Coenzyme-induced Changes in the Optical Rotatory Dispersion Properties of Glyceraldehyde 3-Phosphate Dehydrogenase*

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The high binding affinity of diphosphopyridine nucleotide for glyceraldehyde 3-phosphate dehydrogenase was first estimated quantitatively by Velick, Hayes, and Harting (1) by using an ultracentrifugation separation method. A dissociation constant of the order of 10^-7 M (2) with a stoichiometry of 3 to 4 moles of DPN+ per mole of enzyme was determined (3). Several characteristics of the apoenzyme differ from those of the holoenzyme. The apoenzyme exhibits a greater thermal lability than the native enzyme (4), has resisted crystallization (5) (except in an acylated form (3)), and is more susceptible to proteolysis (6). Also, Boyer and Schulz (7) have demonstrated an increase in negative specific rotation after removal of DPN+. Optical rotatory dispersion techniques have been used extensively for the determination of protein structure (8, 9). Recently, similar techniques have been used to study cofactor-protein interactions (10–12). Thus, for liver alcohol dehydrogenase, Ulmer, Li, and Vallee (13) described an extrinsic Cotton effect at 327 mÅ that was dependent upon binding of reduced pyridine nucleotide. Modifications of optical rotatory properties in the presence of specific inhibitors (14, 15), metal chelators (16), and coenzyme analogues (17) also have been investigated.

The indications already cited that coenzyme-dependent changes occur in the properties of glyceraldehyde 3-phosphate dehydrogenase suggested that changes in optical rotatory dispersion properties may also occur. In this study we report that, unlike the case with liver alcohol dehydrogenase, no extrinsic Cotton effect in the 330 mÅ region was produced by DPNH binding to glyceraldehyde 3-phosphate dehydrogenase. However, other effects related to coenzyme binding were observed, including large changes in parameters of rotatory dispersion and variance in magnitude of an inflection point in the 280 mÅ spectral region.

Materials and Methods

Reagents—DL-Glyceraldehyde 3-phosphate was obtained as the barium salt of the diethylacetal derivative from Sigma and was converted to the free aldehyde according to a previously published method (18). DL-Glyceraldehyde was purchased from Calbiochem. DPN+ (Sigma) and 3-acetylpyridine adenine dinucleotide (Pabst Laboratories) were determined spectrophotometrically as their cyanide complexes (19), and DPNH was determined spectrophotometrically by its absorbance at 340 mÅ with an extinction coefficient of 6.22 x 10^3 M^-1 cm^-1 (20).

Enzyme—Glyceraldehyde 3-phosphate dehydrogenase was prepared from rabbit skeletal muscle, according to the method of Cori, Steim, and Cori (21), with 1 mM EDTA present in all solvents. Aporoemzyme was prepared by treatment of the holoenzyme with charcoal (1) and an A_{280}:A_{340} ratio of 1.94 was used to determine complete removal of nucleotide (22). The extinction coefficients reported by Fox and Dundiger (22) were used to determine protein concentrations. Unless otherwise indicated, solutions were made to contain 1 x 10^-4 M enzyme based on an assumed molecular weight of 140,000 (23). Native and apoenzyme, tested in equal concentrations, gave identical rates of pyridine nucleotide reduction, indicating that the charcoal treatment did not cause any inactivation of the enzyme.

