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# Dissociation of Yeast Enolase into Active Monomers*

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ABSTRACT: Yeast enolase exists as a monomer at concentrations below 0.7 μg/ml, temperatures above 40°, and at pH 7.4, even in the presence of $Mg^{2+}$ and substrate, both of which favor dimer formation. Monomers formed under these conditions, in contrast to those obtained in the presence of high concentrations of $Br^-$ or $Cl^-$, are fully active. Standard thermodynamic parameters estimated for the dissociation process are $\Delta H^\circ = 80$ kcal and $\Delta S^\circ = 221$ eu.

The glycolytic enzyme enolase, isolated from yeast cells, is reportedly a dimeric molecule (Brewer and Weber, 1968). Dissociation into monomers has been achieved in the presence of 1 M $Br^-$ or $Cl^-$ with a concommitant loss of activity (Gawronski and Westhead, 1969). Thus, it could be assumed that the dimeric structure is essential for the proper functioning of the enzyme. An alternative possibility is that this observed inactivation is the consequence of the high salt concentrations used to effect dissociation and not a result of the dissociation process *per se*. If dissociation could be brought about under very mild conditions, the question whether or not the monomers are active could be resolved. One such mild procedure for dissociation is the lowering of the total protein concentration. However, molecular weight determinations in very dilute solutions are difficult to perform with most physical techniques. Of these, Sephadex chromatography provides the most effective approach, since elution volumes, reflecting molecular weight, can be detected at extremely low enzyme concentrations by activity measurements. By this procedure, evidence indicating that the monomers are active has been obtained and is presented here along with some observations concerning this monomer–dimer equilibrium.

## Methods and Materials

Yeast enolase and its substrate, the sodium salt of 2-phosphoglycerate, were purchased from the Sigma Chemical Co., lots 27B7290 and 118B1410, respectively. Purified enzyme preparations were obtained by ion-exchange chromatography according to Rosenberg and Lumry (1964).[1] All other chemicals used were of reagent grade.

All column chromatography experiments described herein were performed on a 2 × 50 cm column packed with Sephadex G-75 gel. Two reservoirs, one for sample, one for elution solvent, were connected to the column through a three-way valve. Both column and reservoir system were enclosed in a continuous-jacketing device through which water was circulated from a constant-temperature water bath. The column was operated under a hydrostatic pressure of approximately 80 cm. resulting in a flow rate that varied with temperature from 60 to 125 ml per hr.

The column was calibrated using the zonal analysis technique. Samples (2.5 mg) of various marker molecules in 1-ml volumes were applied to the column. The effluents were monitored at 280 nm by means of a Beckman Model DBG spectrophotometer equipped with a 0.3-ml flow cell of 1.0-cm path length and the absorbance recorded on a Beckman 10-in. recorder. The elution volumes reported are those corresponding to maximal absorptions in the effluents. Calibrations with BSA,[2] ovalbumin, chymotrypsinogen, and Blue Dextran were performed at both 25 and 45°.

To relate elution volumes to distinct protein concentrations, the experiments with yeast enolase were performed by frontal analysis or large volume chromatography technique. For the column described above, a 50-ml sample application was sufficient to produce a plateau region in the elution pattern. The protein concentration in this region is identical with that of the sample applied to the column. In theory, the elution volume is the centroid point of the boundary. For symmetrical or nearly symmetrical boundaries as observed with enolase, this point can be approximated as the half-height of the plateau. Thus, all elution volumes reported here refer to observed half-heights.

Elution volumes of monomeric and dimeric enolase in Tris-HCl buffer (pH 7.4) were determined by frontal analysis and verified by the zonal technique. In both cases, monomers were obtained in the presence of 1 M KBr. The column was equilibrated with the buffer also 1 M in $Br^-$. For the frontal analysis determinations, the protein concentration applied to the column was 50 μg/ml. For zonal experiments, the 1.0-ml sample used contained 2.5 mg of enolase. Effluents were monitored at 280 nm. Dimeric elution volumes were similarly obtained in the absence of bromide.

The effect of dilution on the elution volume of yeast enolase was determined at 25°. Solutions (50 ml) of enzyme were prepared at various dilute concentrations in the assay buffer,

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[1] The specific activity of the preparation was within 5% of that cited by Malmstrom (1957).

[2] Abbreviation used is: BSA, bovine serum albumin.

