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Inhibition of Chicken Pyruvate Kinases by Amino Acids†

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ABSTRACT: Alanine, serine, and phenylalanine behave as inhibitors competitive with phosphoenolpyruvate for the activated forms of the chicken pyruvate kinases. On the other hand, phenylalanine and alanine behave as K-type inhibitors and serine behaves as a heterotropic activator of pyruvate kinase variants which undergo homotropic activation. Tryptophan lowers the V_m and tends to yield complex plots with all variants studied. Kinetic patterns obtained in the presence of phenylalanine also show some characteristics not generally associated with a competitive mechanism. These observations are related to data previously obtained using the rat isozymes and are used to formulate a mechanism which explains the effects of the amino acids. This mechanism hypothesizes that all the effector amino acids bind to the phosphoenolpyruvate site; however, amino acids with nonpolar side chains also interact with a nonpolar region of the T conformer and thereby stabilize it. It is further proposed that there are two such nonpolar regions on the various pyruvate kinases—the one which reacts with the nonbulky side chains, and another which reacts only with relatively bulky side chains. The stabilizing effect of this second nonpolar interaction imparts inhibitory characteristics which are not competitive in nature. Serine and perhaps other polar compounds may also bind at the phosphoenolpyruvate site, but because of their polarity exert a repulsive force at the same nonpolar site with which the nonbulky nonpolar amino acids interact. This repulsion stabilizes the R conformation. Presumably the homotropic activating ef-

fects of phosphoenolpyruvate operate via this same mechanism. The data are also used to support a specific sequential-concerted mechanism for the homotropic activating effect of phosphoenolpyruvate. According to this mechanism, phosphoenolpyruvate adds sequentially to the first two subunits. This interaction causes the respective subunits to convert to the R conformation but, once two subunits are in the R conformation, the remaining two subunits convert in concert.

It has been hypothesized that the three rat pyruvate kinase isozymes have two different allosteric sites for amino acids—one which interacts with amino acids having small nonpolar side chains and one which interacts with amino acids having bulky nonpolar side chains (Ibsen and Trippet, 1974). The data reported herein indicate that these two groups of amino acids also have distinguishable effects on the chicken isozymes. The data further suggest that all the amino acid effectors, positive or negative, act by binding at the catalytic site but interact with different adjacent sites.

Materials and Methods

Enzyme variants were isolated by electrofocusing as described (Ibsen et al., accompanying manuscript). Kinetic assays were performed at 37 °C using the coupled lactate dehydrogenase assay, previously described (Ibsen et al., accompanying manuscript). The enzyme was preincubated at 37 °C with all substrates or effectors at least 5 min prior to initiation of the reaction by adding ADP at a final concentration of 0.5 mM. In no case were appreciable rates obtained prior to the ADP addition. The concentration of all effectors used was 5 mM, except for fru-1,6-P_2[1] which was 5×10^{-5} M. The concentrations of Phe, Trp, ADP, and P-enolpyruvate were checked spectrophotometrically.

The data shown are representative of a rather large body of data obtained in an attempt to sort out the relationships among the various pI variants obtained (Ibsen et al., accompanying manuscript). A minimum of two similar plots was obtained for each case illustrated. These data were selected because they represent enzyme preparations which were subject to a greater number of kinetic manipulations. The graphs shown represent data obtained at 8–13 different concentrations of P-enolpyruvate. Space consideration required elimination of some data points on the illustrations.

Results

Figure 1 illustrates rate plots obtained when the pH 5.0 variant of the K isozyme (Ibsen et al., accompanying manuscript) is studied in the presence of variable concentrations of P-enolpyruvate, in the absence of added effector and in the presence of 5 mM Ser, Trp, Ala, Phe or 5 × 10 M fru-1,6-P_2. Similar data, not shown, were obtained using the homotetrameric K-isozyme variants, namely: the pH 6.6 or 7.2 forms. For all three variants, Ala acts as a typical negative K-type allosteric effector. That is, rate plots in the presence of Ala remain sigmoidal but are shifted to the right while the V_m is unaltered. In contrast, Trp yields more complicated kinetics. The plots obtained using the pH 5.0 and 6.6 enzymes are definitely bimodal and, for all three enzymes, the V_m is reduced at least 50%. Plots obtained in the presence of Phe tend to show characteristics shared by both Trp and Ala. As in the case of Ala, the $K_{0.5S}$ value is raised and the V_m is not altered. On the other hand, the plots obtained in the presence of Phe tend to be biphasic and closely mimic the plot obtained in the presence of Trp, except that it does not level off near $\frac{1}{2}V_m$.

