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INVESTIGATION OF CONFORMATIONAL CHANGES IN YEAST ENOLASE USING DYNAMIC FLUORESCENCE AND STEADY-STATE QUENCHING MEASUREMENTS

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Conformational changes in yeast enolase were investigated using steady state quenching and dynamic (fluorescence decay and fluorescence anisotropy decay) measurements. The tryptophan fluorescence rotational correlation time increases from 24 to 38 ns on subunit association. The acrylamide quenching constant decreases two-fold when the subunits associate. The conformational metal ion effect suggests a more compact molecule. Under conditions of catalysis, the correlation time decreases 25%, though the sedimentation constant does not change (Holleman, 1973). The enzyme may undergo a hinge-bending motion during catalysis.

Yeast enolase (2-phospho-D-glycerate hydrolyase, E.C.4.2.1.11) has been shown to undergo three conformational changes:

a. Subunit association/dissociation. The enzyme as isolated is dimeric (M_r=93,345) (1,2). Subunit interaction is hydrophobic in character (3,4,5). Chaotropic salts induce subunit dissociation in the presence of EDTA. Dissociation is accompanied by a "hydrophilic (red) shift" in the fluorescence properties of the enzyme (3).

b. The enzyme binds one mole/subunit of divalent cation (6,7). This produces a conformational change (3,8) so the metal ion producing this is called "conformational". The conformational change affects subunit association but occurs in monomeric or dimeric enzyme (9,10). The conformational change is accompanied by a "hydrophobic (blue) shift" of tryptophan fluorescence (3). Binding of conformational metal ion is necessary for substrate binding (6,7).

c. Substrate binding to the enzyme-conformational metal ion complex does not result in significant activity (11). If excess divalent metal ion is added, an additional mole of metal ion/mole of subunit will bind (7,11). If the two metal ions bound/subunit produce activity (Mg^{2+} or Zn^{2+}, for example), a conformational change results which involves an increase in tryptophan fluorescence (12). The second metal ion is called

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"catalytic" metal ion. Substrate or analogue must bind before catalytic metal ion will (11; reviewed in ref. 2).

We reinvestigated these conformational changes using dynamic fluorescence methods.

MATERIALS AND METHODS

Yeast enolase was prepared by the method of Westhead and McLain (13) modified as described in reference 7. Tris (Eastman Kodak) was twice recrystallized from 95% ethanol. Potassium chloride was "Ultrapure" fro Alfa Products (Danvers, Mass.). The substrate, 2-phospho-D-glycerate (N salt) was from Sigma. Acrylamide (Kodak) was twice recrystallized from benzene. Other chemical were reagent grade. Water was filtered and deionized (Continental Deionized Water Corp.).

Enzyme solutions were prepared for use immediately before excitation from stock enzyme solutions. The latter were assayed for activity as described by Westhead (14). A Bausch and Lomb Spectronic 200 was used f routine spectrophotometric measurements. Measurement of pH was carried out with an Orion 701A Ionalyzer. Steady state fluorescence measurement were made using a SLM 8000C spectrofluorometer. Fluorescence and fluorescence decay measurements were made using an instrument from Appli Photophysics as described in reference 15.

Protein solutions with or without appropriate ligands are incubated various temperatures of interest and illuminated by short (ca.10 ps) pulses of light. Excitation is at 300 nm or greater wavelengths to minimize energy transfer among tryptophans (16) and to ensure only tryptophans are excited. Samples made up separately and excited gave th same results as samples which were excited then ligands (Mg^{2+} and/or substrate) added and excited again. This suggests absence of photodegredation effects.

Data collection and analysis has been described repeatedly in the literature (17); basically, statistical criteria (chi squared and Durbin-Watson parameter values) are employed to provide acceptable fits t models of fluorescence decay (18). In the case of anisotropy decay, fit are obtained by minimizing chi squared values, and Durbin-Watson parameters are used to indicate the goodness of the fits. Fits of lifetime data are made to the peak channel of the fluorescence signal. W were able to fit the anisotropy decay data to within two or three channe (0.14-0.21 ns) of that of the prompt maximum to a two-exponential model with good statistics. The shorter correlation time (<2 ns) corresponds t segmental motion ("wobble") (not shown). We present only the longer of the two correlation times we obtained, the one reflecting motion of the enolase molecule as a whole.

RESULTS

a. Lifetime measurements

Although yeast enolase has five tryptophans per subunit (19), we have gotten satisfactory fits to lifetime decay profiles with three exponentia terms (Table I). Since even single tryptophans in proteins often give complex decay patterns (20,21), the decay parameters should not be considered to represent single tryptophans.

