4 M NaOH added to give desired pH.

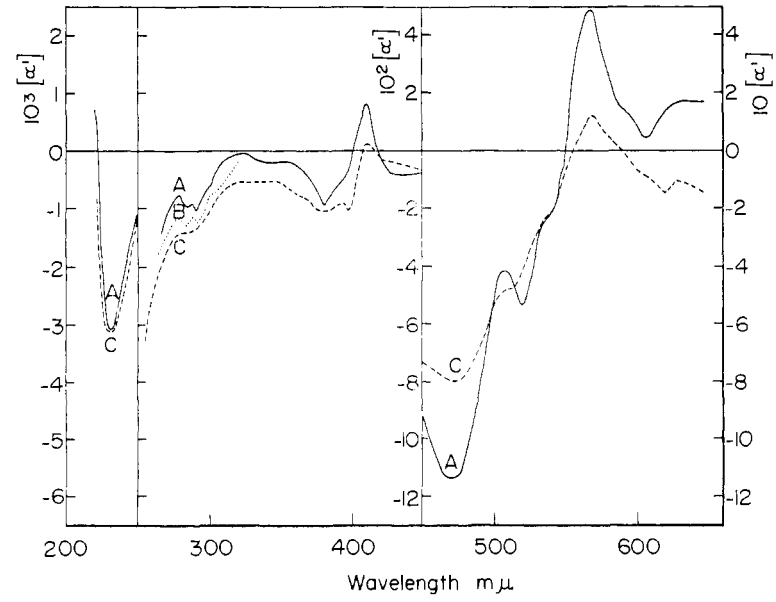

FIGURE 2: Optical rotatory dispersion of yeast iso-2-ferricytochrome *c*. (A) pH 7, (B) 8.1, and (C) 8.8. Solutions in phosphate buffer (0.1 M)–0.8 M NaCl, 4 M NaOH added to give desired pH.

by a linear gradient on Amberlite IRC 50 resin, using the procedure of Margoliash and Walasek (1967). A few drops of mercaptoethanol were added to the cytochrome on the column to ensure that it was in the monomeric form. The purified cytochrome *c* was kept on IRC-50 resin and eluted with 0.8 M sodium chloride in 0.1 M phosphate buffer at pH 6.8 on the same day as it was used. The yeast cytochromes *c* are unstable if left standing in solution. Samples were oxidized with a minimal amount of potassium ferricyanide and reduced with sodium dithionite, potassium borohydride, or mercaptoethanol.

The concentration of cytochrome *c* solutions was measured using a Zeiss PMQ II spectrophotometer or a Cary 14 spectrophotometer. Extinction coefficients of ϵ_{mM} 11.2 at 528 mμ for ferricytochrome *c* were used (Margoliash and Frohwirt, 1959). Optical rotatory dispersion measurements were made with a Jasco UV-ORD 5 spectropolarimeter in the wavelength range 215–700 mμ; 5- and 10-mm cells were used for

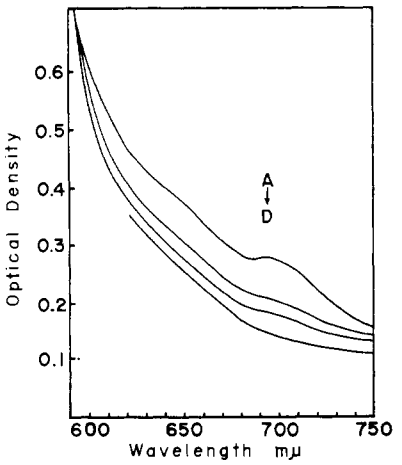

FIGURE 3: Variation of 695-mμ band of iso-1-ferricytochrome *c* with pH. (A) pH 7.2, (B) 8.3, (C) 8.5, and (D) 9.0. Solutions in phosphate buffer (≈0.1 M)–0.8 M NaCl, 4 M NaOH added to give desired pH.

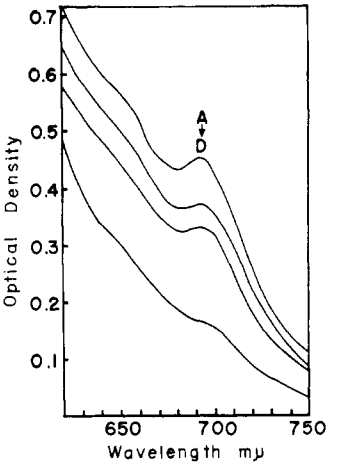

FIGURE 4: Variation of 695-mμ band of iso-2-ferricytochrome *c* with pH. (A) pH 7.3, (B) 7.7, (C) 8.1, and (D) 8.8. Solutions in phosphate buffer (≈0.1 M)–0.8 M NaCl, 4 M NaOH added to give desired pH.

most experiments. pH was measured using a Beckman GS pH meter. A molecular weight of 13,000 fo the yeast isocytochromes *c* was used in calculations of [α'] (Narita *et al.*, 1963).