For all three of these enzyme variants, Ser acted as a heterotropic activator. In the presence of Ser, the lowest P-enolpyruvate levels do not induce as rapid an increase in activity as they do when the enzyme is activated by fru-1,6-P_2. For at least the pH 5.0 and 6.6 enzymes, substrate inhibition in the presence of fru-1,6-P_2 caused the rate plots to cross. Thus, Ser becomes the more effective activator (Figure 1).

When the K isozymes are activated by fru-1,6-P_2, essentially linear double reciprocal plots are obtained. As shown in Figure 2 for the pH 7.2 enzyme, the activating amino acid Ser and the inhibiting amino acid Ala act as though they were weak competitive inhibitors of the fru-1,6-P_2 activated enzyme.

Figure 3 illustrates the effect of variable concentrations of P-enolpyruvate on the suspected K_3M (pH 7.4) isozyme (Ibsen et al., accompanying manuscript) in the presence or absence of the amino acid effectors and fru-1,6-P_2. The data show that, even though relatively linear double reciprocal plots are obtained in the absence of effector, the enzyme is still further activated by fru-1,6-P_2 and Ser. Ser appears to be the more effective activator of the isozyme. Phe and Ala act as negative K-type modulators, causing the enzyme to be homotropically activated by P-enolpyruvate. Extrapolation of data obtained at the lower P-enolpyruvate levels in the presence of 5 mM Trp suggests Trp would act as a typical competitive inhibitor; however, at about $\frac{1}{2}V_m$ the rate abruptly levels off. Under all conditions studied there is a plateau at 0.02–0.0325 mM P-enolpyruvate which is accentuated by the presence of Ala or Phe, the negative effectors of K variants. Similar plateaus were obtained with pH 7.4 enzyme isolated from heart, brain, and embryonic extracts.

Data obtained with the proposed K_2M_2 isozyme are illustrated in Figure 4. Rate plots obtained with this enzyme were basically hyperbolic under all conditions studied, except in the presence of Phe. Fru-1,6-P_2 and Ser did not activate this preparation. However, a tendency for slight activation of K_2M_2 enzyme has been observed in other preparations. As previously observed with the activated form of the K_4 isozyme (Figure 2), Ser inhibited at low P-enolpyruvate levels. Double reciprocal plots obtained in the presence of 5 mM Trp look as if Trp would act as a competitive inhibitor, but again the rate ceases to rise at about $\frac{1}{2}V_m$. In the particular case illustrated, there appears actually to be a reversal. While Ala acts as if it were a classical competitive inhibitor, Phe causes a bimodal plot. At low P-enolpyruvate levels, Phe is a better inhibitor than Trp, and like Trp the rate plateaus at about $\frac{1}{2}V_m$. However, concentrations of P-enolpyruvate greater than 0.065 mM overcome this latter effect of Phe, and once again Phe behaves as if it were a

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[1] Abbreviations used: fru-1,6-P_2, fructose 1,6-diphosphate; P-enolpyruvate, phosphoenolpyruvate. $K_{0.5S}$ = substrate concentration yielding $\frac{1}{2}V_m$.

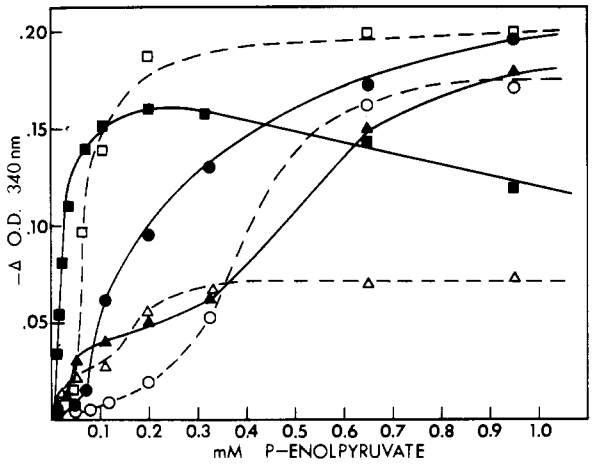

FIGURE 1. Rate plots obtained with the pH 5.0 K-isozyme variant and variable concentrations of P-enolpyruvate in the presence of: no added effector (●); 5 mM Ser (□); 5 mM Phe (▲); 5 mM Trp (△); 5 mM Ala (○); and 5×10^{-5} M fru-1,6-P_2 (■). The enzyme used was obtained from a frozen liver extract by electrofocusing.

