Optical Rotatory Dispersion of Mitochondrial Reduced Diphosphopyridine Nucleotide Dehydrogenase from Cardiac Tissue*

(Received for publication, July 29, 1968)

Tsoo E. King, Peter M. Bayley, and Bruce Mackler

From the Department of Chemistry, State University of New York, Albany, New York 12203

SUMMARY

Optical rotatory dispersion spectra of oxidized and reduced FMN and FAD are presented from 600 to 210 μm and of riboflavin from 600 to 340 μm. Optical rotatory dispersion behavior of soluble DPNH dehydrogenase was studied between 600 and 185 μm with respect to the oxidation state of the enzyme. The optical rotatory dispersion spectra were analyzed and the extrinsic Cotton effects were resolved from the protein backbone rotations. Schechter-Blout's Α' parameters were computed: Α'193 = 147 and Α'225 = -278° for the oxidized enzyme, and Α'193 = 32 and Α'225 = -210° for the reduced. The centers of the resolved extrinsic Cotton effects due to nonprotein chromophores were 437 μm and 350 μm for the oxidized and 436 μm and 346 μm for the reduced enzyme. The rotational strength, amplitude, and band width of these Cotton effects were dependent upon the oxidation state of the enzyme. In addition, anomalous rotations in the region of 550 μm were also observed but were too small for accurate analysis.

These observations indicate that the interactions between the chromophores and the protein are dependent upon the oxidation state of the dehydrogenase. This dependence may be due to changes in geometrical relationships within the system and in the conformation of the protein moiety which is also determined by the oxidation state of the prosthetic groups. These changes may in turn play an intrinsic role in the physiological action of the enzyme for electron transfer in the respiratory chain.

A mechanism for electron transfer in the organized respiratory system has been suggested in terms of a change in the conformation of respiratory enzymes in general and in the geometrical relationships between the prosthetic groups and the apoproteins brought about by a shift of the oxidation state (1). The first requirement to support or discard such a mechanism would be to search for this change in as many respiratory components as possible.

This type of change may be probed by the method of optical rotatory dispersion. Unfortunately, the available methodology does not yield good, reliable results for particulate preparations; thus very few respiratory enzymes have been studied by the use of ORD technique. Recently, samples of carefully prepared DPNH dehydrogenase (2) from cardiac mitochondria have reached a stage suitable for investigation. This enzyme may serve as an example to represent the respiratory chain-linked DPNH dehydrogenase. The present paper describes our results on the oxidation state-dependent change of the ORD behavior of the dehydrogenase. A preliminary report on some facets of this communication has appeared (3).

EXPERIMENTAL PROCEDURE

Enzyme—DPNH dehydrogenase was prepared, with slight modifications, by the method described previously from a particulate DPNH oxidase (2, 4). The starting material was suspended in 0.25 M sucrose. Although a number of steps were involved in the purification, the final preparation was passed, twice, through columns of Sephadex G-75 in order to ascertain that all sucrose was removed. The absence of sucrose was further ensured by dialysis of the sample against 50 mM phosphate buffer, pH 7.8. No change was observed in optical rotatory dispersion of the sample taken before and after dialysis. For ORD measurements, the preparations after Sephadex column treatment were used. The reduction of the enzyme (as well as free flavins) was accomplished by addition of a slight excess of sodium dithionite, and experiments were conducted under helium in specially constructed Thunberg cells. With the exception of the study of concentration effect on ORD behavior, the enzyme was prepared and the experiments were conducted as described previously (2, 4).

1 The abbreviation used is: ORD, optical rotatory dispersion.

* This work was done at Oregon State University, Corvallis, Oregon, and supported by grants from the National Science Foundation, The United States Public Health Service, The American Heart Association, and the Life Insurance Medical Research Fund.

† Permanent address, Department of Pediatrics, University of Washington School of Medicine, Seattle, Washington 98105.

§ National Institutes of Health research career development awardee. Permanent address, Department of Pediatrics, University of Washington School of Medicine, Seattle, Washington 98105.
concentration was generally adjusted to about 2 mg per ml for the region of 600 to 270 mp, 0.75 mg per ml of 370 to 200 mp, and 0.25 mg per ml below 200 mp. The methods for the determination of protein, flavin, and enzymic activity were described previously (2, 4).