Subunit association and Mg^{2+} binding conformational changes are both associated with a hydrophobic (blue) shift in fluorescence suggesting decreased exposure of tryptophans to the solvent (1,8). Addition of Mg^{2+} to dimeric enzyme produces little effect on the parameters (last three lines of Table). ("KOAc" is potassium acetate). Where subunit association results, the longest-lived component increases in magnitude,

Table I. Effect of State of Enolase on Fluorescence and Anisotropy Decay Parameters

Solvent	Addition	State	Intrinsic Fluorescence[a]								Anisotropy[c]	
			α_1	τ_1	α_2	τ_2	α_3	τ_3	τ_{av}	k_q[b]	ϕ ±	SD
1M KCl	EDTA	85% monomer	32	0.7	39	2.5	28	5.1	2.7	1.9	24.1	0.6
1M KCl	Mg	holodimer	27	0.7	41	2.7	32	4.3	2.7	1.0	38	7
1M KCl	Mg,S	turnover	20	0.6	22	2.1	58	4.3	3.1	-	27	0.1
Buffer	EDTA	25% monomer	23	0.6	37	2.5	40	4.7	3.0	1.7	40	1
Buffer	Mg	holodimer	24	0.6	31	2.2	46	4.3	2.8	0.9	47	8
Buffer	Mg,S	turnover	19	0.8	26	2.8	55	4.7	3.5	0.7	35	7
1M KOAc	EDTA	0% monomer	20	0.5	25	2.1	55	4.1	2.9	0.9	49	8
1M KOAc	Mg	holodimer	19	0.6	23	2.1	58	4.1	2.9	0.7	43	5
1M KOAc	Mg,S	turnover	17	1.0	26	3.1	57	4.6	3.6	-	35	1

a) Yeast enolase (6 or 16 μM) in 0.05 ionic strength Tris-HCl, pH 7.8, 0.5 -1mM EDTA, plus additions as noted, was excited at 300, 305 or 308 nm and 22°. Emission was taken, centered at 340 or 349 nm using a 23 nm slitwidth. Final concentrations of Mg^{2+} were 2-4 mM and final substrate concentrations (added as 2-phospho-D-glycerate) were 1 mM. Some samples were made up separately before excitation and some were reexcited after addition of ligands (Mg^{2+} and/or substrate). Fluorescence decay parameters are averages of two to four independent measurement except for the last entry. "α_i" values are percentages of the total decay and "τ_i" values are lifetimes and are in ns. The average lifetimes are calculated from $\Sigma\alpha_i\tau_i/100 = \tau_{av}$ Average lifetimes (τ_{av}) have percentage deviations ranging over 1-13% and average ± 5%. Values of chi squared ranged from 1.00 to 1.42 and Durbin-Watson parameters from 1.65 to 2.06; seventeen of twenty-three values were 1.80 or greater.

b) Average collisional quenching constant, taken from Stern-Volmer plots. The quencher was 0.02-0.20 M acrylamide (four concentrations). Excitation was at 300 nm, and emission was measured at 340 nm. Bandwidths were 2 nm. Values are averages of two measurements taken over a period of over one year and are corrected for the viscosities of the solvents. Deviations from the mean ranged from 0 to 13%, the average being 6%. Units are $M^{-1}s^{-1} \cdot 10^9$.

c) Rotational correlation times ± standard deviations. The correlation times are in ns and are corrected for the viscosity of the solvents. The values were obtained using a two-exponential fit, but only the longer correlation times are given. The shorter ones were all less than two ns (see text). Durbin-Watson parameters ranged from 1.70 to 2.20; all save one were above 1.80. Values of correlation time were obtained from experiments done over a period of more than a year.

ut is shortened (compare lines 1 and 2, 4 and 5). Addition of substrate s well always produces an increase in average lifetime: in ten sets of amples (the Table shows average data) we obtained a 12 ± 5% increase. ddition of substrate in the presence of excess Mg^{2+} produces a 16% ncrease in quantum yield (12). The conformational change involving catalytic" metal ion binding involves a reduction in collisional uenching of tryptophan(s) in the enzyme.

b. Steady-state quenching measurements.

We measured the quenching effectiveness of acrylamide to see if changes in tryptophan exposure could be demonstrated. Quenching constant were calculated from Stern-Volmer plots (18). These are presented for th different protein conformations in Table I.