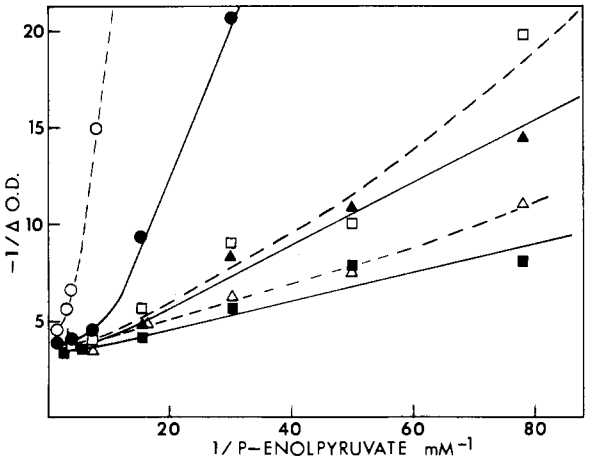

FIGURE 2. Double reciprocal plots obtained with the pH 7.2 enzyme in the absence of effector and in the presence of 5 mM Ser or Ala with and without 5×10^{-5} M fru-1,6-P_2. The symbols used designate the following: (○) Ala without added fru-1,6-P_2; (△) Ala with 5×10^{-5} M fru-1,6-P_2; (●) no effector; (■) fru-1,6-P_2; (□) Ser without added fru-1,6-P_2; (▲) Ser with added fru-1,6-P_2. The enzyme used was a pH 7.2 peak obtained from a fresh extract of liver.

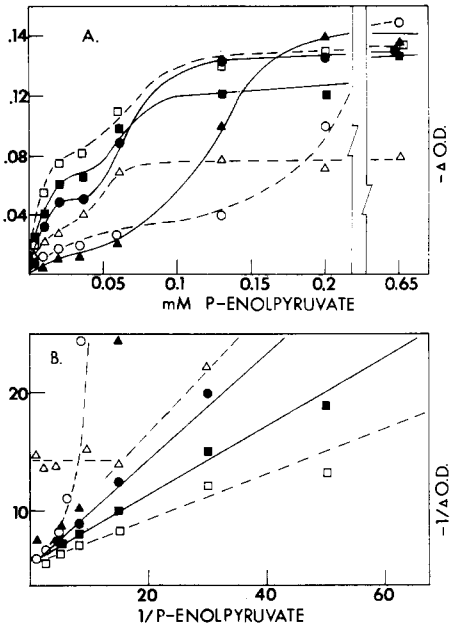

FIGURE 3. Rate plots (panel A) and double reciprocal plots (panel B) obtained using the K_3M isozyme (pI value, 7.3) isolated from a fresh extract of testes. Effector concentrations and symbols are as in Figure 1.

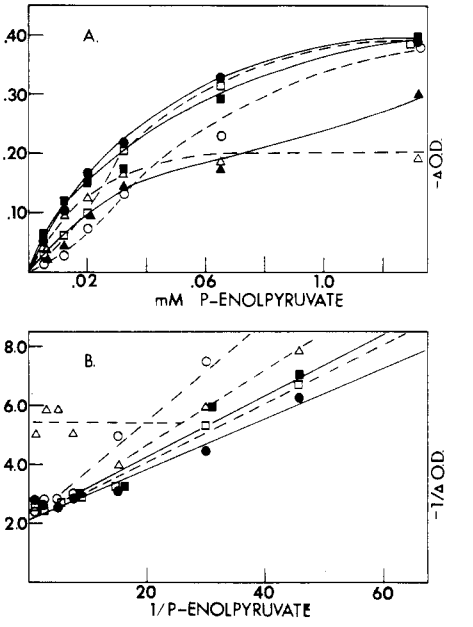

FIGURE 4. Rate plots (panel A) and double reciprocal plots (panel B) obtained using the K_2M_2 isozyme (pI value, 8.0) isolated from a fresh thigh muscle extract. Effector concentrations and symbols are as in Figure 1.

competitive inhibitor. Although this is a suspected K_2M_2 enzyme, a plateau at about $\frac{1}{2}V_m$ was not seen in the presence of any effector but Phe, using this enzyme or K_2M_2 enzyme from any other source.

Figure 5 illustrates data obtained with the suspected KM_3 isozyme. Except in the presence of Trp, the rate plots are almost classical. Ala and Phe act as typical competitive inhibitors of about equal strength. Fru-1,6-P_2 and Ser have no discernible effect. Plots obtained in the presence of Trp are, however, complicated. At lower P-enolpyruvate levels, it behaves as a K-type inhibitor; however, rates again level off at $\frac{1}{2}V_m$. No obvious plateaus at about $\frac{3}{4}V_m$ were discerned in any preparation studied.