Activity Determinations—Both glyceraldehyde and 3-acetylpyridine adenine dinucleotide have been shown to be substrates of glyceraldehyde 3-phosphate dehydrogenase (24, 25). The enzymatically catalyzed oxidation of glyceraldehyde with 3-acetylpyridine adenine dinucleotide as electron acceptor, was sufficiently slow so that rates could be measured easily with micromolar enzyme concentrations. Assay solutions contained 0.10 mM imidazole chloride, pH 8.0, 1.45 x 10^-3 M 3-acetylpyridine adenine dinucleotide, 0.01 M DL-glyceraldehyde, 0.01 M sodium arsenate, and 0.3 to 1.0 x 10^-5 M enzyme, in a final volume of 1.0 ml. When inactivation was expected, as for instance in the p-hydroxymercurebenzoate titration experiments, glyceraldehyde 3-phosphate was used as the substrate to increase the sensitivity of the assays. These solutions contained 0.10 mM amidazole, pH 7.0, 7.2 x 10^-4 M 3-acetylpyridine adenine dinucleotide, 4 x 10^-4 M DL-glyceraldehyde 3-phosphate, 0.01 M sodium arsenate, and 0.3 to 1.0 x 10^-5 M enzyme. After initiation of the reaction by addition of enzyme, the absorbance change at 363 mÅ (19) was recorded with a Gilford model 2000 multiple sample absorbance recorder equipped with a temperature-controlled (24°) cuvette chamber. Values of 0.0535 and 1.21 μmoles of reduced 3-acetylpyridine adenine dinucleotide produced per min per mg of enzyme were observed with the assays containing glyceraldehyde and glyceraldehyde 3-phosphate, respectively. Under different conditions of assay, Velick reported a specific activity (expressed in terms of a second order rate constant) of 8.4 x 10^5 (26). Under these assay conditions...
Optical Rotatory Dispersion Measurements—The optical rotatory dispersion measurements were performed on the Cary model 60 recording spectropolarimeter. The enzyme solutions were placed in a jacketed cell which had fused quartz end plates and a light path of 1 cm. The optical system of the instrument and cell compartment were continuously flushed with prepurified nitrogen. Before measurements were begun, the protein solutions were allowed to equilibrate at 10° for at least 20 min. Enzymatic rates permitted calculation of $b_0$ from the slope and $a_0$ values from the intercept. The $\lambda_\text{e}$ values were calculated from plots of $[\alpha]_\lambda^2$ versus $[\alpha]$ as suggested by Yang and Doty (31). Results obtained with the Shechter and Blout (32) modified two-term Drude equation were essentially identical and, therefore, are not reported. Rotations in the spectral region between 500 and 300 m\textmu are used for the calculation of the foregoing parameters.

RESULTS

Holoenzyme and Apoenzyme—The optical rotatory dispersion curve obtained with rabbit skeletal muscle glyceraldehyde 3-phosphate dehydrogenase is shown in Fig. 1. The holoenzyme has an $A_{260}:A_{280}$ ratio of 1.06, which agrees with the value reported by Fox and Dandiker (22). The optical rotatory dispersion curve is distinguished by a characteristic shoulder or inflection point at 295 to 270 m\textmu. Rotations through and below the trough at 232 m\textmu due to the Cotton effect are shown in Fig. 2. The value of the mean residue rotation, $[M']_{232} = 3900$, agrees with the earlier data of Jirgensons (33), but a peak at 220 to 225 m\textmu was not observed. The rotations shown in Fig. 2 were obtained at $10^{-4}$ and $10^{-2}$ M concentrations of enzyme in 0.1 mM EDTA, pH 7.4, since $10^{-5}$ M protein solutions in 0.01 M imidazole were only transparent to about 235 m\textmu. Enzymatic rates
The optical rotatory dispersion curves for the apoenzyme are also shown in Figs. 1 and 2. The rotations in the spectral region of 600 to 300 mμ are more negative than those observed with the holoenzyme. In addition, the shoulder observed with the holoenzyme, in the case of the apoenzyme, occupies only about a 10-mμ inflection point between 280 and 290 mμ. However, the position of the Cotton effect trough at 232 mμ is the same as observed with the holoenzyme, and the rotations in this spectral region are identical for the two forms of the enzyme (Fig. 2).

The holoenzyme is characterized by a \(-b_o\) value of 240 (Table I), a value somewhat higher than that observed with the many other dehydrogenases (8). After treatment with charcoal to remove the coenzyme, the \(-b_o\) value decreased to about 145, and the \(-a_o\) value increased from 150 to 245. The value \(\lambda_c\) also changed after the enzyme was treated with charcoal, decreasing from 260 mμ for the holoenzyme to 247 mμ for the apoenzyme. A plot of \([\alpha]_c^2\) versus \([\alpha]\) for the native enzyme exhibited a sharp deviation from linearity commencing at 295 mμ, while the apoenzyme exhibited only slight and gradual deviation down to a wave length of 250 mμ. Similar increased deviations from linearity have been observed for polypeptides and proteins containing a large degree of helical structure (31, 34). The Moffitt-Yang plots were linear in the spectral region between 450 and 310 mμ.

The values of the constants obtained after the native and apoenzyme were treated with 6 or 8 m urea solution are shown in Table II. Both the holoenzyme and the apoenzyme were irreversibly denatured in 6 or 8 m urea. The decrease in \(-b_o\) to 70 is in the direction expected for a protein undergoing an unfolding of its ordered structure. The values of \(a_o\) and \(\lambda_c\) were also changed drastically as a result of urea treatment. It should be noted that in urea solution, the optical rotatory dispersion curves of the holoenzyme and apoenzyme were indistinguishable and the inflection point in the 290 mμ region completely disappeared. This latter finding suggests that the shoulder observed in this spectral region with the holoenzyme and to a lesser degree with the apoenzyme may be related to the spatial orientation of the aromatic amino acid residues.

