Hence, the addition of HPO_4^{2-}, AsO_4^{2-}, or NADH[5] to FA enzyme at suboptimal concentrations shows a common slow phase of reaction velocity with a specific rate or approximately 0.01 s^{-1}, as in the slow phase of spectral perturbation. A common limiting rate of turnover has been observed in the saturated rates with glyceraldehyde-3-PO_4 or 1,3 diphosphoglycerate (2) presumably due to a corresponding conformational change in the intermediate 3-phosphoglyceroyl enzyme. In this case, the specific rate is approximately 10^3-fold faster, thus indicating a synergistic effect of acyl-structure and protein tertiary structure.

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STUDIES OF THE ACTIVATION OF YEAST ENOLASE BY METALS USING A "TRANSITION STATE ANALOGUE"

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Yeast enolase, a dimeric protein, binds up to 2 mol of "conformational" Mg, which enables up to 2 mol of substrate or competitive inhibitor to bind, which in turn enables more Mg to bind. The latter Mg produces the actual catalysis. A putative "transition state analogue," aminoenolpyruvic acid-2-phosphate (AEP), synthesized by Spring and Wold, exhibits a large 295-nm difference spectrum upon binding to enolase with Mg present, permitting the monitoring of binding of AEP, conformational Mg, "catalytic" Mg, and other metals. Spectrophotometric titrations and stopped-flow measurements have led to some tentative conclusions:

(*a*) The strength of catalytic Mg and AEP binding is interdependent, consistent with an ordered sequence of addition. With saturating AEP, about two-thirds of the 295 nm absorbance change occurs on addition of "conformational" Mg, the rest on adding catalytic Mg (Fig. 1).

(*b*) The nonactivating metals Ca, Hg, and Ba do not give this 295-nm absorbance change though at least Ca binds at the same sites as Mg, with a similar affinity and effect on the protein. Ni (a weak activator), Mn (intermediate), and Mg (best) give

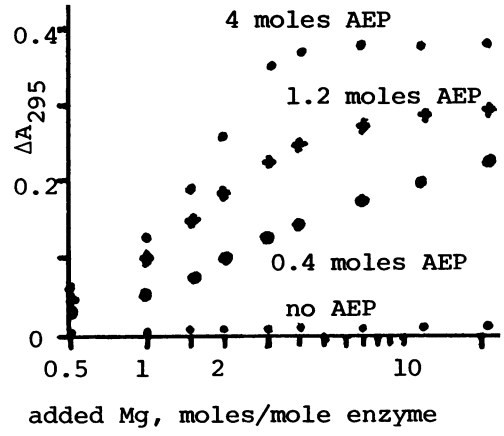

FIGURE 1

the full change—the reactions appear to be all or none (Table I). This suggests that the rate of the reaction is determined by the catalytic metal, a suggestion supported by stopped-flow experiments in which enzyme with Mg, Ca, or Ni is mixed with substrate and additional Ca, Ni, or Mg (Table II). These observations suggest that the conformational metal plays a key role in substrate activation, rather than passively providing only a binding site.

(*c*) Calorimetric measurements of the enthalpy of mixing the competitive inhibitor 3-phosphoglyceric acid with the Ca-enzyme show that the inhibitor does bind. In addition, stopped-flow measurements of the effect of the substrate and AEP on the 295-nm absorbance change produced on adding excess EDTA to the Ca enzyme or the Mg enzyme show that the substrate slows the rate of the reaction from 0.9/s to 0.7/s while AEP reduces it a factor of two, to 0.45/s (Fig. 2). This is consistent with the suggestion that AEP is a transition state analogue and also shows that the different metals behave identically as far as enabling substrate or AEP to bind. The difference between activating and nonactivating metals is that the nonactivating ones do not cause the change in AEP absorbance.

TABLE I

Metal	Activating?	ΔOD_{295}: moles metal/mole enzyme		
		1	2	100
Mg	Yes	0.089	0.189	0.284
Mn	Yes	0.110	0.214	0.298
Ni	Yes	0.098	0.186	0.290
Ca	No	0.014	0.018	0.006
Ba	No	0.009	0.016	0.009
Cu	No	0.012	0.021	—
Hg	No	—	0.001	—

TABLE II

Moles metal/ moles enzyme (initial)	Moles/mole added with substrate	Initial activity
		%
2 Mg	2 Mg	1.16 (100)
2 Mg	2 Ni	0.42 (36)
2 Ni	2 Mg	0.62 (54)
2 Mg	2 Ca	0.38 (32)
2 Ca	2 Mg	0.20 (17)
2 Ni	2 Ni	0.16 (14)

These observations also suggest that the "conformational" metal interacts directly with the substrate in the transition state. This suggestion is supported by stopped-flow experiments in which the AEP is displaced with excess substrate: the rate of the reaction is 0.08/s, lower than that produced by addition of EDTA or excess Mg (to the Ca enzyme) (0.14/s) or Ca (to the Mg enzyme) (0.16/s). And the observed rates of apparent metal loss or displacement in the presence of AEP are much lower than the rates of metal loss in the absence of AEP, monitored with chlorophosphonazo III, a chromophoric metal-chelating agent (2/s).

(*d*) The subunits of enolase dissociated in the absence of magnesium are inactive because the substrate (AEP) binding site has been affected. Stopped-flow measurements indicate that this site is restored only after subunit association (Fig. 3).

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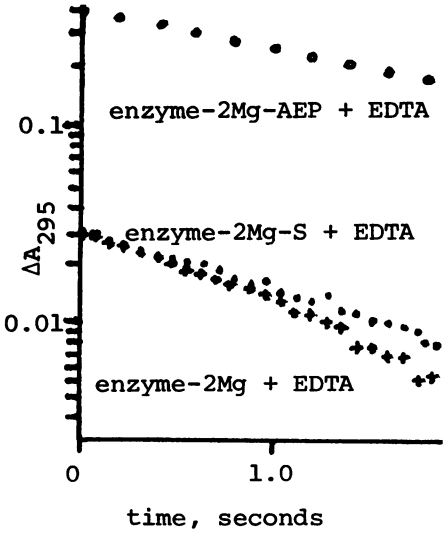

FIGURE 2

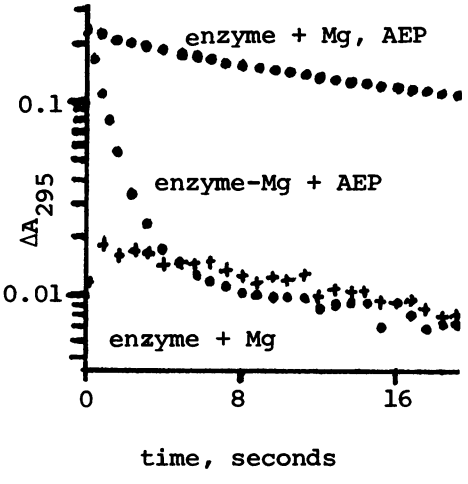

FIGURE 3