Fru-1,6-P_2 did not have a significant effect on the rates of the M_4 isozyme. Therefore in Figure 6, which shows double reciprocal plots obtained with suspected M_4 isozymes, plots obtained in the presence and absence of fru-1,6-P_2 are not illustrated. The data illustrated in panel A show Ala, Ser, and Phe to act as competitive inhibitors. However, the Ala and Ser effects are nonclassical in that they join the control plot rather than intersect at the y axis. Some other preparations, such as the one illustrated in panel B, tend to yield more classical plots. On the other hand, in the latter case Phe appeared to lower the V_m slightly. In both preparations illustrated, Trp again lowered the V_m. The pattern obtained in panel B is the more typical in that the V_m in the presence of Trp was about $\frac{1}{2}V_m$ and the plot was complex. Even though the plot obtained in the presence of Trp illustrated in panel A was linear, it did not intersect with the control plot on the x axis; i.e., it was not typical of a noncompetitive inhibitor.

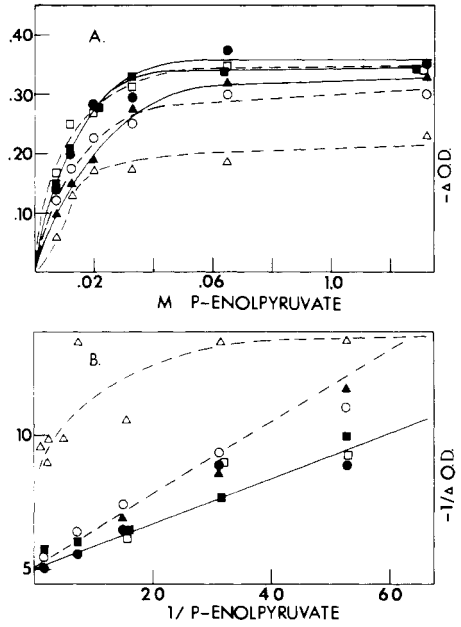

FIGURE 5. Rate plots (panel A) and double reciprocal plots (panel B) obtained using the KM_3 isozyme (pI value 8.5) isolated from a fresh extract of adult brain. The effectors are illustrated as in Figure 1. For simplicity in the double reciprocal plot, a single line was used to trace the data obtained in the presence of no effectors, fru-1,6-P_2, and Ser (the lowest line) and for the data obtained in the presence of Phe and Ala (the middle dashed line).

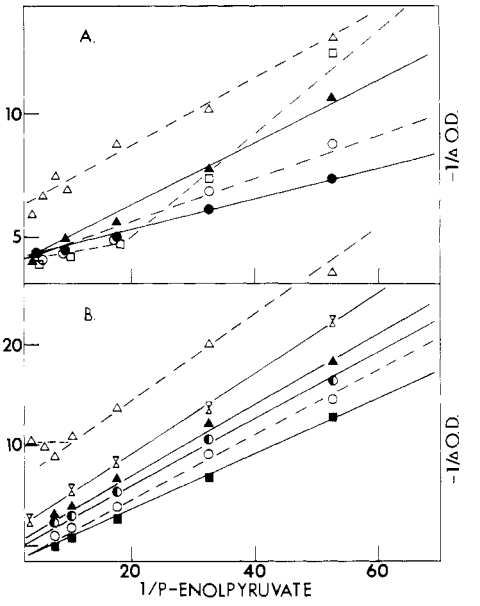

FIGURE 6. Double reciprocal plots obtained using M_4 isozymes. Panel A represents data obtained from a pH 8.8 enzyme isolated from adult breast muscle. Panel B represents data obtained from a pH 9.0 peak isolated from adult brain. In both panels the symbols are as in Figure 1, but in addition the ◐ represent data obtained in the presence of 5mM Ala plus 5mM Pne and the ⧖ represent data obtained in the presence of 5mM Ala plus 5mM Trp. The slope of the curve obtained in the presence of Trp decreases at lower P-enolpyruvate concentrations not shown. Thus the upper part of the curve becomes convex-up.

The data of panel B also illustrate the fact that Ala appears to activate the Phe- or Trp-inhibited M_4 isozyme.

