The Effect of Magnesium on Some Physical Properties of Yeast Enolase*

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SUMMARY
It is shown by sedimentation velocity and polarization of fluorescence measurements that yeast enolase in solution becomes more compact on addition of magnesium. This is associated with changes in absorption, fluorescence emission, and fluorescence-polarization spectra, as well as in the thermal stability of the enzyme. Evidence is presented that this structural change is produced by the binding of 1 mole of metal per mole of protein. The dissociation constant, as determined by fluorimetric titrations, is \(1.8 \pm 0.5 \times 10^{-8}\) M.

Warburg and Christian (1) observed that magnesium was required for yeast enolase (2-phospho-d-glycerate hydrolyase, EC 4.2.1.11) activity. Subsequent kinetic studies, notably by Malmstrom (2) and co-workers and by Wold and Ballou (3), showed that the metal functioned by activating the enzyme, rather than by forming an "active complex" with the substrate. It was also known that the enzyme was far more heat-stable in the presence of magnesium (1, 4). These facts suggested the possibility that the metal stabilizes a particular conformation of the enzyme.

While exploring the effects of substrate on the fluorescence of yeast enolase, we noticed that when tightly bound magnesium was removed with excess ethylenediaminetetraacetate, characteristic changes in the fluorescence of the enzymes would occur. This paper is the result of further investigations of this phenomenon. A preliminary account of these findings has been presented (5).

MATERIALS AND METHODS
Enolase was prepared from brewers' yeast by the method of Westhead and McLain (6). It gave a single symmetrical peak in the analytical ultracentrifuge and an asymmetrical peak on TEAE-cellulose chromatography. The specific activity of the enzyme used in these experiments was only 93% of that reported by Westhead and McLain, under similar assay conditions. In addition, electrophoresis on starch gel at pH 8.6 or in acrylamide gel at pH 7.7 or 8.9 showed this enzyme to have two major and one minor components, in addition to traces of four other components (6). However, key experiments were checked with electrophoretically homogeneous enzyme obtained by preparative disk electrophoresis. No significant difference in results was observed.

1-Dimethylaminonaphthalene-5-sulfonyl-enolase was prepared by adding a 5-fold molar excess of the sulfonyl chloride (in methyl Cellosolve) to a solution of yeast enolase (at 0°), which was 0.05 to 0.1 M in Tris-HCl, pH 8.5. In most preparations, 0.01 M magnesium acetate and 0.001 M 2-phosphoglyceric acid were present when the sulfonyl chloride was added. The latter conditions had no effect on the specific activity of the product. The final concentration of methyl Cellosolve was 1 to 3%. After the mixture of dye and protein was allowed to stand overnight, it was dialyzed for 18 to 24 hours against three changes of 0.01 M Tris-HCl, pH 7.5. For facilitation of removal of unbound dye, a few milliliters of a thick water suspension of Dowex 2 (Cl-) were added to the dialysis fluid. After dialysis, the labeled enzyme solution was centrifuged at 105,000 \(\times g\) for 4 hour.

1-Dimethylaminonaphthalene-5-sulfonyl chloride was prepared by allowing the sulfonic acid to react with phosphorus pentachloride according to the method of Weber (7).

1-Dimethylaminonaphthalene-5-sulfonyl chloride was prepared by allowing the sulfonic acid to react with phosphorus pentachloride according to the method of Weber (7).

2-Phosphoglyceric acid was purchased from Sigma and crystallized from aqueous acetone at least once before use.

Sephadex G-25 (coarse) was purchased from Pharmacia. Tris was obtained from Sigma and recrystallized twice from 95% ethanol before use. Other reagents were made with analytical reagent grade chemicals. Glass-distilled water was used for all solutions. Reagents were treated with Chelex 100 (obtained from Calbiochem) before pH adjustment, addition of magnesium, or storage. Solutions other than those made up daily were stored in polyethylene bottles.

