# Intermediates in Enolase-Catalyzed Reactions†

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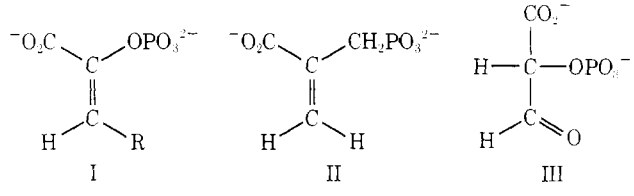
ABSTRACT: Temperature-jump, stopped-flow, combined stopped-flow temperature-jump and steady-state kinetic methods have been used to study reactions catalyzed by enolase (EC 4.2.1.11) and interactions of inactive 2-phosphoenolpyruvate (PEP) derivatives with the enzyme. Fast unimolecular steps are observed in the spectral region 230–260 nm with the substrates PEP ($t_{1/2} \simeq 1$ msec, 11°), (*Z*)-phosphoenol-3-fluoropyruvate ($t_{1/2} \simeq 30$ msec, 4°) and (*Z*)-α-(dihydroxyphosphinylmethyl)acrylate ($t_{1/2} \simeq 10$ msec, 4°). D-Tartronate semialdehyde phosphate (TSP) binding is consistent with the two-step mechanism: E + TSP ↔ E·TSP ↔ E·TSP′, with $K_1 = 7.7 \times 10^{-5}$ M, $k_2 = 0.19$ sec$^{-1}$, $k_{-2} = 0.01$ sec$^{-1}$, 4°. The competitive inhibitors (*Z*)-phosphoenol-α-ketobutyrate (3-$CH_3$PEP) and 3-aminoenolpyruvate 2-phosphate (AEP) bind according to one-step association-dissociation mechanisms: E + I ↔ E·I. For 3-$CH_3$PEP, $k_1 = 2.8 \times 10^5$ M$^{-1}$sec$^{-1}$, $k_{-1} = 7$ sec$^{-1}$, 4°; for AEP, $k_1 = 2.5 \times 10^4$ M$^{-1}$sec$^{-1}$, $k_{-1} < 10^{-2}$ sec$^{-1}$, 4°. No evidence for intermediary carbanions (Healy, M. J., and Christen, P. (1973), *Biochemistry 12*, 35) was found in trapping experiments employing easily reducible dyes, either at pH 7.8 or 9.2. Results are interpreted in terms of a molecular mechanism for catalysis, the salient feature of which is the induction of strain in enzyme-bound substrate by isomerization of the initially formed enzyme–substrate complex.

Molecular mechanisms for catalytic action embodying one or another of virtually every conceivable factor as central to reactivity have been proposed (*cf.* Jencks, 1969). Resolution of alternative models in specific enzyme-catalyzed reactions has proved difficult since information on the structural and dynamic properties of reactive intermediates is generally not available and data from steady-state kinetic studies and structural determinations of unreactive enzyme–inhibitor complexes can be applied only obliquely to this point.

Fast kinetic methods are well suited to the study of intermediates in enzyme-catalyzed reactions (*cf.* Hammes, 1968); both rate and structural information can often be had (*cf.* Hammes and Haslam, 1969). In this paper we examine the behavior of detectable intermediates in reactions catalyzed by yeast enolase (phosphoenolpyruvate hydratase or 2-phospho-D-glycerate hydrolyase, EC 4.2.1.11). Transient kinetic binding patterns and intermediate spectral properties of phosphoenolpyruvate (PEP)[1] and modified derivatives are correlated with thermodynamic data for analog binding to provide evidence for a molecular mechanism in which activation is caused by substrate distortion in the active site arising from isomerization of the initially formed enzyme–substrate complex. Structures of analogs used in this study are



I II III

for I, R = H, 2-phosphoenolpyruvate (PEP); R = F, (*Z*)-phosphoenol-3-fluoropyruvate (3-FPEP); R = $CH_3$, (*Z*)-phosphoenol-α-ketobutyrate (3-$CH_3$PEP); R = $NH_2$, 3-aminoenolpyruvate 2-phosphate[2] (AEP). II, (*Z*)-α-(dihydroxyphosphinylmethyl)acrylate ($CH_2$PEP). III, D-tartronate semialdehyde phosphate.

## Experimental Section

### *Materials*

Enolase was isolated and purified from baker's yeast (Fleishman's) by the method of Westhead and McLain (1964). Enzyme preparations were initiated on the day of arrival of fresh yeast at the local distributor's warehouse. Specific activities for purified enolase were 100–110% that originally reported (Westhead and McLain, 1964); residual activities of metal-free enzyme were <1% of the specific activities in assay solutions. Atomic absorption analyses of purified apoenzyme solutions gave a ratio of $Mg^{2+}$ to enolase active sites of *ca.* 0.07, *i.e.*, 15% $Mg^{2+}$ bound to the first metal binding site (Hanlon and Westhead, 1969a).