TABLE I: Elution Volumes for Marker Molecules at 25 and 45°.

| | Mol Wt[a] × $10^3$ | $V_e$ 25° (ml) | $V_e$ 45° (ml) |
|---|---|---|---|
| Blue Dextran | | 50.0 | 50.0 |
| Rabbit muscle enolase | 84 | 55.0 | |
| BSA | 67 | 57.0 | 57.0 |
| Ovalbumin | 44 | 67.5 | 67.0 |
| Chymotrypsinogen | 25 | 83.0 | 83.0 |

[a] Determann and Michel (1966).

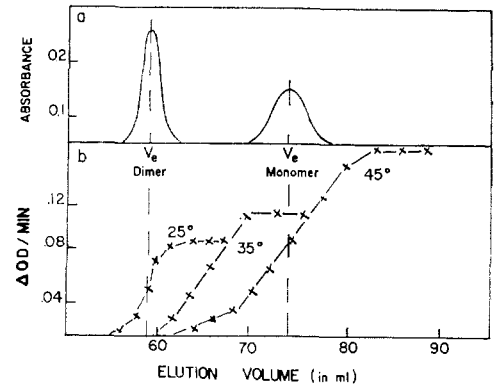


FIGURE 1: (a) Zonal elution patterns for dimeric and monomeric yeast enolase on Sephadex G-75 (2.0 × 50 cm) column. The monomer is obtained in the presence of 1.0 M $Br^-$. Absorbance of the effluent is recorded at 280 nm. Elution solvent is Tris-HCl buffer (pH 7.4), ionic strnegth 0.025 in $Cl^-$, and $10^{-3}$ M $Mg^{2+}$, 1 M in KBr. (b) Reaction velocity frontal elution patterns obtained with 0.7 μg/ml of yeast enolase at various temperatures. Column size as in part a. Elution solvent is Tris-HCl buffer (pH 7.4), ionic strength 0.025 in $Cl^-$, $10^{-3}$ M $Mg^{2+}$, and $10^{-3}$ M substrate.

a 0.025 ionic strength Tris-HCl buffer–$10^{-3}$ M $Mg^{2+}$ (pH 7.4). The column was equilibrated with this buffer. The effluent was collected in 2-ml fractions and assayed. This assay consisted of taking 1-ml aliquots of collected enzyme already at proper pH and $Mg^{2+}$ concentration and adding 10 μl of substrate sufficiently concentrated so that the final conditions were equal to those as described by Malmstrom (1961). Optical densities were monitored at 240 nm using a Beckman DU spectrophotometer. Enzyme concentrations, expressed at ΔOD/min, were plotted against volume to obtain an elution pattern.

In another set of experiments, the effect of temperature on the dissociation of the enzyme was studied. Solutions (50 ml), 0.7 μg/ml in enzyme, were prepared in assay buffer also containing $10^{-3}$ M substrate. Such solutions were passed through the column preequilibrated with buffer and substrate at various temperatures between 25 and 45°. Fractions (2 ml) were collected into tubes immersed in a water bath at the desired temperature. The enzyme activity of each fraction was measured in 1.0-ml cuvets of 0.5-cm path length on a Beckman DU spectrophotometer equipped with a temperature-controlled cell compartment set at the temperature of chromatography. Optical densities at 240 nm were recorded at 30-sec intervals. Although the initial absorbances of each tube varied, the ΔOD/min remained strictly proportional to the enzyme present. The resulting plot of velocity *vs.* volume yielded a frontal elution pattern.

## Results

Elution volumes for the various marker molecules at 25 and 45° are listed in Table I. The $V_e/V_0$ values obtained fit rather well on the calibration curve prepared and published by Determann (1969) on the basis of all available literature data. In addition, since the elution volumes are independent of temperature, this calibration curve can be used to estimate molecular weights over the given temperature range. Elution volumes for yeast enolase in the absence and presence of $Br^-$ as obtained both by zonal and frontal techniques were found to be 58.5 and 73 ml, respectively (see Figure 1a). Such elution volumes are consistent with molecular weights of approximately 66,000 and 33,000.

The results of molecular weight determinations performed at various enzyme concentrations indicate only a single elution volume corresponding to a molecular weight of about 66,000 over a concentration range from 50.0 to 0.01 μg per ml.