Glassware and cuvettes were cleaned successively with ethanolic potassium hydroxide, sodium dichromate cleaning solution, nitric acid, and glass-distilled water before use. Polyethylene ware was washed with dilute detergent, 0.1 M sodium hydroxide, 0.1 M HCl, 0.001 M EDTA, and glass-distilled water.

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1 A generous gift of the Anheuser-Busch Company, St. Louis, Missouri.

2 The abbreviations used are: TEAE-, triethylaminoethyl (-C\(\text{H}_3\)-N\(+\)-(C\(\text{H}_5\)\text{NI})); DNS-, 1-dimethylaminonaphthalene-5-sulfonyl-.
Fluorescence emission and polarization of fluorescence measurements were made with instruments previously described (8, 9). The instrument used to measure fluorescence polarization spectra is described by Weber and Bablouzian (24). Assays of magnesium by emission spectroscopy were performed with the machine described by Malmstadt, Barnes, and Rodriguez (10) with a Zener power supply substituted for the batteries. Sedimentation velocity measurements were done on a Spinco model E ultracentrifuge at 20° with the use of schlieren optics with higher protein concentrations and interference optics for concentrations of 2 mg per ml and below. Assays of enzyme activity and determinations of difference spectra were made on a Cary model 15 recording spectrophotometer. For measuring enzyme activity at high concentrations of protein, a stopped flow apparatus, described by Gibson and Milnes (11), was used. pH measurements were made at room temperature on a Beckman 15 recording spectrophotometer. For measuring enzyme activity at high concentrations of protein, a stopped flow apparatus, described by Gibson and Milnes (11), was used. pH measurements were made at room temperature on a Beckman model 76 pH meter. The temperature of solutions during the measurements of polarization of fluorescence and of fluorescence spectra was held at 25 ± 0.1° by a Forma refrigerated constant temperature bath.

RESULTS

Increasing amounts of magnesium added to purified enzyme solution produce proportional increases in activity. This may be used as an empirical criterion to determine the amount of endogenous magnesium present in the preparation. By the use of this criterion, it is concluded that the purification procedure of Westhead and McLain yields an enzyme which contains variable amounts of magnesium. Estimates of the magnesium left in several preparations after various periods of dialysis are listed in Table I. As shown in the table, the results from the activity measurements were in good agreement with those from direct assays for magnesium by emission spectroscopy.

It is evident that some of the endogenous metal is tightly bound. For a study of the effects of removal of magnesium, molar ratios of EDTA to enzyme of 10 to 50 were required. The fluorescence emission spectra reproduced in Fig. 1 are those successively obtained from a solution of yeast enolase: (a) in the presence of endogenous magnesium, estimated from the total dialysis time to be about 3 to 6 moles per mole of enzyme (Curve 1); (b) after addition of a saturating excess of EDTA (Curve 2); (c) after addition of magnesium to 0.01 M (Curve 3); and (d) on addition of substrate as well (Curve 4).

![Figure 1](http://www.jbc.org/)  
**Fig. 1.** The effect of magnesium and substrate on the fluorescence emission spectrum of yeast enolase. The fluorescence emission of a solution containing 0.06 M Tris-HCl, pH 7.8, and 4.2 × 10⁻⁴ M yeast enolase in a total volume of 2.0 ml was scanned (Curve 1), then the effect of adding 1 µl of 1 M EDTA on the fluorescence at 335 nm was measured. Another 1 µl of 1 M EDTA had no effect; therefore, the emission spectrum was scanned again (Curve 2). Then 10 µl of 2 M MgCl₂ were mixed with the solution, and the scan was repeated (Curve 3). For the final scan (Curve 4), 10 µl of 0.2 M 2-phosphoglyceric acid were added. The excitation wavelength was 280 nm. The excitation and emission band widths were 2.5 and 3.3 nm, respectively. The spectra are uncorrected for monochromator transmission of photomultiplier spectral response. These corrections are very small in our case since the cathode surface of the photomultiplier has almost constant quantum response in the ultraviolet and the grating of the monochromator is blazed at about 300 nm.