Discussion

Mechanism for Amino Acids Effectors. Previous work (Ibsen and Trippet, 1974) performed with the rat isozymes led to the conclusion that inhibitory amino acids with bulky nonpolar side chains were bound to a different site than inhibitory amino acids with nonbulky nonpolar side chains. Since these binding sites were generally presumed to be allosteric and since Mg-ATP, fru-1,6-P_2, and Ser seemed likely to act at still different sites, there appeared to be a surfeit of binding sites. The data obtained in the present study also suggest a difference in binding among amino acids depending upon the size of their side chains and, in addition, suggest a hypothesis which explains the action of the amino acids without evoking the need for a multiplicity of sites. The concept is as follows. The carboxyl, α and β carbons of the amino acids fit into the P-enolpyruvate binding site. Bound amino acids with small nonpolar side chains further interact with a nonpolar group on pyruvate kinase isozymes in the T conformation. This interaction stabilizes the T conformation and makes these amino acids more effective inhibitors. Nonetheless, the basic mode of inhibition remains competitive with P-enolpyruvate. As the nonpolar side chain increases modestly in bulk, it becomes more difficult for the amino acids to interact at the catalytic site, thus decreasing their effectiveness as inhibitors. There is, however, a critical side chain size, which, if exceeded, actually increases the effectiveness of the amino acid as an inhibitor (Ibsen and Trippet, 1974). Presumably in this latter case, interaction of the bulky nonpolar side chain with a second nonpolar region on the enzyme stabilizes the complex sufficiently to overcome the steric hindrance. The resulting inhibition would thus be competitive with respect to the first three-carbon moieties, but not competitive with respect to the bulky nonpolar side chain. The overall kinetic pattern obtained would depend upon the relative strength of interaction at the two sites. Thus in these studies Trp would be presumed to bind more tightly at the second nonpolar site since the basic mechanism is not competitive, while Phe is presumed to bind more weakly at the second site since the mode of inhibition is basically competitive. The hypothesis further suggests that the more polar amino acids with nonbulky side chains also bind at the P-enolpyruvate site, but because of their polarity, they repel the nonpolar region of the T conformer. This repulsion causes the enzyme to favor the R conformation. Presumably P-enolpyruvate activates by this same mechanism. Thus the differences among the various pyruvate kinases with respect to susceptibility to different amino acid effectors may be related to the relative availability to the effector of the nonpolar regions near the P-enolpyruvate binding site. Evidence supporting these concepts is presented below.

Ala and Ser as Competitive Inhibitors. Double reciprocal plots of data obtained in the presence of Ala and Ser indicate these effectors act as relatively typical competitors for P-enolpyruvate, provided the enzyme is in the active conformation (Figures 2 and 4–6). These data plus the structural similarity between these amino acids and P-enolpyruvate strongly suggest they can bind at the catalytic site. The interaction between Ala and less activated forms of the enzyme yield sigmoidal rate plots (e.g., Figures 1–3) but is also basically competitive: i.e., the $K_{0.5S}$ value is raised; the V_{max} is unaltered. The increase in n_n value induced by Ala (Tables I and III, Ibsen et al., accompanying manuscript) is a natural consequence of the fact that Ala is the more potent inhibitor of T conformers; i.e., P-enolpyruvate binding takes on an added dimension of cooperativity—the conversion of the enzyme from a form more sensitive to inhibitor to a form less sensitive. Similarly, the ability of fru-1,6-P_2

Table I: Relationship among the Experimentally Obtained $K_{0.5S}$ Values and Those Expected for Hybrid Forms Assuming No Interaction among Subunits.

System		$K_{0.5S}$ Value, mM P-enolpyruvate[a]				
		K_4[b]	K_3M	K_2M_2	KM_3	M_4
No effector	expected value[c]		0.16	0.11	0.066	
	% R form[d]		25	50	75	
	obtained value	0.21	0.053	0.033	0.029	0.019
	% R form[d]		82	93	95	100
Plus 5 mM Ala	expected value[c]		0.31	0.22	0.12	
	% R form[d]		25	50	75	
	obtained value	0.41	0.18	0.025	0.043	0.023
	% R form[d]		54	99	95	100
Plus 5mM Phe	expected value[c]		0.53	0.36	0.20	
	% R form[d]		25	50	75	
	obtained value	0.69	0.21	0.046	0.050	0.035
	% R form[d]		74	98	98	100
Mean % R form obtained			72	97	96	100

[a] Mean value. [b] Values obtained using both the pH 6.6 and 7.2 forms. [c] Calculated by assuming each subunit has the same $K_{0.5S}$ value as the K_4 or M_4 forms and there is no interaction. [d] Percent R type = $[(K_{0.5S}$ K type) − ($K_{0.5S}$ hybrid)$]/[(K_{0.5S}$ K type) − ($K_{0.5S}$M type)$] \times 100$. assuming that M subunit and R form are equivalent.

to counteract Ala inhibition would be a reflection of the lesser affinity of Ala for the R conformer.