Results

Rotations were plotted as [α'], the reduced specific rotation. Correction for the refractive index of the solvent was made as indicated previously (Mirsky and George, 1966). The optical rotatory dispersion spectra of the iso-1- and iso-2-cytochromes *c* are almost identical with those of horse heart, chicken heart, and tuna heart cytochrome *c*, although the magnitude of the different Cotton effects varies somewhat (Myer and Harbury, 1965; Mirsky and George, 1966). In the ferricytochrome *c* the positive rotation from 680 to 610 mμ, the complex Cotton effect centered around 528 mμ, the complex effect centered at 410 mμ, consisting of at least two parts, the shape of the 380–300-mμ portion, and significantly the double peaks seen at 278 and 287 mμ are almost identical with the optical rotatory dispersion curves obtained from other species. Similarly, the same changes are seen at alkaline pH in the 270–700-mμ range as are seen with horse heart cytochrome *c*. However these changes occur in the pH range 7.8–8.8 at high salt concentration in the yeast isocytochromes rather than in the pH range 8.5–10 at which they occur in horse heart cytochrome *c* (see Figures 1 and 2). In horse heart cytochrome *c* below pH 9.5 optical rotatory dispersion curves do not vary in solutions of ionic strengths varying between 0.05 and 1 M (Mirsky and George, 1966).

The most important changes in optical rotatory dispersion above pH 7.8 were a decrease in positive rotation in the 680–610-mμ region, a decrease in size in all parts of the complex effect centered around the 528-mμ optical absorption peak, a change in shape of the Soret optical rotatory dispersion peak so that it is more clearly split into two components, with maxima at 410 and 385 mμ, and the disappearance of the sharply defined peaks at 287 and 278 mμ, particularly the latter. Ulmer has shown that the peak at 278 mμ is due to the single tryptophan residue in the cytochrome chain (Ulmer, 1966a). The relative helical content of both isocytochromes as reflected by the 232-mμ trough is much lower than in horse heart cytochrome *c*, reflecting differences in tertiary structure. Iso-2 has slightly more rotation at 232 mμ than iso-1. The rotation at 232 mμ did not change between pH 6 and 9.8 in the ferricytochromes. The 695-mμ optical absorption band disappears over exactly the same pH range as the optical rotatory dispersion changes occur in both isocytochromes (see Figures 3 and 4).

The optical rotatory dispersion spectra of the reduced isocytochromes at pH 7 are almost identical and differ significantly from that of horse heart cytochrome *c* only in the 500–540-mμ region, where the size of the Cotton effect corresponding to the 520-mμ β-absorption band varies between iso-1, iso-2, and horse heart (see Figures 5 and 6). The size of the 289-mμ peak is very small in the yeast cytochromes. Reduced iso-1-cytochrome *c* is not as stable at alkaline pH as horse heart cytochrome *c* or iso-2-cytochrome *c*. At pH 9.8 there is a change in the shape of the optical rotatory dispersion Soret peaks quite similar to the change seen in horse heart cytochrome *c* at pH 12.5 when it is left standing several hours before reduction (Mirsky and George, 1966). The changes in the 450–680-mμ region are, however, quite different from the changes in horse heart ferrocytochrome at pH 12.5 and are similar to the spectrum of the undecaheme-peptide of cytochrome *c* and to dicarboxymethylated horse heart cytochrome *c* (Ulmer, 1966b; Mirsky and George, 1967). The depth of the 232-mμ trough is

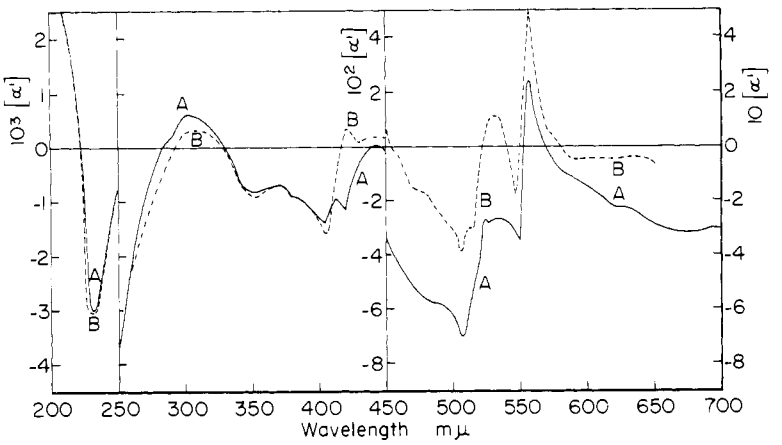

FIGURE 5: Optical rotatory dispersion of yeast iso-1-ferrocytochrome *c*. (A) pH 7 and (B) 9.8. Solutions in phosphate buffer (≈ 0.1 M)–0.8 M NaCl, 4 M NaOH added to give desired pH.

