*THE REVERSIBLE DISSOCIATION OF YEAST ENOLASE**

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Warburg and Christian[1] showed that yeast enolase crystallized as the mercury salt had an equivalent weight of 67,000. The subsequent finding of Malmstrom *et al.*[2] that yeast enolase had one N-terminal alanine per 67,000 mol wt and an unquantitated amount of C-terminal leucine was interpreted as indicating that the yeast enolase molecule was a single polypeptide chain. This interpretation was strengthened by the observation of Westhead[3] that the number of peptides produced by tryptic digestion of yeast enolase was close to the number predicted from the lysine and arginine content.

In studying the effects of high salt concentrations on some physical properties of the enzyme, we observed a loss of activity in enzyme preincubated in 1 *M* KCl and an excess of ethylenediaminetetraacetic acid (EDTA). This was recoverable by addition of magnesium. The loss of activity and the kinetics of recovery appeared to be concentration-dependent, and additional experiments revealed that these phenomena were correlated with a dissociation and reassociation of the enzyme.

Materials and Methods.—Yeast enolase was prepared by the method of Westhead and McLain.[4] Potassium chloride, potassium acetate (both Baker A. R. grade) and tris (from Boehringer) were recrystallized twice from 0.001 *M* EDTA, 50 per cent ethanol, and 95 per cent ethanol, respectively. "Bicine" (bis-diethanolglycine) was prepared as described by Good *et al.*[5] It was twice recrystallized from absolute ethanol. Fluorescein isothiocyanate was purchased from Calbiochem. The preparation of other reagents and cleaning of glassware were described in reference 6.

Fluorescein-labeled enolase was prepared essentially as described in reference 6, substituting 0.2 *M* "bicine"–KOH, pH 8.6, for tris buffer. No substrate or competitive inhibitor was present during labeling, and the specific activity of the labeled enzyme decreased with increasing covalently bound dye.

Sedimentation equilibrium experiments were performed with a Beckman-Spinco model E ultracentrifuge using absorption optics or interference optics with either a conventional double-sector cell or a multichannel cell. In experiments using interference optics, concentrations of protein at the meniscus were measured by the method of Labar.[7]

Activity measurements were made using a Cary 15 recording spectrophotometer. Fluorescence and polarization of fluorescence measurements were made using the instruments described in references 8 and 9. Stopped-flow absorption measurements were made with the apparatus described by Gibson and Milnes.[10]

Results.—Sedimentation equilibrium: The results from two determinations of the molecular weight of yeast enolase in 1 *M* KCl and EDTA with or without excess magnesium are plotted in Figure 1. The maximum, average, and minimum weight-average molecular weights from these and other similar experiments

are given in Table 1. It is evident that the enzyme is largely dissociated in 1 *M* KCl and excess EDTA, and that the subsequent addition of excess magnesium suppresses the dissociation. It is also evident that little dissociation occurs in the presence of excess EDTA alone, or in the presence of excess EDTA and equivalent concentrations of potassium acetate.

TABLE 1. *Average, maximum, and minimum weight-average molecular weights of yeast enolase in various solvents.*

Solvent	Molecular Weight			
	Minimum	Average		Maximum
1 *M* KCl, excess EDTA*	34,200	38,724		41,300
1 *M* KCl, excess magnesium*	51,100	61,578		70,100
2 Moles Mg^{++}/mole E†, ‡	—	67,500	(70,100; 64,500)	—
Excess EDTA†	—	55,300	(71,900; 63,300)	—
1 *M* KOAc and excess EDTA†	—	62,300	(73,800; 77,000)	
Excess Mg^{++}†	—	68,000	(68,000)	—
1 *M* KCl, excess EDTA†	—	44,200	(38,500)	—

Concentrations of protein, EDTA, and magnesium, and the buffer used are given in the legend to Fig. 1. The data were plotted and the average molecular weights determined by a computer using a least-squares treatment. Maximum and minimum weights were calculated from the plotted data by hand. The numbers in parentheses were obtained with absorption optics, using a protein concentration of 0.4 mg/ml. The reliability of the valves obtained using the multichannel cell is estimated to be ±10%; those obtained with the double-sector cell, ±5%.

* Data plotted Fig. 1.

† Multichannel cell used.

‡ Cf. ref. 6.