D(+)-2-Phosphoglyceric acid (PGA) and phosphoenolpyruvic acid (PEP) were generally purchased as their trisodium salts. Stock solutions of substrate ($6 \times 10^{-3}$ M), stored at pH 7 and 4°, were prepared fresh every few days. In some experiments barium salts of PGA were used as an alternative source of substrate. Reagent solutions were prepared by removal of barium by cation exchange on Bio-Rad AG50W-X8 resin in the hydrogen form. No differences in the behavior between the two sources of PGA were noted, either in the standard assay solution or in other kinetic experiments.

Cyclohexylammonium dihydrogen phosphoenol-α-ketobutyrate [*Z* isomer] (3-$CH_3$PEP) was a generous gift from Dr. George L. Kenyon.

Cyclohexylammonium dihydrogen phosphoenol-3-fluoropyruvate [*Z* isomer] (3-FPEP) was synthesized by the method of Stubbe and Kenyon (1972). The proton magnetic resonance (pmr) spectrum was in agreement with published values for the *Z* isomer, but indicated a few per cent contamination by the *E*

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[1] Abbreviations used are: PGA, 2-D-phosphoglycerate; PEP, 2-phosphoenolpyruvate; 3-FPEP, (*Z*)-phosphoenol-3-fluoropyruvate; 3-$CH_3$PEP, (*Z*)-phosphoenol-α-ketobutyrate; $CH_2$PEP, (*Z*)-α-(dihydroxyphosphinylmethyl)acrylate; AEP, 3-aminoenolpyruvate 2-phosphate; TSP, D-tartronate semialdehyde phosphate.

[2] *Z,E*-isomeric composition unknown (Spring and Wold, 1971a).

isomer. Several recrystallizations from methanol–ether failed to alter the percentage contamination. α-(Dihydroxyphosphinylmethyl)acrylic acid ($CH_2$PEP) was synthesized by the method of Stubbe and Kenyon (1972). Proton magnetic resonance data were in agreement with their published spectra. D-Tartronate semialdehyde phosphate (TSP) and 3-aminoenolpyruvate 2-phosphate (AEP) were prepared by the methods of Spring and Wold (1971a). TSP and AEP concentration levels in reaction solutions were determined from their ultraviolet (uv) absorption spectra (Spring and Wold, 1971a).

Repeated attempted preparations of D-erythronic acid 3-phosphate by the method of Wold and Barker (1964) were unsuccessful.

Phosphoglycolic acid, porphyrexide, porphyrindine, potassium hexacyanoferrate(III), 2,6-dichlorophenolindophenol, Tris, metal salts, and other reagents were obtained from commercial sources and used without further purification. All water was passed through a Millipore filter system prior to use.

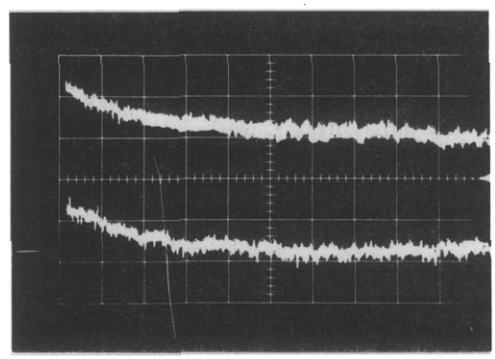

FIGURE 1: Oscilloscope traces for relaxations observed at 255 nm in enolase–substrate solutions. Amplitude: 5 mV/cm; sweeptime: 500 μsec/cm. Upper curve: combined stopped-flow temperature-jump measurement, (enolase) = $4.5 \times 10^{-5}$ M, (PEP) = $1.0 \times 10^{-3}$ M, ($Mg^{2+}$) = $1.0 \times 10^{-2}$ M, pH 7.7, 12°, perturbation applied 100 msec after mixing. Lower curve: temperature-jump measurement of the equilibrated solution under identical conditions.

*Methods*

Enolase, TSP, AEP and porphyrexide concentrations were determined spectrophotometrically. For enolase, active-site concentrations were calculated assuming $\epsilon_{280} = 3.9 \times 10^5$ $M^{-1}$ $cm^{-1}$, based upon an absorbance of 0.89 for 1.0-mg/ml solutions of protein (Warburg and Christian, 1941) and a subunit molecular weight of 44,000 (Mann *et al.*, 1970); TSP was determined as its enolate ion in 1 M sodium hydroxide, for which $\epsilon_{260} = 1.2 \times 10^4$ $M^{-1}$ $cm^{-1}$ (Spring and Wold, 1971a); for AEP, $\epsilon_{260} = 8.4 \times 10^3$ $M^{-1}$ $cm^{-1}$ in alkaline solutions (Spring and Wold, 1971a); for porphyrexide, $\epsilon_{420} = 670$ $M^{-1}$ $cm^{-1}$ (pH 8.0) (Healy and Christen, 1973). Concentrations of other reagents were calculated from weighed samples dissolved and diluted to measured volumes. Ultraviolet spectral measurements, enzyme assays, and steady-state kinetic runs were made on a Cary Model 16 spectrophotometer equipped with accessories for continuous recording and thermostatted for accurate temperature control.