\(p\)-Hydroxymercuribenzoate has been shown to remove the DPN\(^+\) which is bound tightly to glyceraldehyde 3-phosphate dehydrogenase and, at the same time, to inactivate the enzyme (2, 35). Table II summarizes the optical rotatory dispersion parameters and enzymatic activities observed with increasing concentrations of \(p\)-hydroxymercuribenzoate. After addition of

### Table I

**Optical rotatory dispersion parameters of glyceraldehyde 3-phosphate dehydrogenase**

<table>
<thead>
<tr>
<th>Preparation and conditions of measurements</th>
<th>(-b_o)</th>
<th>(-a_o)</th>
<th>(\lambda_c)</th>
<th>(-[\alpha]_a)</th>
<th>Residual activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Holoenzyme at pH 7.4, 10°</td>
<td>240</td>
<td>150</td>
<td>260</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>2. Apoenzyme at pH 7.4, 10°</td>
<td>145</td>
<td>245</td>
<td>247</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>3. Holoenzyme at pH 7.4, 30°</td>
<td>220</td>
<td>140</td>
<td>260</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>4. Holoenzyme* at pH 8.6, 10°</td>
<td>290</td>
<td>180</td>
<td>250</td>
<td>136</td>
<td></td>
</tr>
</tbody>
</table>

*Measurements made in 0.1 m Tris-chloride, 1.0 m μ EDTA, pH 8.6.

The optical rotatory dispersion curves for the apoenzyme are also shown in Figs. 1 and 2. The rotations in the spectral region of 600 to 300 mμ are more negative than those observed with the holoenzyme. In addition, the shoulder observed with the holoenzyme, in the case of the apoenzyme, occupies only about a 10-mμ inflection point between 280 and 290 mμ. However, the position of the Cotton effect trough at 232 mμ is the same as observed with the holoenzyme, and the rotations in this spectral region are identical for the two forms of the enzyme (Fig. 2).

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### Table II

**Effects of various treatments on enzymatic activity and optical rotatory dispersion parameters of glyceraldehyde 3-phosphate dehydrogenase**

<table>
<thead>
<tr>
<th>Preparations and conditions of measurements</th>
<th>(-b_o)</th>
<th>(-a_o)</th>
<th>(\lambda_c)</th>
<th>(-[\alpha]_a)</th>
<th>Residual activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Holoenzyme in 8 m urea………….</td>
<td>70</td>
<td>625</td>
<td>220</td>
<td>338</td>
<td>&lt;7</td>
</tr>
<tr>
<td>2. Apoenzyme in 6 m urea………….</td>
<td>70</td>
<td>625</td>
<td>220</td>
<td>338</td>
<td>&lt;10</td>
</tr>
<tr>
<td>3. Holoenzyme plus 4 eq of (p)-hydroxymercuribenzoate….</td>
<td>180</td>
<td>225</td>
<td>250</td>
<td>129</td>
<td>50</td>
</tr>
<tr>
<td>4. Holoenzyme plus 10 eq of (p)-hydroxymercuribenzoate….</td>
<td>140</td>
<td>325</td>
<td>232</td>
<td>174</td>
<td>0</td>
</tr>
<tr>
<td>5. Commercial preparation………….</td>
<td>225</td>
<td>150</td>
<td>254</td>
<td>118</td>
<td>49</td>
</tr>
</tbody>
</table>

Preparations and conditions of measurements:

- 1. Holoenzyme in 8 m urea
- 2. Apoenzyme in 6 m urea
- 3. Holoenzyme plus 4 eq of \(p\)-hydroxymercuribenzoate
- 4. Holoenzyme plus 10 eq of \(p\)-hydroxymercuribenzoate
- 5. Commercial preparation

*Measurements made in 0.1 m Tris-chloride, 1.0 m μ EDTA, pH 8.6.

Fig. 3. Coenzyme-dependent changes in the optical rotatory dispersion parameters of glyceraldehyde 3-phosphate dehydrogenase. O—O, \(-a_o\) values; □—□, \(-b_o\) values. The apoenzyme concentration was 1 X 10^{-5} m. A, changes after addition of DPN; Δ, values of \(-a_o\) and \(-b_o\) obtained for holoenzyme. B, changes after addition of DPNH; Δ, values of \(-a_o\) and \(-b_o\) obtained for the DPNH-holoenzyme formed by addition of 1.6 X 10^{-4} m glyceraldehyde 3-phosphate to native holoenzyme.