Examinations of the molecular weights of proteins in high concentrations of solutes have sometimes been complicated by the existence of preferential interactions between the protein and solute.[11] The magnitude of such interactions between the potassium chloride and enolase does not appear to be very different in the presence or absence of magnesium, since measurements of the fringe drop in sedimentation velocity experiments carried out in EDTA and 1 *M* potassium chloride showed no significant change upon rerunning the experiment after addition of excess magnesium. Consequently, we expect that the relative calculated molecular weights in Table 1 are not invalidated by preferential interactions with the solute.

Fluorescence depolarization: Hybridization of fluorescein-labeled enolase with unlabeled enzyme can be demonstrated by polarization of fluorescence measurements (Fig. 2). In these experiments, the polarization of the fluorescence of several preparations of fluorescein-enolase conjugates is measured in solutions containing EDTA and 1 *M* KCl before and after addition of magnesium, as a function of the relative amount of unlabeled enolase added. The increase in fluorescence polarization upon hybridization with unlabeled enzyme is due to suppression of electronic energy transfer between oscillator labels that were located in different subunits of the same molecule before hybridization. A calculation of the magnitude of the effects expected may be made for an ideal system on the assumptions:

(1) Poisson distribution of the label among reactive groups in number large compared with the average number of labels/molecule.

(2) A single transfer is responsible for the depolarization.

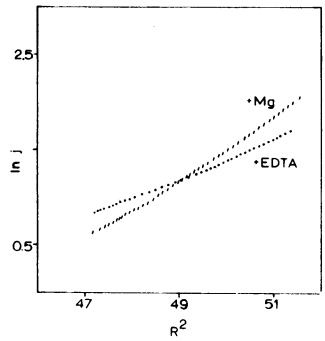

FIG. 1.—Plot of equilibrium distribution of enolase in 1 *M* KCl in the presence (*short lines*) and absence (*dots*) of excess magnesium. Both solutions contained 1 mg/ml yeast enolase, 1 *M* KCl, 3×10^{-3} *M* EDTA, and 0.05 *M* tris-HCl, pH 7.8. The "+Mg" solution also contained 0.02 *M* $MgCl_2$. The temperature was 20.0°C. The ultracentrifuge was run 24–50 hr at 10,000 rpm before the pictures were taken, and then accelerated to 29,500 rpm for 5–8 hr until no protein remained at the meniscus.[7] A second set of pictures was then taken.

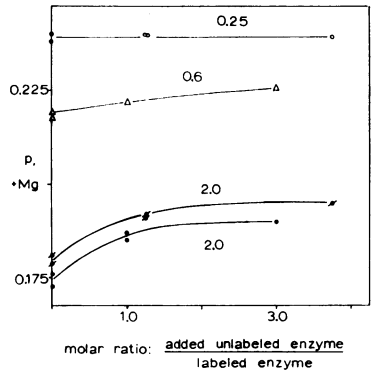

FIG. 2.—Polarization of the fluorescence of fluorescein-labeled enolase preparations in 1 *M* KCl, excess magnesium, and various molar excesses of unlabeled enzyme. The composition of the solutions was as described in the legend to Fig. 1, save that the concentration of labeled protein was either 0.16 or 0.33 mg/ml. The molar ratios of fluorescein to enolase in the four preparations examined are given in the figure. The specific activities ranged from about 75% of the original (0.25 moles/moles) to 33% (2 moles/mole).

(3) The radiation that emerges after one transfer is characterized by having an average emission anisotropy A_T, which is the same for transfer between label in different subunits or the same subunit.

(4) The probability of transfer ν is on the average the same for transfers between labels in the same or different subunits.

The mode of calculation is similar to that described by Weber and Daniel (1966)[12] for labels adsorbed at different points of the molecule. From these premises it is seen that complete replacement of the accompanying labeled subunit for an unlabeled one will affect the polarization by altering the distribution of the labels.

To determine the magnitude of the expected effects after hybridization, we have used as an equivalent system one in which the probability of transfer and that of emission are equal ($\nu/\lambda = 1$), $A_T = 0.125$, and $A_1 = 0.274$ (see ref. 12). The values of A as function of $\bar{n}$ (number of labels/molecule) calculated for such systems are very close to the experimentally observed values before hybridization. Complete replacement in the equivalent system results in an increase ΔA in anisotropy of emission. Table 2 gives the values of the relative increase $\Delta A/A$ and compares them with the experimental findings. At $\bar{n} = 0.25$, the replacement effect should be below experimental detection, at $\bar{n} = 0.6$, 3.2 per cent is expected and observed. Since the precision of the measurements is here ±0.5 per cent, the result is certainly significant. At $\bar{n} = 2$, $\Delta A/A$ = 9–10 per cent or

TABLE 2. *Increase in anisotrophy of emission A observed on hybridization with excess of unlabeled enzyme (complete replacement).*

	$\Delta A/A$	
	Observed (%)	Expected (%)
$\bar{n} = 0.2$	0	<0.2
$\bar{n} = 0.6$	3.2	3.3
$\bar{n} = 2$	8.9	14.5
$\bar{n} = 2$	9.8	14.5

two thirds of the expected 14.5 per cent. We can safely conclude that the observed polarization changes upon hybridization are of the order that we can expect from simple probability considerations.

