Investigations of Yeast L-Lactate Dehydrogenase
(Cytochrome $b_2$)

V. CIRCULAR DICHROISM OF THE FLAVIN MONONUCLEOTIDE-FREE APOENZYME*

(Received for publication, December 27, 1968)

TIAN YOW TSONG AND JULIAN M. STURTEVANT‡
From the Department of Chemistry, Yale University, New Haven, Connecticut 06520

SUMMARY

An apoenzyme, free of flavin mononucleotide, can be prepared by several different methods from cytochrome $b_2$, L(+)lactate-cytochrome $c$ oxidoreductase (EC 1.1.2.3) of bakers' yeast. This material shows a complex circular dichroism (CD) spectrum which is strongly dependent on the oxidation state of the heme group. The numerous CD extrema in the region of absorption by the heme group result from interactions of the normally optically inactive protoheme with the protein environment. The CD spectrum below 250 mp, which is of the type usually ascribed to the a-helical structure, is completely independent of the oxidation state of the heme group.

Freshly prepared apoenzyme, on treatment with excess FMN and L-lactate, recovers about 80% of the original enzymic activity and has a CD spectrum very nearly identical with that of the original enzyme. FMN has no effect on the CD extrema below 250 mp.

Aging of the apoenzyme leads to denatured material with impaired ability to yield active enzyme on treatment with FMN and L-lactate. Even completely inactive material has an essentially unchanged CD spectrum above 250 mp but a significantly altered one below this wave length. It has been found that both inactivated enzyme and inactivated apoenzyme can be partially reactivated by treatment with $p$-chloromercuribenzenesulfonate followed by mercaptoethanol. If partially reactivated apoenzyme is treated with FMN and L-lactate, the resultant material has a CD spectrum above 250 mp which approaches fairly closely to that of the native enzyme, while the spectrum below 250 mp remains unaffected by the reactivation.

The various observations reported in this and in previous papers suggest that a direct heme-FMN interaction is responsible for the catalytic and optical properties of cytochrome $b_2$.

* This investigation was supported in part by Research Grant GM-04725 from the National Institutes of Health, United States Public Health Service. The preceding paper in this series is Reference 1.

‡ To whom inquiries should be addressed.

Cytochrome $b_2$, the L(+)lactate-cytochrome $c$ oxidoreductase (EC 1.1.2.3) of aerobic bakers' yeast, was first isolated by Bach, Dixon, and Zerfas (2), and was purified and crystallized by Appleby and Morton (3). The enzyme crystallized by the method of Appleby and Morton is a deoxyribonucleoprotein containing protoheme and flavin mononucleotide groups in equimolar amounts, and about 15 DNA residues per heme group (4). Studies by Morton (5) of various enzyme modifications indicated that both the protoheme and the FMN groups are essential to the dehydrogenase activity, whereas removal of the nucleic acid component has no significant effect on the catalytic properties of the enzyme. Among the nonprotein groups in the enzyme molecule, the FMN moiety is particularly easily removable, either by ammonium sulfate precipitation at pH 3.2 (5), or by the $p$-chloromercuribenzenesulfonate method as reported by Pajot, Pell, and Sturtevant (6). The enzyme freed of FMN (referred to hereafter as apoenzyme) shows no enzymic activity. On addition of FMN, however, the original enzymic activity can be nearly completely restored (6, 7). Extensive studies have been carried out in several laboratories on the function of the FMN group in the catalytic processes of cytochrome $b_2$ (8, 9), on the fluorescence of the apoprotein and the FMN (1, 67), and on the spectral effects of the binding of FMN to the apoprotein (1, 6, 7).