### Table I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein concentration mg/ml</th>
<th>Total dialysis time hrs</th>
<th>Magnesium remaining molar/mole protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>10a (DNS-enolase)</td>
<td>10</td>
<td>42</td>
<td>3</td>
</tr>
<tr>
<td>11a (DNS-enolase)</td>
<td>7</td>
<td>42</td>
<td>3</td>
</tr>
<tr>
<td>12a (DNS-enolase)</td>
<td>10</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>1 (native enolase)</td>
<td>40</td>
<td>30</td>
<td>6 (6)*</td>
</tr>
<tr>
<td>2 (native enolase)</td>
<td>30</td>
<td>30</td>
<td>4 (4)*</td>
</tr>
<tr>
<td>2 (native enolase)</td>
<td>30</td>
<td>30 + 60 (against water)</td>
<td>[2.3]*</td>
</tr>
<tr>
<td>3 (native enolase)</td>
<td>30</td>
<td>48</td>
<td>4 (4)*</td>
</tr>
<tr>
<td>3 (native enolase)</td>
<td>48 + 1000-fold excess of Chelex 100</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>4 (native enolase)</td>
<td>2.4</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>5 (native enolase)</td>
<td>4.5</td>
<td>18</td>
<td>-</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are magnesium content as determined by emission spectroscopy. Known amounts of metal were added to the enzyme solution as in the kinetic measurements described above.

* Obtained by neutron activation analysis.
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Estimation of binding constants from titration curves

The dissociation constant was determined on the assumption that a magnesium-dependent equilibrium exists:

\[ E + Mg \rightarrow EMg \]  

\[ K_d = \frac{(E_{free})(Mg_{free})}{(EMg)} = \frac{(E_{free})(Mg_{added}) - EMg \rightarrow EMg}{(EMg)} \]  

At 50% of the total change:

\[ K_d = \frac{(Mg_{added}) - (E_{total})}{(E_{total})} \]

Measured "fluorescence" refers to the fluorescence of the enzyme at 313 or 316 nm (the exciting wave length is 280 nm); "absorption," the absorption at 296 nm; "DNS-conjugate fluorescence," the fluorescence yield of the label, as excited by the isolated 366 nm line of a mercury arc and measured on a null point galvanometer (9); "polarization of fluorescence" refers to the angle \( \theta \) read on the instrument (9), which is related to the polarization of fluorescence.

<table>
<thead>
<tr>
<th>Measured</th>
<th>Inhibition point</th>
<th>Protein concentration</th>
<th>Dissociation constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 2 Fluorescence of protein</td>
<td>( 7.3 \times 10^{-6} )</td>
<td>( 1.1 \times 10^{-6} ) (unlabeled enzyme)</td>
<td>( 1.8 \times 0.5 )</td>
</tr>
<tr>
<td>Fig. 9 Fluorescence of protein Absorption</td>
<td>( 9.3 )</td>
<td>( 3.7 \times 10^{-6} ) (DNS-conjugate)</td>
<td>( 0.5 \times 0.7 )</td>
</tr>
<tr>
<td>Fluorescence of DNS-conjugate Polarization of fluorescence</td>
<td>( 3.4 \times 10^{-6} ) (DNS-conjugate)</td>
<td>( 3.7 \times 10^{-6} ) (DNS-conjugate)</td>
<td>( 1.7 \times 0.1 )</td>
</tr>
<tr>
<td>Polarization of fluorescence</td>
<td>( 4.1 \times 10^{-6} ) (DNS-conjugate)</td>
<td>( 3.2 \times 10^{-6} ) (DNS-conjugate)</td>
<td>( 2.8 \times 0.1 )</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are estimates of uncertainty, calculated from the total amount of noise present in any two measurements divided by the total change in the property being measured.

The dissociation constant is found to be \( 1.8 \pm 0.5 \times 10^{-5} \) at 25°C and pH 7.8, and in the absence of added potassium chloride (Table II).