The variation in elution volume as a function of temperature for the enzyme in the presence of substrate is illustrated in Figure 1b. The observed elution volume increases with increasing temperature until 43°, where an elution volume corresponding to that obtained in 1 M $Br^-$ is reached. Then above 43° at a concentration of 0.7 μg/ml, yeast enolase is completely dissociated. Since the enzyme was detected by activity measurements, the monomeric species must be active.

Figure 1b also illustrates that the elution patterns obtained are of a single-waved nature. The monomer–dimer equilibrium then is rapid with respect to the rate of separation on the column and the measured elution volumes represent weight-average molecular weights of monomer–dimer mixtures. The concentration of monomer and dimer present may be calculated from

$$\overline{MW} = (\mathrm{M}) \times MW_{\mathrm{M}} + (\mathrm{D}) \times MW_{\mathrm{D}}/(\mathrm{M}) + (\mathrm{D})$$

where (M) and (D) are the concentrations of monomer and dimer, respectively, $MW_{\mathrm{M}}$ and $MW_{\mathrm{D}}$, the molecular weights of monomer and dimer, respectively, and $\overline{MW}$, the weight-average molecular weight of the mixture. For the purpose of calculation only, the dimeric and monomeric molecular weights are taken as 66,000 and 33,000 (see Discussion). From the equilibrium concentrations of monomer and dimer and their variation with temperature, thermodynamic parameters can be calculated by the usual procedures (Brandts and Lumry, 1963).

## Discussion

The results presented here give sufficient evidence that the monomer is an active form of the yeast enolase enzyme. The inactivity previously reported seems a likely consequence of the high salt concentrations used to affect dissociation. Whenever dissociation is brought about by a denaturant, the structure and properties of the monomers so formed may be significantly altered. Employing low protein concentrations and somewhat elevated temperatures, we endeavored to simulate the natural milieu to the extent that was experimentally feasible. Dissociation occurred in the presence of $Mg^{2+}$ and substrate; both known to favor the dimeric state (Gawronski and Westhead, 1969). That an Arrhenius plot derived from the observed velocities in the plateau regions at various temperatures (see Table II) is linear in accord with previous work

TABLE II: Dependence of Elution Volume on Temperature for Enolase at 0.7 μg/ml.

| Temp (°C) | Plateau Reaction Velocity, ΔOD/min | $V_e$ (ml) | $\overline{MW} \times 10^{-3}$ | $K_{eq} \times 10^{-9}$ (Moles of Monomer/l.) |
|---|---|---|---|---|
| 25 | 0.07 | 58.5 | 66.0 | |
| 30 | 0.08 | 58.5 | 66.0 | |
| 35 | 0.11 | 62.5 | 53.0 | 5 |
| 38 | 0.13 | 64.5 | 50.0 | 91 |
| 40 | 0.16 | 68.5 | 43.8 | 275 |
| 43 | 0.18 | 73.0 | 33.0 | |
| 45 | 0.19 | 73.0 | 33.0 | |

(Westhead and Malmstrom, 1957) seems to indicate that the enzyme has identical activity in both dimeric and monomeric forms.

The thermodynamic parameters derived from the dissociation process can only be considered approximations for the following reasons. There appears to be some controversy as to the molecular weight of the yeast enolase molecule. It has long been considered to be 66,000 on the basis of sedimentation (Bergold, 1946) and light-scattering (Bücher, 1943) measurements. Recently, Mann and coworkers (1970) have reported a value of 88,000. Since our experiments were not designed to resolve this question, for the purpose of thermodynamic calculation only, we have elected to use 66,000 as the dimeric molecular weight because this value was directly measured on our column.

Since the temperature range over which dissociation occurs is small, it is difficult to obtain a sufficient number of experimental points to ensure a high degree of accuracy. A further complication is that $\Delta H°$ and $\Delta S°$ may be temperature-dependent quantities as proposed by Brandts (1967) for the thermal denaturation of proteins. Because of such difficulties, the values given in Table II must be considered approximate. Still the calculated equilibrium constant at 25° indicates that a protein concentration of $10^{-4}$ μg/ml would be required to effect 50% dissociation and at $10^{-2}$ μg/ml, no appreciable dissociation would be expected in agreement with our experimental observations.

Finally, we wish to reemphasize the general utility of frontal gel filtration chromatography in affecting protein dissociation, since other enzyme subunits, previously thought inactive, may be shown capable of catalysis when studied by this technique.

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