The CMS* titration of the enzyme carried out by Pajot et al. (6) led to the conclusion that the --SH groups which react with the mercurial, five in the oxidized form of the enzyme and six in the reduced form, are not directly involved in the catalytic mechanism or in binding the FMN group, and that the introduction of mercurial groups appears to cause a conformational change which stabilizes the enzyme, decreases the strength of FMN binding, and interferes with the heme-FMN interaction which appears to be necessary for catalytic activity. In a subsequent report on optical rotary dispersion studies of cytochrome $b_2$ (1), it was shown that the Cotton effect in the region of the Soret


2 The abbreviations used are: CMS, $p$-chloromercuribenzenesulfonate; ORD, optical rotatory dispersion; CD, circular dichroism.
Fig. 1. The CD spectra at 27° of the oxidized (-----) and H2-Pt reduced (-----) forms of the apoenzyme derived from Type II cytochrome b2 by removal of the FMN group, and of the reconstituted enzyme (- - -) obtained by treatment of the apoenzyme with excess FMN and L-lactate. Apoenzyme concentration, 10.2 μM in 0.05 M phosphate buffer, pH 7.0, 1-cm light path. In the reconstitution experiment the L-lactate and FMN concentrations were 5 mM and 22.5 μM, respectively; reconstituted activity, 160 sec⁻¹; original activity, 200 sec⁻¹.

EXPERIMENTAL PROCEDURE

Crystalline cytochrome b2 was prepared by the method described by Morton and Shepley (4). The yeast (Saccharomyces cerevisiae) used in this preparation was supplied by Standard Brands Incorporated. The enzyme so prepared is of Type I, which contains DNA and shows in the reduced state an absorption maximum at 265 μm. The ratio of the absorptivity in this band to that in the heme γ-band is 0.82 to 0.80. Passage of Type I enzyme through a DEAE-cellulose column removes the nucleotide component, the resulting product being known as Type II enzyme. The absorption maximum of the band in the ultraviolet shifts to about 277 μm, with an absorptivity approximately 0.44 times that of the γ-band. The apoenzyme used throughout this work was prepared either by ammonium sulfate precipitation at acidic pH (5) or by the CMS method (6), in either case starting with Type II enzyme. Enzyme concentration and activity were estimated as described elsewhere (8).

CD spectra were obtained with a 6001 CD accessory for the Cary 60 recording spectropolarimeter. Details of calibration and operation of this apparatus will be given elsewhere.3 All reagents and miscellaneous apparatus were as described previously (1).

Whenever hydrogen reduction or strictly anaerobic conditions were required, a vacuum system which can be pumped to 10⁻⁴ mm was used. The CD spectra in such cases were taken in a long-necked silica cell with necessary modifications in both the cell holder and the cell itself.

RESULTS AND DISCUSSION

CD Spectra of Apenzyme—The CD spectra of oxidized (dotted line) and reduced (solid line) apoenzyme are given in Fig. 1. Ellipticities (11) in deg cm² per decimole are plotted against wave length. The corresponding absorption spectra are given in Fig. 2. (The absorptivities given in Fig. 2 may be in error by a few percent (12) since we have not obtained a quantitative measure by reference to iron determinations. The absorptivities at the γ maxima were taken to be equal to those of the native enzyme less the contribution of FMN or FMNH₂ at those wave lengths.) The apoenzyme prepared in the presence of air is obtained directly in the oxidized form. The reduced form was obtained by reduction with H₂ and platinum black as catalyst. In the presence of slight residual enzymic activity, as in the present case, the apoenzyme is also readily reduced by L-lactate; no differences

were detected between samples of the apoenzyme reduced by either of these methods.

The only prosthetic group in the apoenzyme, the protoheme group, is optically inactive when uncombined, so that the numerous CD bands exhibited by the apoenzyme at wave lengths longer than 300 mp are the result of protein-heme interactions.

A comparison of the CD and absorption spectra shows that a large number of transitions must be involved. In the case of the oxidized apoenzyme, neither of the apparent extrema in the 500 to 600 mp regions corresponds closely in wave length with the absorption maxima of the α- and β-heme bands. The CD extremum at 440 mp appears to correspond to a small shoulder on the γ-absorption band, and the prominent CD maximum at 413 mp is at very nearly the same wave length as the γ absorption maximum. However the remaining features of the CD spectrum down to 250 mp do not stand in any obvious correlation with the absorption spectrum, except for the double minimum centered at 277 mp which coincides with the single broad absorption peak at this wave length.