Fast kinetic studies (rapid mixing and perturbation relaxation) were made on a combined stopped-flow temperature-jump spectrophotometer, the design of which is similar to that developed by Hammes and coworkers (Faeder, 1970). The mixing dead time for the instrument is *ca.* 15 msec. For temperature-jump experiments, a temperature rise of *ca.* 8° is effected in less than 15 μsec when a 10-kV pulse from a 0.1-μF capacitor is passed through solutions of ionic strength, $\mu = 0.1$ M. Relaxations occurring in the time range of 30 μsec to 50 msec are readily amenable to investigation. In combined flow temperature-jump measurements perturbation can be electronically timed to occur from 1 msec to 2 sec after cessation of flow. Absorption spectrophotometry was used to monitor reactions. Monochromatic light was obtained from either of two sources, a 100-W tungsten–iodide lamp or 200-W high-pressure mercury–xenon arc lamp, passed through a Bausch and Lomb high-intensity grating monochromator; the spectral band pass varied from *ca.* 4–22 nm, depending upon solution absorbancies. Relaxation phenomena were sought in the ultraviolet spectral region (230–300 nm); additionally, the indicator dye Phenol Red ($\lambda_{max}$ 558 nm) was used to monitor rapid changes in solution acidities.

Relaxation times were determined graphically or by curve matching with an exponential curve generator attached to an oscilloscope. In the latter case, the original stored oscilloscope trace of a relaxation process was reproduced on positive transparency film, the image being identical in size with the original trace. Adjustments were made in the amplitude and time constant of an exponential curve displayed on the screen from the curve generator until a best fit with the superimposed transparency was obtained. The curve generator was calibrated so that relaxation times could be read directly from the position of the potentiometer dial controlling the line shape. Duplicate analyses of a given curve were reproducible to within 5% by this method. Rate constants from conventional and stopped-flow kinetic runs were determined graphically; steady-state rates for enolase-catalyzed reactions were determined from initial slopes of reaction curves.

Results

*Interactions of Metal Ions with Enolase.* A single fast relaxation ($\tau \sim 60$ μsec) of very low amplitude (<0.1% total signal) was detected at 295 nm for solutions nominally containing only apoenzyme, (E) = $9 \times 10^{-5}$ M. (Enzyme concentrations are expressed in terms of active-site concentrations assumed to be twice the molar concentrations; Wold, 1971.) The wavelength and direction of amplitude change corresponded to that of maximum absorbance observed in the enolase–$Mg^{2+}$ ion difference spectrum (Brewer and Weber, 1966) and attributed to $Mg^{2+}$ ion binding to the first metal-binding site on the enzyme (Hanlon and Westhead, 1969a). A similar relaxation of low amplitude was observed in enolase solutions containing Phenol Red. Addition of $Mg^{2+}$ in concentrations sufficient to saturate the first metal-binding site, ($Mg^{2+}$) $\geq 10^{-4}$ M, caused both relaxations to disappear. Quantitative study of the phenomenon was obviated by the low amplitudes of the effects, which we tentatively identify with metal binding to enolase. Attempts to observe binding of $Mn^{2+}$, $Co^{2+}$, $Zn^{2+}$, $Ni^{2+}$, or $Ca^{2+}$ ions to the apoenzyme were unsuccessful. No detectable pH changes accompanied perturbation of solutions containing ($Mg^{2+}$) = $10^{-5}$–$10^{-3}$ M, (Phenol Red) = $2 \times 10^{-5}$ M, (KCl) = 0.1 M.

*Interactions of Substrates (PGA, PEP) with Enolase.* Relatively slow relaxations ($\tau_1 = 0.9 \pm 0.4$ msec) of moderate amplitude (~1% total signal) were observed in the spectral region 230–260 nm when solutions containing enolase, $Mg^{2+}$ ion, and equilibrium concentrations of substrates were perturbed (Figure 1). Relaxations were concentration-independent over the ranges, (E) = 4–9 $\times 10^{-5}$ M, ($Mg^{2+}$) = 0.5–20 $\times 10^{-3}$ M, [(PGA) + (PEP)] = 0.6–24 $\times 10^{-4}$ M, pH 7.6–7.8. Loss of amplitude precluded quantitative study of the relaxation at lower enzyme and substrate concentrations. The effect was not observed with Phenol Red indicator.

Combined stopped-flow temperature-jump measurements made under conditions where little (<10%) net conversion of

TABLE I: Stopped-Flow Temperature-Jump Experiments.[a]

| (PGA) Initial (mM) | (PEP) Initial (mM) | (Enolase)[b] (μM) | Delay (msec) | $\tau$ ($msec^{-1}$) |
|---|---|---|---|---|
| 0.95 | | 50 | 25 | |
| 0.95 | | 50 | 2000 | ~1 |
| | 1.0 | 50 | 25 | ~1 |
| | 1.0 | 50 | 2000 | ~1 |

[a] $(Mg^{2+})$ = 1.0 × $10^{-2}$ M; (KCl) = 0.1 M; at 11°, pH 7.8. [b] Concentration of active sites.

substrate had occurred established that the relaxation corresponds to a transient associated with PEP binding to the enzyme. When solutions containing $Mg^{2+}$ and PEP were rapidly mixed with solutions containing $Mg^{2+}$ and enolase, and perturbation applied within a few milliseconds after mixing, the relaxation was observed (Figure 1). In identical experiments with PGA as substrate the relaxation was not observed, but if sufficient time was allowed after mixing for appreciable conversion to PEP to occur, the relaxation was again seen. Experimental conditions are given in Table I.