The activity of the enzyme as a function of magnesium concentration is also presented. The \( K_m \) is \( 6 \times 10^{-5} \) M at an enzyme concentration of about \( 1.0 \times 10^{-4} \) M (determined with a Cary spectrophotometer) and \( 2.4 \times 10^{-5} \) M at \( 4 \times 10^{-6} \) M enzyme (determined with a stopped flow apparatus) which is comparable to the concentrations used in the fluorimetric titrations (see "Discussion").

A plot of the percentage of change in fluorescence upon adding excess magnesium to EDTA-saturated enzyme as a function of wave length is given in Fig. 3. The pattern results from a blue shift and increase in quantum yield of the fluorescence emission of one or more tryptophan molecules, superposed upon a quenching of tyrosine. The quantum yield of the protein increases in

The change in fluorescence emission is complete one to two seconds after addition of EDTA or magnesium. It is also readily and completely reversible (the discrepancy between Curves 1 and 2 is not significant). The emission spectra of enolase in the presence of a 3- to 6- or 1000-fold molar excess of magnesium are essentially identical.

For determination of the minimum number of moles of magnesium needed to produce the observed change, a fluorometric titration of metal-free enzyme with magnesium was performed (Fig. 9). The titration curve shows that only 1 mole of magnesium per mole of protein is required for the fluorescence change. If we assume a magnesium-dependent equilibrium between two forms of the protein, a dissociation constant for the magnesium-protein complex can be calculated. From the midpoint of a plot of fraction of total fluorescence change against the log of the calculated free magnesium concentration (see Fig. 2), the enzyme is considered free of both metal and EDTA since no fluorescence change occurred on addition of EDTA, and since more magnesium was needed to produce changes in fluorescence in preparations known to be contaminated with EDTA.
the presence of magnesium by 13%, from an absolute value of 0.20 to about 0.23. The simultaneous quenching of tyrosine fluorescence can be detected directly, as described by Weber and Young (8), by exciting a solution of the enzyme in the presence and absence of magnesium at 275 and 297 μm. The emission caused by tryptophan is made equal by adjusting the fluorescence emission of the enzyme at 375 μm to a constant value. The difference remaining between the emission spectra produced by excitation at the two wave lengths is the tyrosine fluorescence (see Fig. 3, inset). The fraction of the total absorption of the tyrosine is estimated from the known amino acid composition of enolase and the extinction coefficients of tryptophan and tyrosine. The absolute values of the quantum yields of tyrosine in the absence and presence of magnesium are very low, about 0.0021 and 0.0017, respectively, or a relative change of about 20%. (See "Discussion."

The ultraviolet difference spectrum produced by addition of magnesium is shown in Fig. 4. An increase in absorption, with a peak at 296 μm and a shoulder at 288 μm, occurs when excess magnesium is added to an EDTA-saturated enzyme solution. The molar difference extinction coefficient at 296 μm is of the order of 2000 M⁻¹ cm⁻¹.

The observed fluorescence and absorption changes suggest that the polarity of the environment of one or more tryptophans is decreased when magnesium is added. Both the magnitude and sign of the absorption difference spectrum associated with the metal effect can be duplicated by comparing the absorption of free tryptophan in dioxane-water against that in water (12); electrostatic perturbations (for example, pH changes) produce molar difference extinction coefficients an order of magnitude smaller.

- Fig. 3. Percentage of change in fluorescence on addition of magnesium as a function of wave length of emission. The fluorescence emission spectrum of 2 ml of a 4 × 10⁻⁴ M solution of enolase in 0.1 M Tris-HCl, pH 7.8, and 10⁻⁶ M EDTA was scanned; then 2 mM MgCl₂ was added to a concentration of 0.01 M. The emission spectrum was scanned again. The two curves are duplicate experiments. The band width for excitation and emission were 2.5 and 3.3 μm, respectively. Excitation was at 280 μm. Inset: The fluorescence emission spectra of a 10⁻⁴ M solution of yeast enolase, excited at 275 (upper curve) and 297 μm and adjusted to equal emission at 375 μm. The solution was 10⁻⁴ M in EDTA and 0.1 M in Tris-HCl, pH 7.8. The band width for excitation and emission was 1.67 μm.