It is evident that changing the heme iron from ferri to ferrous has a pronounced influence on the CD spectrum. In the long wave length region, extrema are observed at 590, 560, 550, and 522 mp, whereas only two absorption maxima, at 557 and 528 mp, are observed. A strong maximum and minimum are centered close to the wave length of maximum absorption of the extremely intense Soret band, indicating again that two or more transitions contribute to the Soret band. The features of the CD spectrum in this region are extrema of enhanced molar ellipticities shifted to longer wave lengths as compared with the bands observed with the oxidized apoenzyme.

With all of the profusion of differences between the CD spectra of the apoenzyme in reduced and oxidized states, it is perhaps surprising that the two ultraviolet dichroic bands at 281 and 270 mp show relatively little dependence on the oxidation state of the heme group. The assignment of these two bands is at present difficult since a variety of chromophores may contribute in this region of wave length. Urry's observation of the CD spectrum of a heme undecapeptide (13) has shown that dichroic bands in this region may come from a contribution of heme chromophores. Two similar bands have been reported for β-lactoglobulin by Townend, Kumosinski, and Tinasheff (14) and tentatively assigned to tyrosyl residues in the protein molecule. Similar bands have also been observed in ribonuclease (15) and cytochrome c (16). The suggestion has been made (17) that optical activity in this region may be related to the disulfide chromophore of cysteine residues, and a recent report by Coleman and Blount (18) shows that a dichroic band at 262 mp is indeed observed in some derivatives of l-cysteine. In the present case, the fact that the CD spectra for the oxidized and reduced states of the apoenzyme differ widely except in this region is good indication that these ultraviolet bands are not the result of asymmetric transitions of the heme group. Observations described below make it seem unlikely that the ultraviolet bands are associated with transitions in the disulfide groups of cysteine residues (there are 18 half-cystines per heme unit). It thus seems reasonable to assign these bands to transitions in tyrosyl or tryptophanyl residues (of which there are, respectively, 14 and 3 residues per heme unit).

The CD spectrum of the apoenzyme in the absorption region of the protein backbone is given in Fig. 3 (solid line). Mean residue ellipticities in deg cm² per decimole of amino acid residue are plotted against the wave length. The CD spectrum in this region is practically independent of the oxidation state of the heme group, and indeed is essentially unaltered from that of intact cytochrome b₂. It has the form frequently associated with the α-helical arrangement of peptide units.

Binding of FMN to Apeoenzyme—The dashed line in Fig. 1 shows the CD spectrum of freshly prepared apoenzyme when it is treated with excess FMN in the presence of L(+)-lactate. The spectrum shows all the details of the CD spectrum of intact, reduced Type II enzyme, except that the ellipticities of the heme transitions are about 20% smaller. In the experiment which supplied the data for Fig. 1 about 80% of the original enzymic activity was recovered. The large changes in the CD spectrum resulting from the addition of FMN to the apoenzyme are not unexpected since such a phenomenon has already been observed in our ORD studies (1) on cytochrome b₂.

In all experiments involving the addition of FMN to apoenzyme an approximately 2-fold excess of FMN was used. It is thus necessary to consider the possibility of contributions from unbound FMN to certain of the CD spectra reported in this paper. In the region above 250 mp the only significant features of the CD spectrum of FMN are a negative extremum at 270 mp with a maximum molar ellipticity of $-6 \times 10^3$ deg cm² per decimole, and a positive extremum at 340 mp with a maximum molar ellipticity of about half this magnitude. These results are in substantial agreement with those published by Miles and Urry (19). Neither of these bands can give an important contribution to the CD spectra reported.

The dichroic bands in the region of the heme γ-band undergo a reversal of sign, with the extrema changing to about 440 mp and 425 mp on addition of FMN. This change is accompanied by the appearance of a broad band at 380 mp. The pronounced changes produced by FMN in the region of 230 to 300 mp are much more obvious in the CD than in the ORD spectrum (1) because of the large contribution in the latter from the Cotton effects associated with transitions involving the backbone peptide groups.