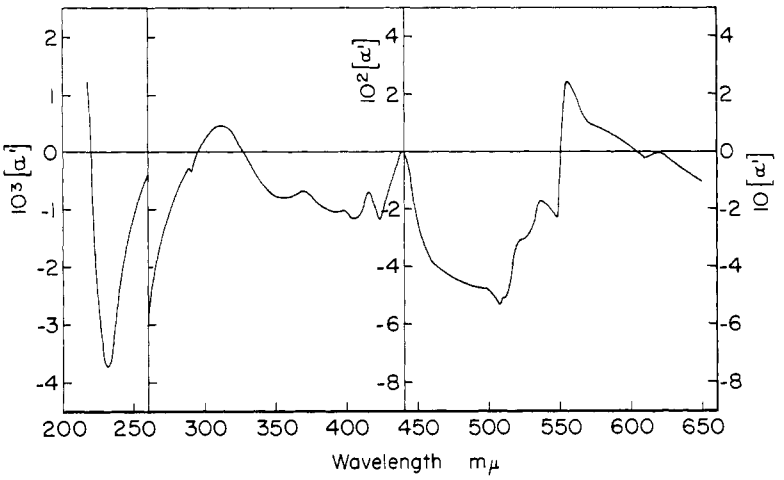

FIGURE 6: Optical rotatory dispersion of yeast iso-2-ferrocytochrome *c*. Optical rotatory dispersion curve remains unchanged over pH range 7.0–10.0. pH 7–8.5: solutions in phosphate buffer (≈0.1 M)–0.8 M NaCl, 4 M NaOH added to give desired pH. pH 8–10.0: solutions in borate buffer (≈0.1 M)–0.8 M NaCl, 4 M NaOH added to give desired pH.

deeper in iso-2-ferrocytochrome *c* than in iso-2-ferricytochrome *c*, as with horse heart, but no significant difference could be detected between oxidized and reduced forms of iso-1-cytochrome *c*. The 232-mμ values for both oxidized and reduced iso-2 were greater than those for iso-1.

Discussion

The over-all similarity between the optical rotatory dispersion of the yeast cytochromes in the 270–680-mμ region and the optical rotatory dispersion of other cytochromes supports the conclusion that the heme environment in all these species is substantially the same. The small variations observed may indicate small differences in interactions in the neighborhood of the heme, but the iron–ligand bonds must be identical in all three types, since replacement of one ligand by, for instance, cyanide changes the optical rotatory dispersion considerably in the neighborhood of the optical absorption bands (Myer and Harbury, 1966).

The 695-mμ band is present in all cytochromes *c* and is a good criterion of the nativity of the protein at pH 7 (Schejter and George, 1964). Eaton and Hochstrasser (1967) have recently shown that in crystalline ferricytochrome *c* this optical absorption band is polarized in a direction perpendicular to the heme plane whereas other bands are polarized in the heme plane. They suggest that the 695-mμ absorption involves a porphyrin–iron (π–d_{z^2}) transition which is extremely sensitive to the placement of the fifth (or sixth) ligand. Greenwood and Palmer (1965) have given evidence for a second form of ferricytochrome *c* in alkaline solution which is reduced more slowly than the species existing at pH 7. The decrease in the absorption at 280 mμ and the change in the optical

rotatory dispersion in the 270–290-mμ region suggest that the single tryptophan residue at position 59, and perhaps some of the tyrosyl residues, are involved also. There are three invariant tyrosines in the amino acid sequence of cytochrome *c*, in positions 48, 67, and 74 (Margoliash and Schejter, 1966). Since the invariant amino acid sequence from positions 70 to 80 has been implicated as being positioned near the heme, the tyrosine residue at position 74 is the most likely contributor to the optical rotatory dispersion effect at 287 mμ. There seems to be no contribution from the four phenylalanine residues present in iso-1-cytochrome *c* which are absent in horse heart cytochrome *c*. The pH range over which the transition occurs is probably dependent on the amino acid sequence of the particular cytochrome *c*, since the immediate environment of the heme seems to be identical in all the cytochromes *c*, and yet the p*K* of the transition varies between horse heart and yeast cytochromes. The effect of neighboring groups in altering the p*K*'s of charged groups is well known.

The differences between apparent helical content of the isocytochromes and other cytochromes cannot be measured quantitatively since the use of polyglutamic acid as a standard for measurement of absolute values of helical content, using the trough at 232 mμ, is unreliable (Yang and McCabe, 1965). The fact that no differences could be detected between the values of the 232-mμ trough in iso-1-ferri- and -ferrocytochrome *c* is in accord with the results from side-group ionization ratio experiments which show differences in structural rigidity between mammalian ferri- and ferrocytochromes, but not for the yeast iso-1-cytochrome (Margoliash and Schejter, 1966). The difference between the oxidized and reduced values of the 232-mμ trough in the iso-2-cytochrome *c* suggest that in this respect it is similar to horse heart cytochrome *c*.

The differences in magnitude of the Cotton effects in the 300–680-mμ region between the different cytochromes are not unexpected since the intensity of the heme–protein interaction will vary with the detailed amino acid sequence. However, the optical rotatory dispersion and absorption spectra show that the heme environment in all these proteins is very similar. The absence of the histidine residue in position 26 in yeast iso-2-cytochrome *c* seems not to affect the optical rotatory dispersion spectra at all. This suggests that histidine 26 does not normally participate in heme–ligand interaction.

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