An additional very slow step ($t_{1/2} \geq 100$ msec) was observed when either of the above pairs of solutions were mixed. Reaction could be monitored either in the uv region, 230–260 nm, or with indicator dye. Amplitudes of the effect were in opposite directions when PGA or PEP were present, uv changes corresponding to the appearance or disappearance of the PEP chromophore, respectively. The time scale is appropriate for the step corresponding to net conversion of substrates. The effect was not observed if any reactant (enolase, $Mg^{2+}$, or substrate) was omitted and quite reasonably can be ascribed to the rate-determining step in the catalyzed reaction.

In the absence of added activator metal ion, solutions of enolase, (E) = 3–5 × $10^{-5}$ M and substrates, [(PGA) + (PEP)] = 0.6–30 × $10^{-4}$ M, exhibited no relaxation phenomena with either uv absorption or indicator dye methods of detection. No relaxations were observed in combined flow-perturbation measurements of solutions of enzyme (5 × $10^{-5}$ M) and PEP (1 × $10^{-3}$ M), but not containing activator metal ions.

*Effect of Divalent Metal Cations.* Solutions of enolase and substrates at equilibrium exhibited fast concentration independent relaxations in the presence of the activator metal ions $Mn^{2+}$, $Co^{2+}$, $Zn^{2+}$, and $Ni^{2+}$. No effect was observed in the presence of $Ca^{2+}$, which is inhibitory. With $Zn^{2+}$ and $Ni^{2+}$ relaxation amplitudes were too low for accurate determination of $\tau$ values; relaxation times in the other reactions are dependent upon the identity of divalent activator ion present. Results are summarized in Table II.

TABLE II: Effects of Divalent Metal Cations on Metal–Enzyme–Substrate Relaxation Process.[a]

| (Metal Ion) (mM) | (Enolase)[b] (μM) | (Substrate) Total (μM) | $\tau$ (msec) |
|---|---|---|---|
| $Mg^{2+}$ (0.5–20) | 40–90 | 60–240 | ~1 |
| $Mn^{2+}$ (0.5–1) | 50 | 70 | ~2 |
| $Co^{2+}$ (0.5–1) | 40 | 60 | ~3 |
| $Zn^{2+}$ (0.5–1)[c] | 40 | 60 | *d* |
| $Ni^{2+}$ (0.1) | 40 | 60 | *d* |
| $Ca^{2+}$ (0.5–1) | 40 | 60 | |

[a] (KCl) = 0.1 M; at 11°, pH 7.5–7.8. [b] Concentration of active sites. [c] 0.05 M imidazole buffer. [d] Relaxation observed, but with very low amplitude.

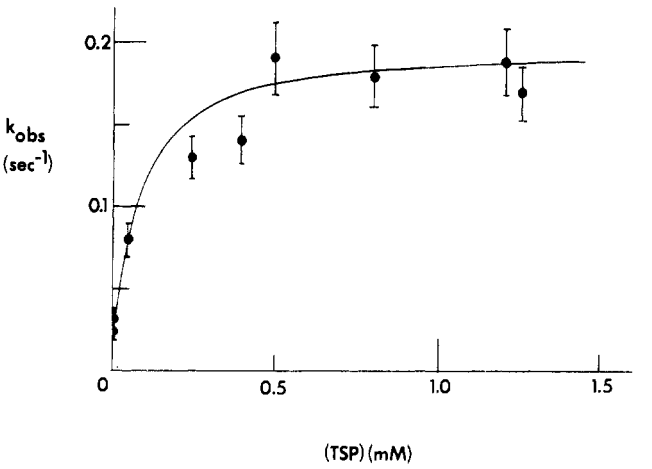


FIGURE 2: Kinetics of TSP binding to $Mg^{2+}$-enolase. The solid line is the theoretical curve for $K_1$ = 7.7 × $10^{-5}$ M, $k_2$ = 0.19 $sec^{-1}$, $k_{-2}$ = 0.01 $sec^{-1}$. Filled circles are averaged values, with error bars indicating the experimental range of individual determinations. Experimental conditions are given in the text.

*Kinetics of Substrate Analog Binding.* Interaction of several substrate analogs with $Mg^{2+}$-enolase were investigated. No relaxation effects were seen in temperature-jump studies over the accessible time range using uv detection methods (230–300 nm). Binding kinetics were observed in certain instances in stopped-flow measurements. In these experiments sufficient excess of analog was used ((S) > 10 (E)), so that pseudo-first-order conditions were obtained.