The values of average quenching constant are similar to those obtained using acrylamide with several native globular proteins such as bovine serum albumin (15,22,23). The data suggest that binding conformational Mg^{2+} does reduce quenching constants ca. 18% (lines 7 and 8). Subunit association is accompanied by a two-fold decrease in quenching constant (lines 1 and 2), indicating sharply decreased exposure of tryptophan(s). Trp-367 lies at the subunit interface (Lebioda et al., in preparation).

c. Fluorescence anisotropy decay

Tryptophanyl- anisotropy decay measurements were also made in these solvents. Correlation times are also presented in Table I. All are corrected to the temperature and viscosity of water at 22°.

The rotational correlation time of the enzyme calculated from M_r 93,345, V=0.742 and the relatively precise sedimentation constant (5.87 ± 0.03 S) (2) is 46 ± 0.9 ns in water at 22°. This is in good agreement with the average correlation time (Mg^{2+} in buffer only) of 47 ± 8 ns (four measurements). These correlation times agree with previously measured hydrodynamic properties of the enzyme.

The effect of subunit association is clear. On adding excess Mg^{2+} to enzyme in 1M KCl and 1 mM EDTA, the correlation time increases from 24.1 ± 0.6 ns (three measurements) to 38.4 ± 7.3 ns (four measurements). In the presence of buffer only, addition of excess Mg^{2+} produces a smaller increase, as expected.

The effect of conformational Mg^{2+} on yeast enolase has been suggested to involve a contraction or increase in rigidity of the protein (8). The enzyme is fully dimeric in 1M potassium acetate. In three sets of measurements, the correlation times of enzyme in 1M potassium acetate and excess Mg^{2+} were 89 ± 10% relative to values in 1M potassium acetate and excess EDTA obtained in the same series of experiments. This is consistent with previous findings of a 13% decrease in correlation time c binding conformational Mg^{2+} (8). It is not consistent with increased overall rigidity, which would produce an increase in correlation time. This effect would counteract the effects of Mg^{2+} - induced subunit association in 1M KCl and in buffer only.

On addition of substrate to the enzyme in the presence of excess (1-4 mM) Mg^{2+}, catalysis results (8,11,12). This is always accompanied by a lower correlation time (twelve times in twelve attempts). If we measure correlation times in the presence of excess Mg^{2+} and substrate relative to correlation times in the presence of excess Mg^{2+} only obtained in the

same experimental series, we find the average relative correlation time is 75 ± 10% in buffer only (four measurements), 76 ± 18% in 1M potassium chloride (three measurements) and 85 ± 11% (two measurements) in 1M potassium acetate. The enzyme is inhibited 50-75% in those salt solutions with 1 mM Mg^{2+}, but this is partly due to an increase in Mg^{2+} Michaelis constant. Use of the same experimental series as the basis of comparison has been employed previously (3,8,12).

We carried out additional measurements to confirm this finding (not shown in Table I). We measured the correlation times in the normal assay medium, with or without 2-phosphoglycerate, at 22°, the normal assay temperature in this laboratory and at 30°, the temperature used by Westhead (14). At 22°, we obtained a longer correlation time of 50.3 ns in the presence of Mg^{2+} only and 37.3 ns with substrate also, which was 74% of the former value. At 30°, we obtained 45.7 ns (corrected to 22°), in the presence of Mg^{2+}. With substrate also, the observed longer correlation time was 31.1 ns (corrected to 22°). The latter value was 70% of the control value, in the presence of Mg^{2+} only. We conclude catalysis reduces the correlation time of the enzyme by about 25%.

DISCUSSION

We believe this is the first time subunit association has been demonstrated using intrinsic fluorescence anisotropy decay. We believe this is also the first demonstration of the effect of catalysis using this technique.

We find these natural tryptophan emission anisotropy data are of comparable precision, an average standard deviation (excess Mg^{2+} present) of 16% compared with 19% for dansyl conjugates (2,3,8). This was despite the relatively short lifetime of excited state of the tryptophans in this protein, ca. 3 ns, compared with average lifetimes in other proteins (24). It is noteworthy that without the capability for making lifetime measurements then we were unable to demonstrate even the effect of subunit association (3).

The origin of the lower correlation time under conditions of catalysis could be from subunit dissociation, from increased energy transfer between tryptophans or from acquisition of flexibility. Subunit dissociation does not seem to be a factor. The sedimentation constant under conditions of catalysis is not significantly different from that in its absence (25; J.M. Brewer, unpublished observations). Energy transfer should be low at 300 nm. However, we obtained the same effect on excitation at 305 or 308 nm (Table I) and energy transfer fails at the red edge of absorption bands (16).

Enolase monomers are organized into two domains (26). If a "hinge bending" flexibility developed on substrate and catalytic Mg^{2+} binding,

this would explain our results. This would involve movement of a substantial part of each subunit relative to the rest of the dimer. Hing bending domain movements, especially during catalysis, are subjects of intense interest (27,28).

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