The observation that Ser does not activate K isozymes at the lowest P-enolpyruvate levels (Figures 1 and 2) could also indicate Ser and P-enolpyruvate are competitors. That is, Ser converts the enzyme to the R conformation at all P-enolpyruvate levels but effectively blocks P-enolpyruvate binding to the R conformer at the low concentrations of substrate. Evidence for this concept is provided by forms of the enzyme known to be activated, which behave similarly (Figures 4 and 6).

An observation not expected to result from the binding of Ala at the catalytic site is that Ala promotes dissociation of mouse K isozyme (Sparmann et al., 1973). On the other hand, electrofocusing data obtained with the chicken (Ibsen et al., accompanying manuscript) and rat K isozymes (Ibsen and Trippet, 1972) indicate that the apparent T-tetrameric conformer is readily converted to a dimer. Thus, Ala would be expected to promote dissociation if the T conformer and this dimer were in equilibrium.

Competitive and Noncompetitive Effects of Phe and Trp. Because Ala, Phe, and Trp have identical aliphatic sequences, it is possible to suggest they all bind at the P-enolpyruvate site. The structural significance of the three-carbon aliphatic portion of the Phe molecule is supported by the observation that α-aminophenyl acetate is not a potent inhibitor of rat isozymes (Schwark et al., 1971; Ibsen and Trippet, 1974). Moreover, Ala activates the rat M isozyme in a manner competitive to Phe inhibitors (Ibsen and Trippet, 1974) and binding studies show Ala and Phe act as competitors for the rabbit M isozyme (Kayne and Price, 1973). However, these latter studies could also indicate that the two amino acids were competing for different conformers.

Although the general characteristic of the plots obtained in this study in the presence of Phe is primarily competitive, diphasic plots (Figures 1, 3, and 4), a tendency to decrease V_{max} (Figures 3, 4, and 6), and a tendency to lower the n_H value below 1 (Tables I and III, Ibsen et al., accompanying manuscript) were also observed in the presence of Phe. The various modes of action ascribed to Phe with respect to the mammalian M isozyme, namely, competitive (Weber, 1969; Vijayvargiya et al., 1969; Schwark et al., 1971), mixed competitive–noncompetitive with linear double reciprocal plots (Carminatti et al., 1971; Figure 1), and allosteric (Carminatti et al., 1971; Kayne and Price, 1972), may suggest a complex mode of interaction between Phe and the mammalian isozymes. Moreover, the effects of Phe on the rat K isozyme differ from those of Ala in the following respects: when the enzyme is activated with H^+ or P-enolpyruvate, the inhibition caused by Ala is overcome more readily than is inhibition induced by Phe, but the reverse is true when the enzyme is activated by Ser (Ibsen and Trippet, 1974); and increasing levels of the amino acid cause a reduction of activity to zero with Phe, but not with Ala (Jiménez de Asúa et al., 1971; Van Berkel et al., 1973; Ibsen and Trippet, 1974). In addition, binding studies conducted with rabbit M isozyme and Phe show cooperativity, whereas those conducted with Ala do not (Kayne and Price, 1973). Thus in sum these data indicate that, although Ala and Phe effects have much in common, there are obvious differences. This might be expected if both amino acids bound at the catalytic site but Phe also bound at a second site.

The structural resemblance between Trp and Phe suggest these two amino acids would act by similar mechanisms. The facts that Trp tends to behave competitively at low P-enolpyruvate levels (Figures 1, 3, and 4) or that Phe effects tend to mimic Trp effects by promoting a discontinuity at $\frac{1}{2}V_m$ (Figures 1 and 4) further support the concept of analogous reaction mechanisms. For at least the chicken enzymes, it seems plausible to suggest that Trp shows less of a competitive effect because it binds more avidly at the second nonpolar site, perhaps because of its greater bulk. That a similar relationship might hold for the rat enzyme is suggested by the observation that Trp inhibition of the rat K isozyme could only be released by a much greater concentration of fru-1,6-P_2 than is usually required to activate the enzyme (Ibsen and Trippet, 1974).