Methods—ORD experiments were conducted in a Cary model 60 spectropolarimeter at 23 ± 0.5°, the temperature of the sample. Fused silica cells (optical cells) of 0.1-, 1.0-, and 5-cm light path were used. The slit width of the instrument was programmed for a bandwidth of 15 Å in order to give a constant light intensity over the wave length range from 185 mp to 600 mp. The polarimeter was calibrated with sucrose and potassium dichromate (5). During experiments, the polarimeter was continuously purged with nitrogen at about 14 cubic feet per hour. The solution was in general agreement with those obtained in other laboratories with one exception. We observed with FAD in the region of 240 mp a small but relatively well formed Cotton effect which is much less discernible in the previously reported curves. The difference might have arisen from the fact that we usually scanned in this region with a very slow speed at a time period of 10 so that the signal to noise ratio was more favorable.

Because of an extremely low solubility, reduced riboflavin was measured only down to about 340 mp. In general, upon reduction, the ORD profiles of these flavins completely changed. The most dramatic were the anomalous rotations below 400 mp. With riboflavin, the Cotton effects in the usual 440 mp region were abolished. The complicated transitions for all of these flavins in both the oxidized and reduced forms preclude any precise assignment of the optical origin for each chromophore at present. Despite the long history of study, even electronic spectra have not been satisfactorily analyzed.

The samples of DPNH dehydrogenase used in this study contained 1 mole of FMN and 2 atoms of non-heme iron per mole of protein of molecular weight of about 82,000. Less than 5% of impurities was revealed by sedimentation experiments. As shown previously (2, 4), no polymerization or aggregation was observed from the ultracentrifuge study.

The ORD behavior of four samples of DPNH dehydrogenase with comparable activity and purity was studied between 185 and 600 mp in the oxidized and reduced forms. The results were essentially the same for all samples. ORD spectra were independent of the concentrations tested between 0.2 and 4 mg of protein per ml. The absorption spectra of DPNH dehydrogenase (as shown in Fig. 2) remained the same after the ORD measurements. Likewise, no decrease of enzymic activities was observed after ORD experiments even for as long as 3 hours at 23°.

Fig. 3 is a representative example of the ORD spectra for DPNH dehydrogenase. Two regions of anomalous rotations were observed corresponding to transitions of nonprotein chromophores. The enzyme showed a positive Cotton effect centered at about 435 mp in the oxidized form. The amplitude in the region of 435 mp increased significantly upon reduction but the position of the apparent inflection point remained essentially the same as the oxidized form. In contrast, the reduced enzyme showed much lower light absorbance in this region (cf. Fig. 2).

Materials—All other chemicals in best purity available were purchased from commercial sources. The phosphate buffer used throughout this study was prepared from KH₂PO₄ and Na₂HPO₄; the molarity was referred to phosphorus.

RESULTS

Since on prosthetic group of DPNH dehydrogenase is a flavin, we also studied the ORD behavior of oxidized and reduced riboflavin, FMN, and FAD. The ORD profiles are shown in Fig. 1; some main peak and trough values together with molar amplitudes are summarized in Table I. ORD studies of these flavins have been reported on the oxidized form but not on the reduced form (12-14). Our results for the oxidized flavins are in general agreement with those obtained in other laboratories with one exception. We observed with FAD in the region of 240 mp a small but relatively well formed Cotton effect which is much less discernible in the previously reported curves. The difference might have arisen from the fact that we usually scanned in this region with a very slow speed at a time period of 10 so that the signal to noise ratio was more favorable.

Because of an extremely low solubility, reduced riboflavin was measured only down to about 340 mp. In general, upon reduction, the ORD profiles of these flavins completely changed. The most dramatic were the anomalous rotations below 400 mp. With riboflavin, the Cotton effects in the usual 440 mp region were abolished. The complicated transitions for all of these flavins in both the oxidized and reduced forms preclude any precise assignment of the optical origin for each chromophore at present. Despite the long history of study, even electronic spectra have not been satisfactorily analyzed.

The samples of DPNH dehydrogenase used in this study contained 1 mole of FMN and 2 atoms of non-heme iron per mole of protein of molecular weight of about 82,000. Less than 5% of impurities was revealed by sedimentation experiments. As shown previously (2, 4), no polymerization or aggregation was observed from the ultracentrifuge study.