4 moles of \(p\)-hydroxymercuribenzoate per mole of enzyme, the activity of the enzyme was decreased to 49% of the initial activity. The optical rotatory dispersion parameters indicated removal of DPN\(^+\) from the enzyme. Velick has reported that the binding of only 1 mole of \(p\)-hydroxymercuribenzoate is sufficient to alter the DPN\(^+\)-binding ability of the holoenzyme (2). Increasing the \(p\)-hydroxymercuribenzoate concentration to 10 times the concentration of the holoenzyme caused complete loss of activity. It should be noted that this concentration of \(p\)-hydroxymercuribenzoate altered both the \(-b_o\) and \(\lambda_c\) values from those normally observed with the apoenzyme. The inflection point around 290 mμ observed with the \(p\)-hydroxymercuribenzoate-treated enzyme was even less than that observed with charcoal-treated enzyme; however, the inflection was not completely absent as after urea treatment.

**Titrations with Coenzyme**—The changes in \(-b_o\) and \(-a_o\) that occurred when apoenzyme was titrated with DPN\(^+\) are shown in Fig. 3A. Addition of only 1 mole equivalent of DPN\(^+\) was re-
Variations in specific rotation at a single wavelength for holo and apoenzyme of glyceraldehyde 3-phosphate dehydrogenase have been reported (7). However, caution should be exercised in relating these observed rotations to protein conformational changes. This is best demonstrated by results which were obtained in a study with holoenzyme in two different buffers. The [\alpha]_200 in 0.1 M Tris-chloride, pH 8.6, was -136 (Table I), which by comparison to the results at pH 7.4 in imidazole ([\alpha]_200 = -110), would indicate a conformation containing less ordered structure. However, the \(-b_0\) value of 290 at pH 8.6 (compared to a value of 240 at pH 7.4) is more compatible with the conclusion that the holoenzyme at pH 8.6 exists in a more ordered form. Such a discrepancy might arise from a difference in solvation of the amino acid side chains at pH 7.4 and 8.6, in imidazole and Tris buffers, respectively. Varying degrees of ionization of the side chains in the enzyme would also contribute to changes in specific rotation but would not necessarily influence the degree of order (37).

**DISCUSSION**

The decrease in \(-b_0\) and \(\lambda_\infty\) values and increase in \(-a_0\) value after DPN+ removal from glyceraldehyde 3-phosphate dehydrogenase is always accompanied by a diminished inflection point in the 280 nm region of the optical rotatory dispersion curve. The calculation of the Moffitt-Yang constants may be influenced by the magnitude of the 280 nm shoulder (38, 39), because the effects of such an inflection point are not considered in the derivation of their equation. Therefore, the \(-b_0\) values cannot be related to the degree of helicity exclusively and the exact nature of the rotational changes produced by the removal of DPN+ is not clear. Nonetheless, the rotatory parameters afforded a convenient quantitative index of the various effects studied. The significant changes in the Moffitt-Yang constants described in the present study may reflect either structural changes in the enzyme induced by the binding of coenzyme or result from the binding of a chromophore to an asymmetric molecule without conformational changes.

The 280 nm inflection point may be a result of either an intrinsic Cotton effect associated with the spatial orientation of the aromatic amino acids or an extrinsic Cotton effect resulting from the asymmetric alignment of the bound coenzyme. The binding of DPNH to liver alcohol dehydrogenase (13) and of ATP or ADP to creatine phosphokinase (40) generate extrinsic Cotton effects in the 327 nm and 259 nm regions, respectively. With the glyceraldehyde 3-phosphate dehydrogenase holoenzyme, however, the extended shoulder is in the 280 nm region, which is significantly removed from the 290 nm absorption maxima of the adenine moiety of the coenzyme. Fig. 5 represents the difference between the optical rotatory dispersion curves of the holoenzyme and apoenzyme. The curve is characterized by a peak at 275 nm and a trough at 295 nm with the inflection point at 280 nm and resembles the type of curves obtained with tyrosine (41) and polytyrosine (42) in this spectral region. Although the position of the Cotton effect in the difference curve is shifted from the absorption maximum of the adenine moiety of DPN+, the possibility of an extrinsic Cotton effect in this spectral region due to the asymmetric alignment of adenine cannot be excluded.