Binding of TSP to $Mg^{2+}$-enolase, monitored at 254 nm, was observed to be a two-step process (Figure 2). The initial bimolecular association–dissociation step occurred too rapidly to be measured directly on the stopped-flow time scale. Kinetic parameters were determined assuming the mechanism

$$E + TSP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot TSP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E \cdot TSP'$$

Under pseudo-first-order conditions, with the first step in rapid equilibrium, $k_{obsd} = k_{-2} + k_2/(1 + K_1/(TSP))$, with $K_1 = k_{-1}/k_1$. Rate data were analyzed by plotting $[k_{obsd} - k_{-2}]^{-1}$ *vs.* $(TSP)^{-1}$, values for $k_{-2}$ being assumed in trial-and-error fashion until a linear plot was obtained. The calculated rate parameters are: $K_1$ = 7.7 × $10^{-5}$ M, $k_2$ = 0.19 $sec^{-1}$, $k_{-2}$ = $10^{-2}$ sec. The overall dissociation constant, $K_d \simeq 4 \times 10^{-6}$ M, at 4°, calculated from $K_d = K_1/(1 + k_2/k_{-2})$, is in reasonable agreement with the spectrophotometrically determined dissociation constant, $K_d$ = 1.5 × $10^{-5}$ M, determined at 25° by Spring and Wold (1971b).

The substrate analogs 3-MPEP and AEP were found to be competitive inhibitors of PEP in the enolase-catalyzed reaction. Inhibition constants, determined from Lineweaver–Burke plots of initial velocities at 4° in the standard assay medium, are given in Table III. Inhibitor binding was studied using the stopped-flow method. Changes in absorbance were followed at wavelength maxima in the E + I *vs.* EI difference spectra, 245 and 295 nm for 3-$CH_3$PEP and AEP, respectively. In both cases, inhibitor binding to the enzyme followed strictly first-order kinetics, *i.e.*, was adequately accounted for by the binding scheme

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI$$

Rate constants were calculated from plots of $k_{obsd}$ *vs.* (I), where $k_{obsd} = k_1$ (I) + $k_{-1}$ (Figure 3). Dissociation constants,

TABLE III: Parameters for PEP and Analog Binding to $Mg^{2+}$–Enolase.[a]

| Substrate | $K_d$ ($\mu$M) | $k_1$ ($M^{-1}$ $sec^{-1}$) | $k_{-1}$ ($sec^{-1}$) | $k_{-1}/k_1$ ($\mu$M) | $k_2$ ($sec^{-1}$) | $k_{-2}$ ($sec^{-1}$) |
|---|---|---|---|---|---|---|
| PEP | 220[b] | | | | $(k_2 + k_{-2})$[c] $\simeq 10^3$ | |
| 3-FPEP | 20[d] | | | | $(k_2 + k_{-2}) \simeq 24$ | |
| $CH_2PEP$ | 8[e] (250[d]) | | | | $(k_2 + k_{-2}) \simeq 70$ | |
| 3-$CH_3PEP$ | 12[f] | $2.8 \times 10^5$ | 7 | 25 | | |
| AEP | 0.09[f] (0.5[g]) | $2.5 \times 10^4$ | $<10^{-2}$ | <0.4 | | |
| TSP | 4 (15[g]) | | | 77 | 0.19 | 0.01 |

[a] $(Mg^{2+}) = 10^{-3}$ M, (Tris-HCl) $\simeq$ 0.05 M, 4°, pH 7.8, except where otherwise indicated. [b] $K_m$, 25° (Hanlon and Westhead, 1969b). [c] Conditions given in Table I, except at 4°. [d] $K_m$, rabbit muscle, 25° (Stubbe and Kenyon, 1972). [e] $Mn^{2+}$–enolase, (KCl) = 0.5 M, 25°, pH 7.5 (Nowak *et al.*, 1973). [f] $K_I$, this work. [g] 25° (Spring and Wold, 1971b).

calculated from $K_d = k_{-1}/k_1$, agree well with constants for competitive inhibition determined from steady-state kinetic measurements (Table III).

Single concentration-independent reactions were observed at 240 nm upon rapid mixing of the analogs 3-FPEP or $CH_2PEP$ with $Mg^{2+}$-enolase. Results are summarized in Table III; substrate concentrations were varied over the ranges (3-FPEP) = $(0.05\text{–}10) \times 10^{-4}$ M, $CH_2PEP = 1.3\text{–}2.5 \times 10^{-5}$ M, with (E) $= 5 \times 10^{-6}$ M. Bimolecular association–dissociation steps were not detected.

No evidence of reaction of phosphoglycolate with $Mg^{2+}$–enolase could be found in stopped-flow measurements.

*Carbanion Trapping Experiments.* Indirect detection of enzyme–substrate carbanion intermediates in the catalyzed reaction was attempted. Observation of reduction of oxidant dyes introduced into assay solutions which is rapid relative to reaction in solutions of components of the enolase system not undergoing catalysis would constitute evidence for the existence of such intermediates (Riordan and Christen, 1969; Healy and Christen, 1973).