Activity: The dissociation in 1 *M* KCl is accompanied by a loss of activity (Fig. 3). The figure shows drawings of oscilloscope tracings of the optical

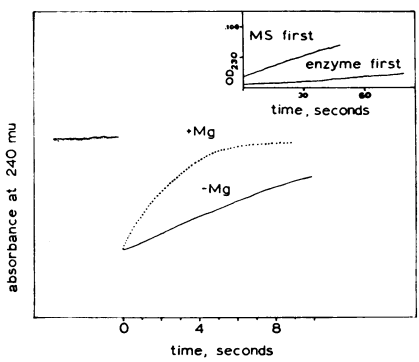

FIG. 3.—Absorbance changes produced by 3×10^{-6} *M* enolase after preincubation in 1 *M* KCl with (*dotted line*) or without (*solid line*) magnesium. Drawings of oscilloscope tracings of the absorbance at 240 mμ vs. time of solutions mixed in a stopped-flow apparatus. All solutions were 0.05 *M* tris-HCl, pH 7.8, 1 *M* KCl, and 2.10^{-3} *M* EDTA. Solutions containing 0.4 mg/ml enolase with or without 0.01 *M* $MgCl_2$ present were mixed with solutions containing 0.01 or 0.02 *M* $MgCl_2$, respectively, and 3×10^{-3} *M* 2-phosphoglyceric acid. The temperature was 25°C. The actual absorbancies of the solutions were not measured.

Inset.—Effect of prior dilution without magnesium on the activity of enolase. Two μliters of a solution containing 1 mg/ml enolase, 0.05 *M* tris-HCl, pH 7.8, and 3×10^{-3} *M* EDTA were mixed with 3 ml of 0.1 *M* tris-HCl, pH 7.8, either before (*enzyme first*) or after (*MS first*) 0.03 ml of 0.1 *M* $MgCl_2$ and 0.01 *M* 2-phosphoglyceric acid was mixed with the buffer. After addition of magnesium and substrate, the absorption at 230 mμ was recorded as a function of time. The temperature was 25°C.

density changes at 240 mμ produced by 0.2 mg/ml enolase, preincubated in 1 *M* KCl with or without excess magnesium, on mixing with magnesium and substrate in the stopped-flow apparatus. The initial rate of change in optical density produced by the enzyme preincubated without magnesium is only 17 per cent of that of the enzyme preincubated with metal.

Even in the absence of potassium chloride, a loss of activity upon preincubation without magnesium can be demonstrated at sufficiently low protein concentrations (Table 3). In one instance (MS first), the enzyme was diluted 3000-fold (to a final concentration of 10^{-8} *M*) into assay buffer containing magnesium and substrate. In the other instance (enzyme first), the same final concentration of enzyme was obtained in buffer before addition of magnesium and substrate. Thus, in the first case the enzyme would have little chance of dissociating before coming into contact with magnesium. In the second case, dissociation would proceed and addition of magnesium might be expected to reverse it only with a perceptible delay. The inset to Figure 3 compares the enzyme activities in the

TABLE 3. *Dependence of activity of enolase on prior dilution.*

Present before dilution	Initial Activities*	
	MS added first	Enzyme added first
0.01 *M* $MgCl_2$	0.179	0.141
Excess EDTA	0.152	0.016
1 *M* KCl, 0.01 *M* $MgCl_2$	0.148	0.148
1 *M* KCl, excess EDTA	0.122	0.018

Aliquots of enzyme from the solutions listed were mixed with the assay buffer before addition of magnesium and substrate (second column) or after addition (first column). Other details are given in the legend to Fig. 3.

* As ΔD_{230}/min.

two instances, and show that this was indeed the case. Preincubation of the enzyme with as little as 7×10^{-6} *M* magnesium largely prevents the loss of activity, which should be considered in the light of the previous finding[6] that in the absence of KCl one mole of tightly bound magnesium produces significant changes in the molecule. The presence of 3×10^{-6} *M* bovine serum albumin in the assay medium or a coating of dichlorodimethylsilane on the cuvettes does not alter these results, so significant adsorption of protein to the walls of the cuvette is not thought to occur.