A shoulder in the 280 nm region similar to that observed with glyceraldehyde 3-phosphate dehydrogenase holoenzyme has been noted with tobacco mosaic virus protein (43) and carbonic
anhydrase (44). In these studies the shoulder was attributed to an intrinsic Cotton effect associated with the absorption of specifically oriented aromatic amino acids. In agreement with the investigations on tobacco mosaic virus protein and carbonyl anhydrase (43, 44), the 280 μm inflection point in the glyceraldehyde 3-phosphate dehydrogenase optical rotatory dispersion curve disappeared after exposure of the protein to extremes of pH or urea. Indeed, changes of this nature in the 280 μm region of the optical rotatory dispersion curves of proteins may be a more sensitive index to certain conformational changes than the 233 μm Cotton effect (45, 46) which in the present study was shown to be unaffected by coenzyme binding or changes of pH.

Although p-hydroxymercuribenzoate causes release of bound pyridine nucleotides (2), the experiments reported here do not reveal whether the changed parameters after treatment with this reagent reflect removal of coenzyme or inactivation of enzyme, or both. Examination of an enzyme preparation that is inactive and yet contains firmly bound DPN+ might reveal the effects of inactivation on the optical rotatory parameters. A commercial preparation of glyceraldehyde 3-phosphate dehydrogenase (Table II) that existed in a form having only 50% of the specific activity of the native enzyme had rotatory dispersion parameters very close to those of the prepared holoenzyme. The $A_{280} : A_{250}$ ratio indicated that the commercial preparation contained 2.3 moles of firmly bound DPN+, and the reported $b_0$ values are consistent with this determination. It is therefore concluded that the major p-hydroxymercuribenzoate-induced rotational changes are a result of the removal of DPN+ associated with the action of this reagent and not of inactivation of the enzyme.

The stoichiometry of pyridine nucleotide binding to glyceraldehyde 3-phosphate dehydrogenase is similar to that previously reported (2, 3); addition of 3 eq of DPN+ or DPNH to the apoenzyme restores the rotational properties of the native enzyme. The fact that the major changes in the optical rotatory dispersion parameters occur after 1 eq of coenzyme has been added is not interpreted to mean that any one of the binding sites is unique or that different sites possess different affinities for the pyridine nucleotide. It merely indicates that the major asymmetric alignment occurs after addition of 1 eq of DPN+ or DPNH. In comparison, changes induced by further addition of coenzyme are small.

Although coenzyme to protein binding may be studied by a variety of methods, specific asymmetric changes must occur for the detection of binding by means of optical rotatory dispersion analysis. Thus, the extrinsic Cotton effects observed with liver alcohol dehydrogenase (13) and creatine phosphokinase (40) and the recently reported changes in $b_0$ value with glutamic dehydrogenase (47, 48) represent instances of rotational changes induced by coenzyme binding. With glyceraldehyde 3-phosphate dehydrogenase, although the addition of DPNH to apoenzyme restores the rotational characteristics of the holoenzyme, there is no extrinsic Cotton effect in the 340 μm spectral region. Indeed, experiments in this laboratory with beef heart and rabbit muscle lactic dehydrogenase revealed no significant changes in optical rotatory properties dependent upon coenzyme binding, although it is known that such binding (with low dissociation constants) does exist (49).

SUMMARY

Changes in the optical rotatory dispersion properties of rabbit skeletal muscle glyceraldehyde 3-phosphate dehydrogenase have been determined as a function of coenzyme binding. Removal of the tightly bound coenzyme results in a lower $b_0$ and $λ_0$ value and an increase in $a_0$ value. Optical rotatory dispersion curves of the native enzyme exhibit a shoulder in the 280 μm spectral region which is diminished in the apoenzyme. The presence of such an inflection in this spectral region precludes a simple correlation between changes in $b_0$ values and helical content of the holo- and apoenzyme. Although addition of 3 eq of diphosphopyridine nucleotide or reduced diphosphopyridine nucleotide to the apoenzyme completely restores the properties of the holoenzyme, the major changes occur after addition of only 1 eq of coenzyme.

Rotational changes induced by p-hydroxymercuribenzoate have been related almost entirely to removal of diphosphopyridine nucleotide rather than to inactivation of the enzyme. Presence of urea in a concentration of 6 or 8 M resulted in additional rotational changes which are related to the unfolding of the protein.

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