Reduction rates of the redox dyes porphyrexide, porphyrindine, 2,6-dichlorophenolindophenol, and the oxidants hexacyanoferrate(III) and tetranitromethane were determined in the following solutions: (a) 0.05 M Tris-HCl, $10^{-3}$ M $MgCl_2$, $2 \times 10^{-5}$ M EDTA (assay medium); (b) assay medium + $2 \times 10^{-3}$ M PGA; (c) assay medium + $5 \times 10^{-6}$ M enolase; (d) assay medium + $2 \times 10^{-3}$ M PGA + $5 \times 10^{-6}$ M enolase. Reactions were run at 30°, both at pH 7.8 and 9.2, with oxidant concentrations at $10^{-3}$ M, except for 2,6-dichlorophenolindophenol, which was $10^{-4}$ M. Only very slow reduction of the dyes occurred. No enhancement of rates in solutions (d), the complete assay systems, was observed; in fact, reduction rates in solutions (d) were somewhat less than the sums of rates in (b) and (c), suggesting possibly slight protection of the enzyme accompanying substrate binding.

Enzyme incubated for 10–15 min in the assay medium, pH 9.2, containing also $10^{-3}$ M oxidant, then diluted into standard assay solution, pH 9.2, containing also $10^{-4}$–$10^{-3}$ M oxidant, showed only slight losses in specific activity compared to reference solutions which contained no oxidant. The lack of enhanced redox dye reactivity in the complete assay systems cannot therefore be attributed to their inhibition of enolase. The reactions studied were: (1) reaction in the presence of $10^{-4}$ M porphyrexide, activity = 95% control; (2) reaction in $10^{-3}$ M ferricyanide, activity = 92% control; (3) reaction in $2 \times 10^{-4}$ M tetranitromethane, activity = 70% control.

## Discussion

The lack of concentration dependence of the relaxation, $\tau_1$, seen in solutions containing $Mg^{2+}$-enolase and phosphoenolpyruvate precludes kinetic identification of the transient. Indirect arguments favoring its assignment as an obligatory intermediate in the catalyzed reaction are: (a) The unimolecular process was not observed in perturbation experiments of solutions containing metal-free, hence inactive, enolase and phosphoenolpyruvate, substrate concentrations being varied over a sufficiently large range to ensure substantial binding to the enzyme active sites (Hanlon and Westhead, 1969b). (b) The relaxation time is sensitive to the identity of divalent activator metal ions present. Magnitudes of time constants and amplitudes vary systematically with the capacity of the various ions to activate the enzyme (Wold and Ballou, 1957); in the extreme with added inhibitory $Ca^{2+}$ ion, no relaxation is seen. (c) Unimolecular steps are detected in stopped-flow studies of binding of the slowly reacting substrates, phosphoenol-3-fluoropyruvate and $\alpha$-(dihydroxyphosphinylmethyl)acrylate, but binding of the competitive inhibitors 3-aminoenolpyruvate phosphate and phosphoenol-$\alpha$-ketobutyrate follows simple second-order kinetics. Thus the transient is detected only under conditions of enolase-catalyzed substrate hydration.

Relaxation is observed only in the spectral region corresponding to strong absorption by the phosphoenolpyruvate chromophore. It seems likely, therefore, that the unimolecular

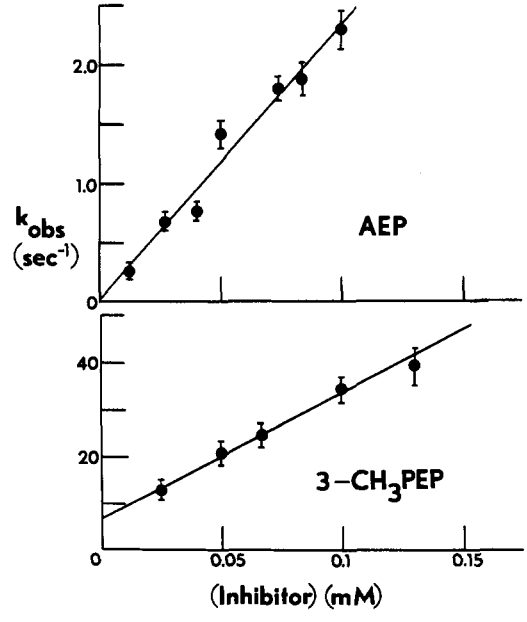


FIGURE 3: Kinetics of inhibitor binding to enolase. For all runs, enzyme concentration was maintained at levels at least 10-fold lower than inhibitor. Each point represents the average of at least three individual runs; error bars are the range of values obtained.

process involves at least partial disruption of the PEP conjugated olefin bond. Unambiguous identification of the intermediate cannot be made with the present data, as the following considerations will show.