The ORD behavior of four samples of DPNH dehydrogenase with comparable activity and purity was studied between 185 and 600 mp in the oxidized and reduced forms. The results were essentially the same for all samples. ORD spectra were independent of the concentrations tested between 0.2 and 4 mg of protein per ml. The absorption spectra of DPNH dehydrogenase (as shown in Fig. 2) remained the same after the ORD measurements. Likewise, no decrease of enzymic activities was observed after ORD experiments even for as long as 3 hours at 23°.

Fig. 3 is a representative example of the ORD spectra for DPNH dehydrogenase. Two regions of anomalous rotations were observed corresponding to transitions of nonprotein chromophores. The enzyme showed a positive Cotton effect centered at about 435 mp in the oxidized form. The amplitude in the region of 435 mp increased significantly upon reduction but the position of the apparent inflection point remained essentially the same as the oxidized form. In contrast, the reduced enzyme showed much lower light absorbance in this region (cf. Fig. 2).

Simpson and Vallee (13) and Listowsky et al. (14) have reported their results of FAD, which differ from the ORD spectra of Gascoigne and Radla (12). The difference is actually due to a mislabeling of the wave length in the paper originally published by Gascoigne and Radla (12).
FIG. 1. ORD of oxidized and reduced flavins. A, riboflavin; B, FMN; C, FAD. The ordinate is the reduced molar rotation, [M']. With the exception of B, the ordinate on the right is for 300 to 600 mp and on the left for 210 to 300 mp. The concentrations of the flavin were determined from absorption spectra by means of the following millimolar extinction coefficients (A_x - A_y):

<table>
<thead>
<tr>
<th>Flavin</th>
<th>Oxidized [M']</th>
<th>Reduced [M']</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>4.7</td>
<td>2.8</td>
</tr>
<tr>
<td>FMN</td>
<td>4.0</td>
<td>2.3</td>
</tr>
<tr>
<td>FAD</td>
<td>4.6</td>
<td>2.4</td>
</tr>
</tbody>
</table>

The behavior of the Cotton effects certainly does not parallel that of the absorption spectra. This disparity may serve as a good example for those rotations which are dominated by magnetic moments. It is known (e.g. Reference 15) that optical rotation is a function of the scalar product of electric and magnetic moments of the transition. Although the electric moments are quite different between oxidized and reduced DPNH dehydrogenase as reflected in the difference of the absorption spectra, the magnetic moments and the orientation (cos θ) may compensate for the difference of electric moments owing to the change of the oxidation state of the enzyme.

Another anomalous rotation centered around 350 mp was also observed in both oxidized and reduced forms of the enzyme. The resolved Cotton effect in this region (see Table II and “Discussion”) showed much more negative rotational strength and larger amplitude in the reduced than in the oxidized enzyme.
Fig. 2. Absolute absorption spectra of oxidized and reduced DPNH dehydrogenase. The enzyme was in 10 mM Sörensen phosphate buffer, pH 7.8, at 1.3 mg of protein per ml and 12.2 mmoles of FMN per mg of protein. The spectra are corrected for the base line. A, visible; B, ultraviolet. The enzymic activities were 96 for ferriyanide and 7.3 for cytochrome c in terms of micromoles of DPNH oxidized per min per mg of enzyme.

The transitions at approximately 440 and 350 μm (corresponding to the absorption maxima in these regions) are evidently due to FMN in the dehydrogenase perturbed by the protein—although the less likely possibility of the interactions from non-heme iron and the apoprotein cannot be conclusively ruled out. In addition, much smaller Cotton effects in the 550 μm region were discernible. The anomalous rotations in this region are probably due to transitions of non-heme iron (perhaps in a form coordinated with labile sulfide) in the asymmetrical environment. At present, analysis of the contribution of optical activity due to iron sulfide is not possible since the anomalous rotations in the region of 550 μm in DPNH dehydrogenase are much smaller than the Cotton effects with broad band widths (see “Discussion”) due to the flavin. Further information may be obtained from circular dichroic experiments which will be pursued. Several investigators (16-18) have found anomalous rotations in the region at about 550 μm in addition to the regions at about 630, 500, and below 300 μm in non-heme iron proteins such as ferredoxins and adrenodoxins which do not contain any flavin.

DPNH dehydrogenase also showed a trough at 233 μm and a sharp maximum at 198 μm. These rotations evidently result mainly from the structural conformation of the backbone peptide.