Addition of FMN to the apoenzyme in the absence of L-lactate leads to a CD spectrum which resembles that of oxidized Type II cytochrome b₂. As was reported in our earlier publication (1), the amplitude of

![Fig. 2. The absorption spectra of the oxidized (-----) and the H₃P₃ reduced (---) forms of the apoenzyme in 0.05 M phosphate buffer, pHe 7.0, 25°.](http://www.jbc.org/content/45/6/2399/F2.large.jpg)
Investigations of Yeast L-Lactate Dehydrogenase. V

Vol. 244, No. 9

The replacement of two bands at 270 and 281 mp by a pronounced 269 mp band deserves more attention. This band does not result from a transition in the bound FMNH₂ group, although, as mentioned above, we have observed such a dichroic band in FMN at 267 mp (cf. Reference 19); it is replaced in the reduced state by a dichroic band at 250 mp, whereas in the CD spectra of reconstituted enzyme the position of the 269 mp band is the same whether the enzyme is in the reduced or oxidized state, with only a slight decrease of rotational strength in the latter.²

Iwatsubo and di Francò¹ have reported that the protein fluorescence of apocytochrome b₅ and the fluorescence of FMN are quenched when the FMN group is bound, and that the intensities of the flavin and protein fluorescence increase in a parallel manner when CMS is added to cytochrome b₅. Such observations indicate that the bound FMN group interacts in some way with certain tyrosyl or tryptophanyl residues in the protein molecule. Our CD observations make it seem likely that the appearance of the 269 mp dichroic band is another result of this interaction.

Intact and Denatured Apoenzyme—Apoenzyme, even when stored at Dry Ice temperature, gradually loses its ability to bind FMN. It is not known whether this denaturation process is similar to that which leads to the inactivation of cytochrome b₅ on storage. In Fig. 4 the solid line shows the CD spectrum of a sample of reduced apoenzyme in the presence of 5 mM l(-)-lactate; the activity and the CD spectrum of this sample were 80% recovered on treatment with FMN in the presence of L-lactate. The dotted line in Fig. 4 is the spectrum of a sample of apoenzyme which was denatured by storage under hydrogen in the refrigerator for 3 days in a sealed cell, and had completely lost its ability to bind FMN with formation of active enzyme. As is evident in the figure, this denaturation had no significant effect on the CD spectrum above 250 mp. The spectra below 250 mp for these two samples are shown in Fig. 3. Inactivation of the apoenzyme causes a loss in apparent helical content which is about the same as that produced in reduced cytochrome b₅ by 2 M urea with nearly complete loss of enzymic activity.³

Urry (20) concluded on the basis of the application of the sum rule of optical activity that some of the Cotton effects observed with ferricytochrome c are due to α helix-heme interaction. That such interactions are not operative in cytochrome b₅ is clearly shown by the complete independence of the CD spectra above and below 250 mp.

Although this sample of apoenzyme gave no recovery of enzymic activity on addition of FMN and L-lactate, it was observed that FMN was bound sufficiently to produce the change in the CD spectrum shown by the heavy dotted line in Fig. 4, with partial recovery of the negative band at 269 mp. But no other detectable changes.

The spectrum shown by the dashed line in Fig. 4 was obtained with the product of the addition of FMN and L-lactate to a sample of apoenzyme which was stored at Dry Ice temperature for 3 days. This product had an enzymic activity approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approximately 40% of that of the enzyme from which it was prepared. The CD spectrum of this reconstituted enzyme approxi...
The spectra discussed in this section show that various aspects of the behavior of the apoenzyme are differently affected by aging. Thus a significant change can be observed in the CD spectrum in the region of absorption by the backbone peptide chromophores, although no change is observed above 250 nm. When partially inactivated apoenzyme is treated with FMN and L-lactate, there is no recovery of the original (cytochrome b$_2$) CD spectrum below 250 nm, although the negative CD peak at 269 nm is completely recovered and the CD spectrum above 300 nm is recovered to a lesser extent. Enzymic activity is recovered to an even lesser extent than the heme CD spectrum.