Dinovo and Boyer (1971) have concluded from extensive kinetic isotope studies on rabbit muscle enolase that conversion to 2-phosphoglycerate proceeds with rate-limiting attack of bound hydroxide on the PEP 3-carbon position, with concurrent $sp^2$ to $sp^3$ hybridization to tetrahedral geometry. Assuming the applicability of their reaction scheme to yeast enolase, we infer the existence of an additional intermediate in the reaction pathway between PEP binding and carbanion formation, *i.e.*, the transient step, being unimolecular, cannot be attributed to initial binding, nor can the step follow the rate-limiting process, here carbanion formation. With this interpretation, binding is represented as a two-step process: $E + PEP \leftrightarrow X_1 \leftrightarrow X_2$, the second, unimolecular step being isomerization of the initially formed enzyme–substrate complex.

Hydrogen isotope effects have been reported for the yeast enolase catalyzed dehydration of 2-PGA (Shen and Westhead, 1973). At pH 7.8, the maximal reaction velocity for 2-[$^2$H]PGA was substantially less than undeuterated PGA ($k_H/k_D = 2$) and rapid isotope exchange in the 2-carbon proton of unreacted PGA during catalysis was shown not to occur. At more alkaline pH values, rapid 2-carbon proton exchange commenced and the kinetic hydrogen isotope effect disappeared. The results were interpreted as partially rate-limiting proton abstraction at the reaction pH optimum, with other steps becoming increasingly rate limiting with increasing pH. An alternative interpretation consistent with the postulate of rate-limiting $sp^3$–$sp^2$ conversion at the pH optimum is that the kinetic isotope effect originates in the preequilibrium distribution of isotopes between the initially formed enolase–PGA complex and carbanion intermediate and that the potentially exchangeable C-2 proton is sequestered in the intermediate. In this case, both equilibrium isotope effect and proton sequestration must be pH dependent. Should, however, the interpretation of Shen and Westhead (1971) prove correct, or should another discrete step between proton abstraction and $sp^3$–$sp^2$ hybridization of the C-3 carbon be rate-limiting,[3] *e.g.*, conformational change of the enzyme–carbanion intermediate, then the relaxation we observe with PEP binding might be attributed to equilibration of enolase-bound PEP and hydroxide ion with the carbanion. Spectral dependence of the relaxation is also consistent with this interpretation. The unimolecular processes ($t_{1/2}$ = 10–30 msec) observed for 3-FPEP and $CH_2$PEP binding are likewise consistent with rapid approach of carbanion intermediates to their quasiequilibrium steady-state concentration levels or two-step binding with rate-limiting carbanion formation.

Bimolecular association–dissociation was observed for the competitive inhibitors, 3-aminoenolpyruvate phosphate and (*Z*)-phosphoenol-$\alpha$-ketobutyrate. Our data on AEP binding measured at 4° agree well with previously published rate and thermodynamic measurements made at 25° by Spring and Wold (1971b). We found no evidence in flow or perturbation measurements supporting their suggestion of isomerization of enzyme-bound AEP. Second-order rate constants for binding of modified substrates or inhibitors to other enzymes have been found to be considerably smaller than rate constants for association of physiological substrates, which generally approach the diffusion-controlled limit (*cf.* Hammes and Schimmel, 1970). Data are scanty, but suggest that precise geometrical and spatial relationships between enzyme active site and substrate play an important role in determining association rates. In the present studies, the increased size of amino or methyl substituents on the 3-carbon position of the inhibitors may constitute a significant steric barrier to proper binding, resulting in greater activation free energies and slower bimolecular rates than for the smaller analogs. Consistent with this suggestion, equilibrium binding of PGA derivatives containing bulky substituents on the 3-carbon position is weaker than that for the physiological substrate (Wold, 1971).

D-Tartronate semialdehyde phosphate binding to enolase proceeds in a two-step reaction. The molecular mechanism involving enolization of the initially formed enzyme–TSP complex, previously suggested by Spring and Wold (1971b), is consistent both with our binding kinetics and their spectral observations.

In marked contrast to the behavior of PGA analogs (Wold, 1971), modified PEP derivatives show stronger binding to the enzyme than the physiological substrate (Table III). Proton magnetic resonance studies of analog binding to $Mn^{2+}$-enolase have located the metal binding sites in the vicinity of the C-3 carbon (Nowak *et al.*, 1973). The substrate $CH_2$PEP does not bind in the primary coordination shell of the metal ion. Thus, although it might be argued that the stronger binding of AEP and 3-FPEP, the 3 substituents of which are nucleophiles potentially suited for metal coordination, derives from metal binding at the active site, this explanation is completely untenable for the 3-$CH_3$PEP and $CH_2$PEP analogs. On the other hand, the data appear to be totally consistent with the strain hypothesis, in which favorable binding free energy that would normally accrue from substrate binding is employed in distorting the enzyme–substrate complex towards the transition-state geometry (*cf.* Jencks, 1969). Further, inasmuch as specificity originates in the steady-state catalytic, $V_{max}$, and not the binding terms, $K_m$, and as the enolase transient kinetic data appear to indicate two-step binding for substrates, but not inhibitors, we tentatively identify the observed isomerization process with conversion from an initially inactive to catalytically reactive enzyme–substrate complex. The frequency with which two-step binding occurs has been commented upon (*cf.* Hammes and Schimmel, 1970); evidence that it may account in large measure for substrate recognition in certain systems obtains from quantitative study of the effect of substrate inhibitors on the isomerization step in aspartate transcarbamylase reactions (Hammes *et al.*, 1971) and correlation of maximal velocities with partitioning between isomeric enzyme–substrate forms in nucleotide diphosphate binding to creatine kinase (Hulett and Hurst, to be published). This mechanism for specificity, embodying conformational response of the enzyme active site to substrate binding, is particularly attractive in rationalizing reactivity in systems like enolase where specificity is extremely high and only subtle structural differences in reagents span the range from highly reactive substrate to competitive inhibitor. The available spectral, kinetic, and thermodynamic data are consistent with this mechanism, that in enolase catalysis substrate binding is *followed* by distortion towards a carbanion-like transition state, the conformational change necessary for activation being extremely sensitive to the spatial requirements about the C-3 carbon.