The experimental ORD spectra were fitted with $A_i, A_{215}$, and $A_5, \lambda_i$, and $\Delta_i$ as variable parameters. With two extrinsic Cotton effects, the fitted curves of both the oxidized and reduced
Table II

ORD parameters of DPNH dehydrogenase

<table>
<thead>
<tr>
<th>Cotton effect</th>
<th>Oxidized</th>
<th>Reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Center ((\text{m}_{\mu}))</td>
<td>437</td>
<td>436</td>
</tr>
<tr>
<td>Band width ((\text{m}_{\mu}))</td>
<td>45</td>
<td>53</td>
</tr>
<tr>
<td>Rotational strength (Debye magneton)</td>
<td>0.35</td>
<td>0.60</td>
</tr>
<tr>
<td>Molar amplitude*</td>
<td>21,100</td>
<td>45,400</td>
</tr>
<tr>
<td>II. Center ((\text{m}_{\mu}))</td>
<td>350</td>
<td>346</td>
</tr>
<tr>
<td>Band width ((\text{m}_{\mu}))</td>
<td>19</td>
<td>36</td>
</tr>
<tr>
<td>Rotational strength (Debye magneton)</td>
<td>-0.16</td>
<td>-0.40</td>
</tr>
<tr>
<td>Molar amplitude*</td>
<td>27,510</td>
<td>42,520</td>
</tr>
<tr>
<td>(\alpha'_{193} ) (degrees)*</td>
<td>147</td>
<td>32</td>
</tr>
<tr>
<td>(\alpha'_{225} ) (degrees)*</td>
<td>-278</td>
<td>-210</td>
</tr>
</tbody>
</table>

* In degrees per dm, based on moles of FMN per liter.

Shechter-Blout's parameters for the backbone protein based on reduced mean residue rotation; 115 was taken as the mean residual weight.

Fig. 4. The observed and computed ORD curves of DPNH dehydrogenase. A, oxidized; B, reduced. Circles, the observed data; solid line, the computed.

Fig. 5. The components of resolved Cotton effects of DPNH dehydrogenase. A, oxidized; B, reduced. I and II, Cotton effects described in Table II; III, the resolved protein backbone rotation. Dashed line, the sum of the components.

Emzyme are relatively satisfactory as shown in Fig. 4. There are still, however, slight deviations in the regions of 500 and 380 \(\mu\)m. These deviations might arise from the fact that we have not considered a complicated but small Cotton effect around 500 \(\mu\)m. Under the circumstances, we are satisfied because we are more interested in comparison of the oxidized with the reduced forms rather than the absolute assignment of the optical origins of individual chromophores. With the use of these parameters, the resolutions of the extrinsic Cotton effects from the backbone rotation are depicted in Fig. 5. The ORD parameters for the resolved components are summarized in Table II. It may be noted that the rotational amplitudes of the Cotton effects at about 440 \(\mu\)m are more than one order of magnitude higher than that of FMN. The anomalous rotations in this region do not decrease upon reduction.

Discussion

Respiratory enzymology of soluble DPNH dehydrogenase is currently in a confused state. At least seven or eight different DPNH dehydrogenases (19, 20) have been isolated from cardiac mitochondria, but most probably all of them are derived from the same segment of the respiratory chain (19). This segment may yield soluble DPNH dehydrogenases with characteristics dependent upon the agents and methods used in solubilization (19). The enzyme used in the present ORD study was selected.
from several considerations to represent the respiratory linked DPNH dehydrogenase. It has been isolated in a practically pure form (2, 4, 21) and is extremely stable and optically clear. The absorption spectra and enzymic activity do not change even after prolonged exposure to the light in ORD experiments. We also studied the ORD behavior of the purified DPNH dehydrogenase solubilized by snake venom at 37° (22-24) and at 30° (25, 20) (the so-called 37° enzyme and 30° enzyme (19), respectively). Under the same conditions as described in this paper, the 30° enzyme not only became turbid, but also lost enzymic activity, and the 37° enzyme lost considerable enzymic activity in the course of the experiment; thus no systematic study was further performed.

Basically, the ORD curves of the DPNH dehydrogenase studied are composed of anomalous rotations superimposed on a plain background. Both in the oxidized and reduced forms, these anomalous regions have the form of multiple Cotton effects with broad band widths. The apparent inflection points do not coincide with the absorption maxima. This discrepancy may well be attributed to the poor definition of individual transitions within broad bands and the overlapping of multiple Cotton effects on the backbone rotation.