**Effect of Mercaptoethanol**—Addition of small concentrations of mercaptoethanol or dithiothreitol to a solution of cytochrome b$_2$ has been reported to stabilize the enzyme (1, 6). The effect of these reagents on the enzyme is, however, unclear. We have investigated by means of CD the effect of mercaptoethanol on low activity enzyme and denatured apoenzyme.

A sample of aged enzyme with initial activity 60 sec$^{-1}$ (about one-quarter of that of good enzyme) was used. This enzyme showed a CD spectrum intermediate between those of intact cytochrome b$_2$ and apoenzyme, resembling more closely the latter. CMS (10 eq) were added and mercaptoethanol was then added to 60 mM concentration about 60 min later. After storage for 3 days in the refrigerator and without addition of external FMN, the CD spectrum above 250 nm of this sample changed to become almost identical with that of highly active enzyme, except in the region of the heme α- and β-bands, where the dichroic bands showed little change and remained similar to those in the CD spectrum of the apoenzyme, and the activity was increased to 110 sec$^{-1}$. If the same aged enzyme was kept in 60 mM mercaptoethanol without prior treatment with CMS, such reactivation was very slight.

The same experiment was performed with fully inactivated apoenzyme. In this case 25 μM FMN was added (apoenzyme concentration 10 μM). After treatment with CMS and then mercaptoethanol in the cold for 3 days, the sample had an activity of 50 sec$^{-1}$, and its CD spectrum changed to resemble fairly closely that of intact cytochrome b$_2$. In no case did treatment with CMS and mercaptoethanol reverse the change caused by inactivation in the CD spectrum below 250 nm.

In these experiments, it is shown that although partial reactivation by mercaptoethanol is possible, in neither case was complete reactivation achieved. The mechanism of the reactivation is unknown, but it may be suggested that mercaptoethanol functions at least in part by reducing sulfhydryl groups or disulfide bonds which have been oxidized by exposure to oxygen. The fact noted above that the extent of recovery of the CD spectrum of intact enzyme by these aged samples of apoenzyme significantly exceeds the regeneration of enzyme activity shows that denaturation processes are involved which cannot be reversed by mercaptoethanol. This is further indicated by the inability of mercaptoethanol to affect the CD spectrum of inactivated apoenzyme below 250 nm.

The CD spectrum of the apoenzyme is not changed by the introduction of mercurial groups into the protein molecule or by treatment with mercaptoethanol. This fact, together with the observation that denatured and intact apoenzyme show the same CD spectra, is good support for the conclusion that the dichroic bands at 270 and 281 nm shown by the apoenzyme are not the result of transitions in disulfide groups.
Since the CD spectrum of the apoenzyme shows clearly that various heme transitions become asymmetric as a result of interactions with the protein environment, the possibility presents itself that the influence of the FMN group on the heme transition may be mediated in an indirect manner by way of alterations in the protein conformation which are the direct result of the binding of FMN, rather than by a direct interaction between the FMN and heme groups. Although it is difficult to present a fully convincing argument for either of these alternatives, we favor the latter view for a number of reasons. Although changes in the CD pattern below 250 mp are admittedly a very insensitive diagnostic of conformational changes in the protein, it is perhaps significant that binding of FMN has no detectable effect in this portion of the CD spectrum despite its large effects at higher wave lengths. Furthermore, the change in the low wave length dichroic extrema caused by aging of the apoprotein is completely resistant to reversal by treatment with CMS and mercapto-ethanol. The evidence presently available indicates that both the FMN and heme groups are involved in the catalytic mechanism of cytochrome b_2, and the correlation between CD spectrum and enzymic activity thus suggests that the former as well as the latter depend on a direct heme-flavin interaction.

Acknowledgment—We are indebted to Mr. Arthur Gregory for effective technical assistance.

REFERENCES

Investigations of Yeast l-Lactate Dehydrogenase (Cytochrome b₃) : V.
CIRCULAR DICHLROISM OF THE FLAVIN MONONUCLEOTIDE-FREE
APOENZYME
Tian Yow Tsong and Julian M. Sturtevant

J. Biol. Chem. 1969, 244:2397-2402.

Access the most updated version of this article at http://www.jbc.org/content/244/9/2397

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/244/9/2397.full.html#ref-list-1