In kinetic studies on PGA binding to enolase we had anticipated, but did not observe, a carbanion intermediate generated

[3] Expansion of the minimal reaction scheme presented by Dinovo and Boyer (1971) is also required in this instance. These investigators measured an inverse secondary hydrogen isotope effect in the hydration reaction with isotopic substitution for hydrogen at the C-3 position. The effect arises from either rate-determining or preequilibrium $sp^2$–$sp^3$ conversion.

by proton abstraction from the substrate C-2 position (Dinovo and Boyer, 1971). The existence of detectable amounts of the carbanion under the conditions of our experiments, pH 7.8, is now questionable, although strong evidence for preequilibrium carbanion formation in more alkaline solutions has been presented (Shen and Westhead, 1973). In an effort to gain additional information concerning the magnitude of steady-state carbanion pools in the PGA → PEP dehydration, we initiated carbanion-trapping experiments using the methods of Riordan and Christen (Christen and Riordan, 1968; Riordan and Christen, 1969; Healy and Christen, 1973). These investigators have demonstrated reaction of carbanion-like intermediates in a variety of enzyme-catalyzed reactions with several chromophoric oxidizing agents. In similar studies we were unable to detect any intermediates with pronounced susceptibility toward oxidation, either at pH 7.8 or 9.2. We regard these experiments as inconclusive since appreciable carbanion concentration levels are most likely reached in the alkaline pH range. The insensitivity of the oxidant indicator probes in the enolase reaction is difficult to account for. Perhaps the carbanion is unusually stabilized in the active site or approach of the dyes to the active site is hindered. Given the reactions which are responsive, there is no *a priori* reason for suspecting either of these possibilities.

Arguments for the proposed mechanism for enolase action have relied heavily upon qualitative observations of binding and transient behavior. Current work is directed toward finding means of extending the concentration range of measurement to obtain quantitative information on the elementary reaction steps appropriate for more rigorous testing of the model.

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References

Brewer, J. M., and Weber, G. (1966), *J. Biol. Chem. 241,* 2550.
Christen, P., and Riordan, J. F. (1968), *Biochemistry 7,* 1531.
Dinovo, E. C., and Boyer, P. D. (1971), *J. Biol. Chem. 246,* 4586.
Faeder, E. J. (1970), Ph.D. Thesis, Cornell University.
Hammes, G. G. (1968), *Accounts Chem. Res. 1,* 321.
Hammes, G. G., and Haslam, J. L. (1969), *Biochemistry 8,* 1591.
Hammes, G. G., Porter, R. W., and Stark, G. R. (1971), *Biochemistry, 10,* 1046.
Hammes, G. G., and Schimmel, P. R. (1970), *Enzymes 2,* 67.
Hanlon, D. P., and Westhead, E. W. (1969a), *Biochemistry 8,* 4247.
Hanlon, D. P., and Westhead, E. W. (1969b), *Biochemistry 8,* 4255.
Healy, M. J., and Christen, P. (1973), *Biochemistry 12,* 35.
Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill.
Mann, K. G., Castellino, F. J., and Hargrave, P. A. (1970), *Biochemistry 9,* 4002.
Nowak, T., Mildvan, A. S., and Kenyon, G. L. (1973), *Biochemistry 12,* 1690.
Riordan, J. F., and Christen, P. (1969), *Biochemistry 8,* 2381.
Shen, T. Y. S., and Westhead, E. W. (1973), *Biochemistry 12,* 3333.
Spring, T. G., and Wold, F. (1971a), *Biochemistry 10,* 4649.
Spring, T. G., and Wold, F. (1971b), *Biochemistry 10,* 4655.
Stubbe, J. E., and Kenyon, G. L. (1972), *Biochemistry 11,* 338.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310,* 384.
Westhead, E. W., and McLain, G. (1964), *J. Biol. Chem. 239,* 2464.
Wold, F. (1971), *Enzymes 5,* 499.
Wold, F., and Ballou, C. E. (1957), *J. Biol. Chem. 227,* 313.
Wold, F., and Barker, R. (1964), *Biochim. Biophys. Acta 85,* 475.