The enzyme contains FMN which is optically active and non-heme iron (probably coordinated with labile sulfide) which may become "optically active" in the asymmetrical environment and thus give anomalous rotations. It is desirable, therefore, to separate the extrinsic Cotton effects as much as possible from rotations due to the structural conformation. The method of analysis, which we have used for some hemoproteins with considerable success (10), has been applied. To our knowledge, no report is available on the resolution of extrinsic Cotton effects from the backbone rotations for any flavoenzyme or metallo-flavoenzyme; only a few papers on the general ORD behavior of some flavoproteins have appeared (16, 27-20). The ORD pattern of DPNH dehydrogenase follows closely that of D-amino oxidases (16, 27-29). It differs significantly from xanthine oxidase (16). Upon dithionite reduction, the anomalous rotations of xanthine oxidase (16). Upon dithionite reduction, the anomalous rotations of xanthine oxidase between 300 and 600 μm decreased dramatically. However, all of these flavoproteins reported in the literature (16, 27-29) also show wide band widths of the extrinsic Cotton effects.

From Table II, it can be seen that the rotational strengths of both extrinsic Cotton effects are much larger in the reduced than in the oxidized form. Although similar analysis has not been rigorously made for free FMN because of technical difficulty, visual inspection of the curves (Fig. 1B) does not indicate that the rotational strength for the Cotton effect in the 440 μm region should be larger in the reduced form than the oxidized form. Moreover, the molar amplitude of rotation is at least one order higher when FMN is asymmetrically perturbed in the enzyme than the free FMN. The enhancement of the amplitudes is evidently conformation-dependent and the observed variation in rotational strength would then reflect changes in the electronic relation between the prosthetic groups and the apoprotein. The conformation of the apoprotein is obviously determined also by the oxidation state of the enzyme. These differences may be taken at least as prima facie evidence for the difference of the asymmetrical environment between the oxidized and reduced form. This argument is further substantiated by the significant difference of Shechter-Blout's A' parameters (cf. Table II).

It must be emphasized that the resolved protein spectra do not represent the actual apoenzyme spectra even if the apoenzyme were available for examination. These spectra depict the protein moiety in the enzyme perturbed by the oxidized and reduced prosthetic groups, respectively, as shown in Curve III of Fig. 4, A and B. In other words, the oxidation state of the prosthetic groups determines the ORD behavior of the protein moiety of the dehydrogenase which is evidently the manifestation of the protein conformation as mentioned. In a way, the prosthetic group-dictated conformation of the enzyme may be visualized as a form of allosterism.

It may be also mentioned that, in interpreting changes in the 233 μm and 195 μm regions of ORD spectra, all contributions from side chains, extrinsic chromophores, etc., must be eliminated before the rotations due to protein backbone may be assigned. This task is almost impossible to achieve for complex respiratory enzymes, such as DPNH dehydrogenase reported here. Thus, it is perhaps not surprising that the effective helicity computed from A' parameters (after the resolution of extrinsic Cotton effects) does not conform to the negative rotations at 233 μm. The reduced enzyme shows a deeper trough. The contributions to the rotation at 233 μm by the transitions at 350 μm and at higher wavelengths may be negligible, but it is not possible to discount contributions from lower transitions. In addition to FMN, the non-heme iron and labile sulfide perturbed by the protein may exhibit anomalous rotations. Moreover, even free FMN shows (cf. Fig. 1 and Table I) transitions below 250 μm and the rotations are also dependent upon the oxidation state. Therefore, the use of the 233 μm trough in estimation of helical content should be done with caution.

In summary, these observations reflect that both the absorption spectra and the interactions of the chromophores with the protein are dependent upon the oxidation state of the enzyme. This dependence may well be due to changes in the geometrical relationships within the system and in the enzyme conformation which in turn plays an intrinsic role in the biological action of DPNH dehydrogenase for electron transfer in the respiratory chain.

Acknowledgment—We acknowledge the advice given generously by Dr. J. A. Schellman.

REFERENCES


5. Samejima, T., and Yang, J. T., Biochemistry, 3, 613 (1964).


Optical Rotatory Dispersion of Mitochondrial Reduced Diphosphopyridine Nucleotide Dehydrogenase from Cardiac Tissue
Tsoo E. King, Peter M. Bayley and Bruce Mackler


Access the most updated version of this article at http://www.jbc.org/content/244/7/1890

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/244/7/1890.full.html#ref-list-1