- Fig. 4. Ultraviolet difference absorption spectrum of yeast enolase in the presence of magnesium (sample) and presence of EDTA (reference). The solutions were 5.5 × 10⁻⁴ M in yeast enolase, 0.06 M in Tris-HCl, pH 7.8, and 1.7 × 10⁻³ M in EDTA. Two 1-ml cuvettes with 10-mm path lengths were used. The relative absorbance from 250 to 325 μm was adjusted to a constant value. Then 2 mM MgCl₂ was added to the sample cuvette to a final concentration of 1.4 × 10⁻³ M. An equal volume of water was added to the reference cuvette.

- Fig. 5. Effect of magnesium on the fluorescence polarization spectrum of enolase. Corning 0-54 filters were used to prevent scattered exciting light from reaching the photomultipliers. The solution was 2 × 10⁻⁴ M in enolase and 0.1 M in Tris-HCl, pH 7.8. Originally, 4 moles of magnesium per mole of protein were present (●). O—O, spectrum 45 min after addition of EDTA to 10⁻³ M; X—X, spectrum after subsequent addition of magnesium to 0.01 M. The difference in spectrum produced by EDTA was 60 to 70% complete within 15 sec of addition. The reversal of the change by excess magnesium showed no time dependence.
The fluorescence-polarization spectra (14) of the enzyme with 0 (excess EDTA), 0.01, or 500 moles of magnesium per mole of protein are given in Fig. 5. The polarization of the ultraviolet emission (300 to 400 m\(\mu\)) of the enzyme on excitation from 250 to 300 m\(\mu\) is greater in the presence of magnesium. This effect, particularly the increase seen on excitation at 295 to 300 m\(\mu\) (where only tryptophan is absorbing), is interpreted as a consequence of less freedom of rotation by the indole groups. The increase in polarization cannot result from a shortening of the lifetime of the excited state, since the quantum yield increases on addition of magnesium.

The polarization of fluorescence of a representative preparation of the DNS-conjugate of enolase, as a function of the quantity \(T/\eta\) in the presence of EDTA or magnesium, is given in Fig. 6. The fluorescence-polarization spectra (14) of the intrinsic protein fluorescence) differ by the fact that in the latter the fluorescing indole and phenolic groups have relatively short lifetimes of excited state. They therefore emit light before Brownian diffusion can rotate the entire protein, but not before the fluorescent amino acid residues can themselves rotate relative to the side chains connecting them to the rest of the protein. In the examination of the polarization of protein conjugates, only the fluorescence of the label is measured. The lifetime of excited state of DNS-conjugate is long enough so that some of the depolarization of the fluorescence occurs through the Brownian rotation of the entire molecule. The depolarization due to the rotation of the DNS-conjugate about the bond connecting it to the rest of the protein is nearly constant over the \(T/\eta\) range covered (14).

\[ \frac{1}{p} = \frac{1}{p_e} - \frac{1}{p_s} + \frac{3}{3\tau} - \frac{3}{3\tau} \]

\[ \frac{1}{p_{m\alpha}} = \frac{1}{p_e} - \frac{1}{p_s} \]

\(1/p_e\) is the intercept (cf. Fig. 6); \(1/p_{m\alpha}\) is the value of the polarization at \(T/\eta = 33,300\), with \(\tau = 12\) nsec.