When magnesium is added, the activity is recovered—slowly at assay concentrations (10^{-8} *M*), but more rapidly at higher protein concentrations (Fig. 4). With suitable precautions (above), the initial rates of reaction may be considered a measure of the number of active molecules present.

The recovery of activity upon addition of magnesium is more rapid in the absence of potassium chloride than in its presence (Fig. 4). Figure 4 shows initial

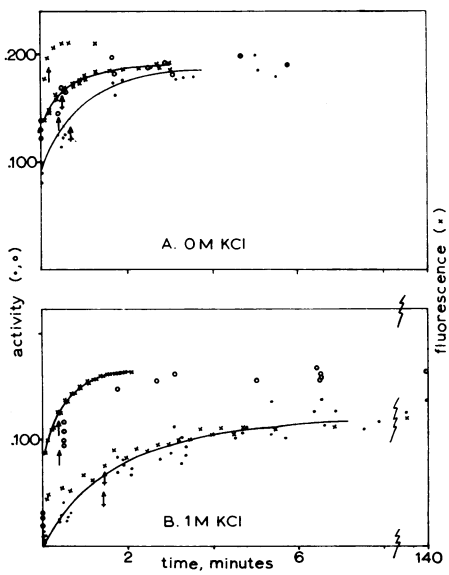

FIG. 4.—Fluorescence and activity of enolase preincubated in excess EDTA as a function of time after addition of excess magnesium. Solutions containing 0.2 mg/ml (*open circles*) or 0.02 mg/ml (*closed circles*) enolase, 0.05 *M* tris-HCl, pH 7.8, 3×10^{-3} *M* EDTA, and 1 *M* KCl where indicated were incubated at room temperature in cellulose test tubes. The activity of the solutions after addition of 2×10^{-2} *M* magnesium was determined at the indicated times by adding aliquots of the preincubated enzyme to 3 ml of the assay medium of Westhead and McLain[4] and determining the initial rate of change of the absorbance at 230 mμ. The concentration of protein in the assay solutions was 10^{-8} *M*. Activities are expressed as ΔD_{230}/min. "0 Time" activities were determined with a stopped-flow apparatus, as described in the legend to Fig. 4. The arrows indicate the half times of the reactions. The solid lines are theoretical curves drawn assuming a first-order reaction with half times as calculated from the data. The assay temperature was about 25°C. The fluorescence changes in identical enolase solutions to which excess magnesium was added (see legend to Fig. 5) are also presented (*crosses*). They are drawn to different scales for comparison with the activity changes. Samples were run in duplicate.

rates of reaction produced by enolase samples preincubated at 0.2 or 0.02 mg/ml in the presence or absence of 1 M KCl as a function of time after addition of excess magnesium. The recovery of activity is slower at the lower protein concentration. The kinetics are approximately first order, and are unaffected by the presence of 10^{-5} M bovine serum albumin. The addition of substrate as well as magnesium, or addition of different amounts of magnesium, to concentrations of 5×10^{-3} to 2×10^{-2} M, also has no effect. Enzyme preincubated with magnesium can be added as well as excess magnesium with no effect on the kinetics. Internal controls showed no significant adsorption of protein to the walls of the cellulose preincubation tubes.

The specific activity of enolase in 1 M KCl decreases somewhat at low protein concentrations even in the presence of excess magnesium (Fig. 4). This is thought to be the result of a partial dissociation of the enzyme occurring at these protein concentrations, and is reversible by dialysis.

A variety of control experiments, including intentional addition of ferrous or ferric iron, calcium, or barium ions to the preincubation solutions, and, conversely, extensive extraction of the buffer and potassium chloride stock solutions with dithizone have indicated that trace metals in the solutions do not influence the results.