The hypothesis can also be used to explain most of the observations relevant to Trp or Phe effects, including the observation that M isozymes tend to be susceptible to inhibition only by amino acids with bulky side chains, but have this inhibition released by other amino acids with nonbulky side chains (Carminatti et al., 1971; Ibsen and Trippet, 1974; Figure 6). In this case, according to the proposed hypothesis, the M isozyme would differ from the other iso-

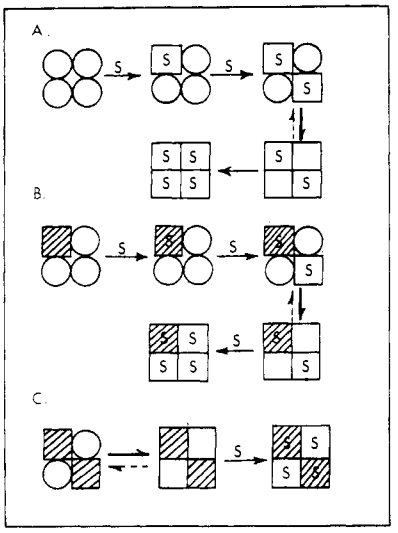

FIGURE 7. Proposed sequence leading to the conversion of T conformers (circles) to R conformers (squares) starting with (A) the K_4, (B) the K_3M, and (C) the K_2M_2 isozymes. Hatched symbols indicate M subunits, open symbols, K subunits. The one-way arrows are used to designate the direction of change caused by increased substrate concentration and do not imply an absence of reversibility. Equilibria between the R and T forms of each subunit are also implicit, but the forms shown are hypothesized to be the predominant ones.

zymes in that it would not be able to stabilize the complex between the T conformer and nonbulky nonpolar side chain amino acids. If it is further assumed that the bulky side chain amino acids have some difficulty in reacting at the P-enolpyruvate site (as evidenced by the minimal effects of amino acids of intermediate bulk), it would seem reasonable to suppose this part of the bond could be broken by smaller amino acids or other compounds binding at the P-enolpyruvate site. In support of this concept is the observation that the activation constants for Ala and Ser are much smaller than the inhibition constant for Phe (Ibsen and Trippet, 1974). A similar intrinsic difference in affinity for the P-enolpyruvate site may account for the previously mentioned observation that Ser activation of the Phe inhibited rat K isozyme occurs at lower concentrations than does activation of the Ala inhibited enzyme. On the other hand, the bulky side chain amino acids would be expected to form more stable complexes with the noncatalytic binding sites of the K isozyme than can those amino acids with nonbulky side chains. This could explain why Phe can produce a more complete inhibition than Ala (Ibsen and Trippet, 1973).

The proposed requirement for stabilization of the T conformer of the M isozyme by simultaneous binding at two sites and the activating tendencies of polar compounds bound to the active site would also explain why Ala activates the Phe-inhibited enzyme but not the 2-phosphoglycerate inhibited enzyme—a phenomenon used by Carminatti et al. (1971) to support the concept of allosteric binding by Phe.

That rate plots obtained in the presence of Trp are not classically noncompetitive is indicated by their tendency to be nonlinear and by the fact that double reciprocal plots never intercept at or close to the x axis. These observations, plus the observations that in most cases the maximum rate obtained in the presence of Trp is close to ½ the V_m, suggest an extreme case of negative cooperativity (Levitzki and Koshland, 1969), to the extent that only two catalytic sites can function. It is felt that the low n_H values and/or otherwise nonclassical patterns obtained in the presence of Phe (Figures 1, 4, and 6) may also suggest negative cooperativity. The proposed hypothesis can also explain negative cooperativity induced by amino acids. It need only be imagined that the conformer stabilized by the inhibitory amino acid is asymmetric so that stabilized complexes can only be formed between two of the catalytic sites with the "back-side" or a noncatalytic area of the other two subunits. Perhaps this stabilized asymmetric form represents an extreme conformational form which then splits into two dimeric sets. If so, Phe- or Trp-inhibited K isozyme should form dimers at a greater rate than the Ala-inhibited enzyme, which in turn would form dimers at a greater rate than the noninhibited or activated enzyme.