\(\eta\) and Methods\).
The rotational relaxation time of this preparation of the DNS-conjugate is 13% lower in the presence of 4 or 500 moles of magnesium per mole of enzyme. The magnitude of the difference varied considerably from preparation to preparation, but in general there was a significantly lower relaxation time in the presence of magnesium (Fig. 7). Individual relaxation times are believed significant to between 4 and 5%, which is the average variation among 14 sets of duplicate samples (data not shown). A lower rotational volume indicates either greater local rotational freedom (a relatively unfolded molecule) or a smaller, more compact structure.

\[
\begin{array}{ccc}
\text{Optics} & \text{Endogenous magnesium} & \text{EDTA added} \\
\text{mg/ml} & (\text{all at pH 7.8}) & \\
\text{Interference} & 0.056 \text{ m KCl-0.07 m Tris-HCl} & 0.04 \text{ m KCl-0.07 m Tris-HCl, pH 7.8; } 2 \times 10^{-2} \text{ m EDTA} \\
1.0-2.0 & 0.1 \text{ m KCl-0.1 m Tris-HCl} & 0.04 \text{ m KCl-0.1 m Tris-HCl, pH 7.8; } 10^{-2} \text{ m EDTA} \\
\text{Schlieren} & 0.05 \text{ m KCl-0.1 m Tris-HCl} & 0.04 \text{ m KCl-0.05 m Tris-HCl, pH 7.8; } 2 \times 10^{-4} \text{ m EDTA} \\
2.4 (1) & 0.08 \text{ m KCl-0.05 m Tris-HCl} & 0.04 \text{ m KCl-0.1 m Tris-HCl, pH 7.8; } 2 \times 10^{-2} \text{ m EDTA} \\
3.0 & 0.05 \text{ m KCl-0.1 m Tris-HCl} & 0.04 \text{ m KCl-0.1 m Tris-HCl, pH 7.8; } 2 \times 10^{-2} \text{ m EDTA} \\
3.0 (2) & 0.05 \text{ m KCl-0.1 m Tris-HCl} & 0.04 \text{ m KCl-0.1 m Tris-HCl, pH 7.8; } 2 \times 10^{-2} \text{ m EDTA} \\
4.8 & 0.05 \text{ m KCl-0.026 m Tris-HCl} & \\
5.9 & & \\
\end{array}
\]

+ (X) and - (O) EDTA samples at 3.0 and 4.8 mg per ml were run simultaneously with the use of a plane and a wedge-windowed cell. The other samples were run separately. A double sector cell was used in the interference optics runs. The corrections for density and viscosity of the solvent (16) increased the observed sedimentation constants by 3 to 5%. The endogenous magnesium is estimated (directly or from the total dialysis time) to be: 1.0 to 2.0 mg per ml, 10 to 50 moles per mole of protein; others, 3 to 6 moles per mole of protein. 1, DNS-conjugate of enolase Preparation 8 (cf. Fig. 7) (1.9 moles of DNS-conjugate per mole of enzyme); 8, DNS-conjugate of enolase Preparation 10 (1.1 moles of DNS-conjugate per mole of enzyme); 8, data of Bergold (17); 4, data of Westhead and McLain (6).

The data in Fig. 8 indicate that addition of EDTA to enzyme containing low molar excesses of magnesium is accompanied by a decrease of about 5% in $s_{20,w}$ (from 5.90 to 5.58 S). Since the enzyme is asserted to be a single chain (14), the decrease in sedimentation constant must reflect an increase in the frictional coefficient of translation, indicating a transition to a less compact form. The lack of dependence of the observed sedimentation constants on concentration shows that interactions between molecules play no significant role in this finding. The over-all evidence from spectral measurements and hydrodynamic properties indicates that relatively low concentrations of magnesium maintain the enzyme in a more compact conformation.

Control experiments have shown that the concentration of magnesium needed to effect changes in polarization of fluorescence...
TABLE III

Effect of various molar ratios of magnesium to protein on thermal stability of protein

A solution of enolase was partially freed of magnesium by absorbing it onto phosphocellulose at pH 6.0 and washing several times with 10⁻² M sodium acetate, pH 6.0, before elution (6). The amount of magnesium remaining with the eluted enzyme was estimated to be 1.5 moles per mole by the kinetic method described in Table I, by flame photometry, and by the fluorescence changes occurring on addition of excess EDTA and magnesium (in comparison with Fig. 2). It was diluted to 2 mg per ml of protein with buffer to 0.03 M Tris-HCl, pH 7.8, and 0.5 ml of the buffered enzyme was added to each of four polypropylene test tubes. Small amounts of EDTA or magnesium acetate solutions were added. The four tubes were then heated in a constant temperature bath for 4 min at 46°C, and the enzyme solutions were subsequently assayed for activity.