Examination of the fluorescence changes[6, 13a] occurring on addition of magnesium reveals a similar dependence of the kinetics upon protein concentration (Fig. 4). In the presence of 1 M potassium chloride, addition of magnesium produces a fast (within 10 msec) initial increase in fluorescence, followed by a slower first-order increase (Figs. 4 and 5). In the presence of 1 M KCl, in which

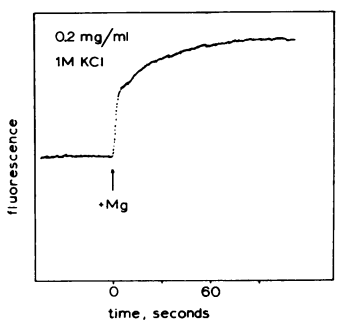

FIG. 5.—Fluorescence changes in enolase preincubated in 1 M KCl after addition of excess magnesium. Two ml of a solution containing 0.2 mg/ml enolase, 1 M KCl, 3×10^{-3} M EDTA, and 0.05 M tris-HCl, pH 7.8, was equilibrated to 25°C in the dark. The solution was then excited at 296 mμ, and the emission at 315 mμ was recorded with time, as 10 μliters of 2 M $MgCl_2$ was added and mixed by hand using a Teflon mixer. The mixing time was 4–7 sec. Control experiments showed that mixing for up to at least 10 sec or mixing by inversion did not alter the results.

the enzyme is largely dissociated, the slower changes are half of the total increase in fluorescence, which under the conditions employed here is about 50 per cent. In the absence of KCl, the extent of the slower changes in fluorescence, which also appear to be first order, increase with decreasing protein concentration. They disappear at concentrations of about 2 mg/ml (data not shown). In both cases, the half times of the fluorescence increase are correlated, within experimental error, with half times of recovery of activity.

Discussion.—We interpret these results as indicating that, in the absence of

magnesium and KCl, enolase at low concentrations dissociates into inactive halves. This dissociation is about 50 per cent complete at 3×10^{-7} M enzyme (Fig. 4) and 85 per cent complete at assay concentrations (10^{-8} M). Potassium chloride increases the subunit dissociation constant to perhaps 10^{-4} M,[13b] apparently by producing an extensive unfolding or swelling of the molecule.[13a] This is reversed to some extent on reassociation. With or without KCl, the dissociation constant is lowered perhaps 1–2 orders of magnitude[13b] by magnesium. This reassociation, which produces the slower fluorescence increases, requires times of up to a few minutes to be completed, depending on the protein concentration.

It has been demonstrated[6] that in the absence of potassium chloride, only one mole of tightly bound magnesium is needed to produce a conformational change in the enzyme. In 1 M potassium chloride, the binding constant of the magnesium producing the change in molecular weight is reduced to 2×10^{-5} M,[13a] but it is still likely that only one mole is required. We propose that one mole of magnesium combines with one of the subunits, converting it into a form with a much higher affinity for the second half of the enolase molecule.

The addition of magnesium to enzyme preincubated in 1 M potassium chloride produces changes in fluorescence and fluorescence-polarization spectra, absorption, polarization of fluorescence, thermal stability, activity, dye binding, sedimentation velocity, and molecular weight.[13a] Using the changes in fluorescence, sedimentation velocity, and activity as criteria, reversibility complete within experimental error has been demonstrated. By alternate additions of excess EDTA and magnesium, complete recovery of activity has been demonstrated over at least four cycles. We therefore feel that the use of equilibrium terminology in describing these results is appropriate.

Data previously published was interpreted by us as showing that yeast enolase at 1–6 mg/ml swells in the absence of high concentrations of potassium chloride upon removal of magnesium. This interpretation was based largely on the finding of a slightly decreased $s^0_{20,w}$ and an increased rotational relaxation time on addition of excess EDTA. The former finding could be explained by a partial dissociation, and on reexamination of our data a slight broadening of the schlieren pattern in ultracentrifugations of samples containing excess EDTA could be detected. However, this could arise from a heterogeneity of shape in what is probably a predissociated state of the molecule. The polarization of fluorescence data also are consistent with a swelling rather than a dissociation. And the low concentration dependence of the sedimentation constants suggests that no appreciable dissociation was occurring over the range of 1–6 mg/ml of protein. Consequently, we feel that the interpretations advanced in reference 6 are still the simplest compatible with the available evidence.

Since enolase from yeast consists of two chains, the data of Westhead mentioned in the introduction can only be interpreted as indicating that the two chains have a different amino acid sequence. It is therefore of possible significance that enolase from rabbit muscle has been shown to consist of two probably dissimilar chains, each of about 42,000 mol wt.[14]

Summary.—Yeast enolase is shown to consist of two inactive subunits of approximately 34,000 mol wt. High concentrations of potassium chloride facilitate dissociation, though equivalent concentrations of potassium acetate do not. Magnesium ion lowers the subunit dissociation constant. From the kinetics of recovery of activity and the fluorescence changes occurring upon addition of magnesium to enzyme in 1 *M* potassium chloride, it is concluded that one mole of magnesium combines with one subunit, producing some change in the half-molecule which increases its affinity for the other subunit.

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