A Possible Mechanism for the Conformational Interconversions. If the subunits interacted independently, the $K_{0.5S}$ value obtained from hybrid forms should be the arithmetical mean of the values for each of the subunits. Table I compares such calculated values with the experimentally derived ones for the three systems in which the $K_{0.5S}$ values were an order of magnitude greater for the K_4 isozymes than for the M_4 isozymes. Clearly the subunits do not behave independently. In fact, the real K_3M isozyme behaves as if it were a KM_3 enzyme which did not show subunit interaction, while the K_2M_2 and KM_3 enzymes behave as though they were almost all in the R form. These data were used to derive the reaction sequence illustrated in Figure 7. The basic concept is that once two subunits are in the R conformation the other two convert spontaneously. Thus, due to the effect of the M subunits, presumed to be in the R conformation, all the subunits of the K_2M_2 and KM_3 isozymes would largely be in the R conformation prior to substrate addition, while the K_3M isozyme would spontaneously convert to the R conformation after only one of the three K subunits is converted by substrate. According to this concept, the addition of substrate to the K_3M isozyme would require the sequential binding of a substrate molecule to the M subunit, then to a K subunit in the T conformation, and then to two K subunits in the R conformation. This sequence would account for the plateau in the rate plot found at 0.02–0.04 mM P-enolpyruvate (Figure 3) and for the fact that the enzyme behaves as though it contained three active subunits and only one less active subunit (Table I).

By analogy with the K_3M isozyme, a sequential addition of substrate to two K subunits followed by spontaneous conversion of the whole molecule to the R conformer is envisioned (Figure 7). This mechanism appears to be similar to the two-step concerted mechanism proposed for the yeast homotetramer (Wieker et al, 1973). The difference between such a two-step concerted and this sequential-concerted mechanism may reduce to semantics for the homotetramer since similar energies of conversion will be required for conversion of the two subunits in each of the two sets of subunits (i.e., the set converted by substrate and set spontaneously converted). Nonetheless, the evidence obtained from the K_3M isozyme certainly suggests a sequential addition of substrate, while still further support for a sequential model is afforded by data obtained with various pyruvate kinases which is not readily explained by a concerted model (reviewed by Seubert and Schoner, 1971; also Ibsen and Trippet, 1973; Rozengurt et al., 1973; Irving and Williams, 1973; Van Berkel, 1974; Ibsen et al., 1975). A sequential mechanism also is consistent with the concept that the nonclassical kinetic patterns obtained in the presence of Trp and Phe are due to negative cooperative effects since nega-

tive cooperativity may be diagnostic of sequential conformational changes (Levitzki and Koshland, 1969).

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Prenyltransferase: The Mechanism of the Reaction†

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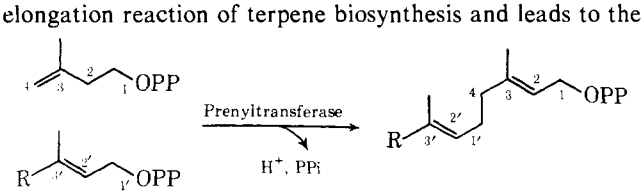
ABSTRACT: The enzyme, prenyltransferase, which normally catalyzes the addition of an allylic pyrophosphate to isopentenyl pyrophosphate, has been found to catalyze the hydrolysis of its allylic substrate. The rate of this hydrolysis is markedly stimulated by inorganic pyrophosphate. Competition experiments with 2-fluoroisopentenyl pyrophosphate and inorganic pyrophosphate demonstrated that inorganic pyrophosphate stimulated hydrolysis by binding at the isopentenyl pyrophosphate site. Hydrolysis carried out in $H_2{}^{18}O$ or with (1*S*)-[1-^{3}H]geranyl pyrophosphate show the C–O bond is broken and the C_1 carbon of geranyl pyrophosphate is inverted in the process. These results are interpreted to favor a carbonium ion mechanism for the prenyltransferase reaction.

Prenyltransferase (EC 2.5.1.1) catalyzes the condensation between C_4 of isopentenyl and C_1' of an allylic pyrophosphate, generating the five-carbon homologue of the allylic pyrophosphate. This condensation is the fundamental chain elongation reaction of terpene biosynthesis and leads to the formation of such diverse products as sterols, carotenes, dolichols, and the hydrocarbon side chains of the respiratory coenzymes.



The mechanisms which have been proposed for prenyl transfer can be grouped into two broad categories—those in which condensation is initiated by heterolytic cleavage of the carbon–oxygen bond of the allylic pyrophosphate, with or without assistance from the double bond of isopentenyl pyrophosphate, yielding cationic intermediates (Lynen et al., 1958; Rilling and Bloch, 1959; Cornforth and Popjak, 1959; Cornforth, 1968), and those in which condensation is initiated by attack of a nucleophilic group at the double bond of isopentenyl pyrophosphate with simultaneous formation of the C_1'–C_4 bond between the two substrates and rupture of the C_1'–oxygen bond (Cornforth et al., 1966; Cornforth, 1968). On the basis of the observation that C_1' is inverted during prenyl transfer, Cornforth and Popjak ar-

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