<table>
<thead>
<tr>
<th>Magnesium</th>
<th>Activity remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (excess EDTA)</td>
<td>28</td>
</tr>
<tr>
<td>1.5</td>
<td>74</td>
</tr>
<tr>
<td>4.5</td>
<td>88</td>
</tr>
<tr>
<td>3.5</td>
<td>99</td>
</tr>
</tbody>
</table>

* Relative to an unheated control.

cence of DNS-conjugates of enolase is quite comparable to that needed to produce the fluorescence changes in the unlabeled enzyme (Fig. 9). The changes in fluorescence emission of protein and of label, absorption at 296 mμ, and a polarization of fluorescence were followed as magnesium was added to a solution of metal-free DNS-enolase. Although the experimental uncertainty is necessarily large, considering the small amounts of enzyme available and the magnitude of some of the differences examined, it is clear that the changes in fluorescence and absorption of the protein, in polarization of fluorescence, and in the fluorescence of the DNS-moiety are correlated. The calculated dissociation constants are given in Table II. They are considered to be in fair agreement and we conclude that the changes are caused in all cases by the binding of 1 mole of magnesium with a dissociation constant of about 2 X 10⁻⁶ M.

Concentrations of magnesium of this order greatly increase the thermal stability of the enzyme (Table III). The correlation between retention of activity after exposure to high temperature, relative concentration of magnesium present, and the titration curves in Figs. 2 and 9 is obvious.

DISCUSSION

Except for the fluorescence-polarization spectra (cf. legend to Fig. 5) and the thermal stability (Table III), no time-dependent changes in any property, including the activity, in labeled or native enolase are found over periods of up to several hours. Except for the thermal denaturation, all the changes described are readily reversible. We conclude that our data are best explained by a magnesium-dependent equilibrium between two conformations of the enzyme.

The data presented show that the conformational change is accompanied by changes in absorption and fluorescence, primarily in the tryptophan residues in the protein. We can not rule out the possibility that the spectral changes are produced by a direct interaction of indole with magnesium. However, the red shift in absorption and blue shift in fluorescence can be more easily accounted for on the basis of changes in the polarity of the environment of the indole residues. The decrease in tyrosine fluorescence when magnesium is added to the protein is not likely to be due to increased energy transfer to tryptophan since the changes in fluorescence shown in Fig. 1 are even greater on excitation of the enzyme at 255 to 300 mμ where tyrosine absorption is virtually absent. This indicates that little of the fluorescence change produced by magnesium is due to changes in energy transfer from tyrosine.

According to Westhead, all the tyrosine residues in magnesium-enolase titrate with an abnormally high pK, indicating perhaps extensive hydrogen bonding to other groups in the enzyme (18). This would account for the very low quantum yield observed (19). An increased separation between tyrosine residues and quenching groups would explain the increase in quantum yield when metal is removed.

Our values of κωg are in agreement both with Westhead’s (18) limiting value of 5.87 S for the metal-saturated enzyme, obtained in a similar solvent, and with that reported for (presumably) metal-free enolase in an ammonium acetate buffer by Bergold (17).

Hanlon and Westhead (20) have recently published the results of an examination by absorption difference spectroscopy of the binding in 0.5 M KCl of several metals including magnesium to the enzyme. Our results, under similar conditions, are in good agreement. More recent work by these authors with the use of equilibrium dialysis techniques has also yielded binding constants in good agreement with values obtained by us (21).

The relaxation times of the DNS-conjugates and the changes in rotational relaxation time produced by addition of magnesium show considerable variability (Fig. 7). Of 13 different preparations examined, 10 showed significant (10% or more) decreases in relaxation time. Three were anomalous: one had a 23% higher relaxation time in the presence of magnesium and in two there was no significant change.

The reason for these differences are not known. There is nearly always some loss (from 8 to 47%) of activity upon labeling, and the labeled enzyme is less stable than the unlabeled in the absence of magnesium. However, the attachment of the DNS-conjugate does not significantly alter the gross physical properties of the enzyme. For example, the sedimentation constants of two “anomalous” preparations (8 and 10) of the labeled enzyme were nearly equal to those of the unlabeled enolase, although the rotational relaxation times in the presence of magnesium differed from each other by nearly 70% (Figs. 7 and 8). Since the same magnesium-induced changes in absorption, fluorescence, and sedimentation constant occur in “anomalous” preparations (8 and 10) of the labeled enzyme and in all active labeled (or native) enzyme preparations examined, the irreproducibilities probably originate in some property of the DNS-enolase conjugates. Variations of 30 to 40% from the average relaxation times of DNS-conjugates of muscle proteins were obtained in an extensive study by Mortonosi and Teale (22). In general, the variability in relaxation times seems to be a characteristic of the particular protein studied since no such variations in the relaxation time of other proteins (e.g. bovine serum albumin) are found.

No correlation has been found between degree of labeling and relaxation times with or without magnesium, degree of labeling

* Data to be published.
and activity, or activity and relaxation times. No correlation has been observed between the relaxation times with and without magnesium and ionic strength of the solutions being measured from $\Gamma/2 = 0.02$ to $0.17$. On the other hand, addition of KCl to 0.05 M seems to increase the difference between relaxation times with and without magnesium (compare Preparations 13a and 13c). Some effect of dialysis time and ionic strength of dialysis solutions exists (Preparations 13b and 13c), suggesting some slow, environmentally sensitive changes occurring in the protein.

The possibility that the results are influenced by variations in the state of aggregation of the labeled protein is discounted. The preparations are routinely centrifuged at 105,000 x $g$ before examination; changes in ionic strength either have no effect on the relaxation time, or have an effect opposite to that expected (Preparations 13a and 13c); no aggregation was seen in labeled enzyme preparations during ultracentrifuge runs.

Variations in lifetime of excited state of the label arising from attachment to different groups in the enzyme and variations in rotational freedom of the DNS-conjugate relative to the protein are probably factors in producing the absolute variability in relaxation times (23). Considerable internal evidence shows, however, that they cannot account for the lower relaxation times usually seen in the presence of magnesium.

In Preparation 3, which was dialyzed against EDTA, and which as a result showed a progressive loss of activity over a period of days, the effect of magnesium on the rotational relaxation time diminished along with the activity and effect of magnesium on the fluorescence. This suggests that the results cited in this paper were not caused by a fraction of the enzyme population which possessed all or most of the label and which was inactive, although responsive to magnesium. Other indirect evidence also suggests that the labeling is reasonably homogeneous: Preparation 9, with 0.5 mole of DNS-conjugate per mole of protein, showed an immediate 40% loss of activity after treatment with EDTA. All this activity was immediately recovered on addition of excess magnesium.$^{10}$

Yeast enolase prepared by crystallization as the mercury salt (1), which is presumably magnesium-free, has been reported to have a low frictional ratio (17), which suggests a nearly spherical shape. If the enzyme does not change greatly in shape when metal is added, a 15% decrease in volume (the average decrease in relaxation time) would produce a decrease in linear dimensions of about 5%, which is roughly what is observed in the sedimentation experiments. The decrease in frictional ratio from Bergold's value of 1.01 (17) would make it 5 or 6% below the theoretical minimum. However, the uncertainty involved in calculating the absolute frictional ratio is probably larger than the relative decrease reported in this paper.

From the data in Fig. 2, it is evident that a concentration of magnesium saturating for the structural change allows less than maximal activity. Other data indicate that the substrate is bound only in the presence of higher magnesium concentrations at a different site (5); therefore, activation of the enzyme by magnesium inevitably produces the conformational change.

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