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Registry No. **3**, 81255-64-3; **4**, 81255-65-4; **5a**, 81255-66-5; **5b**, 81255-67-6; **5c**, 81255-68-7; **5d**, 81255-69-8; **5e**, 81255-70-1; **5f**, 81255-71-2; **6a** (X = Br), 81255-72-3; **6a** (X = I), 81255-73-4; **6b** (X = Br), 81255-74-5; **6b** (X = I), 81255-75-6; **6c** (X = I), 81255-76-7; **6d** (X = Br), 81255-77-8; **6d** (X = I), 81278-79-7; **6e** (X = I), 81255-78-9; **7a**, 81255-79-0; **7c**, 81255-80-3; **7d**, 81255-81-4; **7e**, 81255-82-5; 1,4-dihydrobenzoyl chloride, 3217-88-7; p-$CH_3C_6H_4SO_2NH_2$, 70-55-3.

Utilization of Enolpyruvate by the Carboxybiotin Form of Transcarboxylase: Evidence for a Nonconcerted Mechanism

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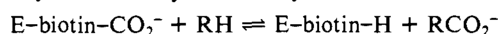
Carboxyl transfers by biotin enzymes



$$\text{E-biotin-CO}_2^- + \text{RH} \rightleftharpoons \text{E-biotin-H} + \text{RCO}_2^-$$

share two features of possible mechanistic significance: proton replacement is always with retention of configuration, and proton activation as measured by isotope exchange between substrate and water does not occur unless the enzyme is in the carboxybiotin form and the reaction conditions are complete.[2] The generality of these properties has been taken as evidence for a coupling of proton abstraction with carboxyl transfer in a concerted electrophilic displacement mechanism.[2,3] Notwithstanding these generalizations and because the proton to be replaced is invariably neighboring to a potential electron-withdrawing center, usually a carbonyl or carboxyl group, a stepwise "carbanion" mechanism is a plausible alternative.[4] The carbanion, enol or aci acid, could then either form a tetrahedral adduct at the carboxy center of the carboxybiotin or directly attack bound CO_2 formed from the carboxybiotin.[5] Stubbe et al.[4] have provided the only evidence for a carbanion mechanism. Transcarboxylase (TC) and propionyl-CoA carboxylase were found to eliminate HF from β-fluoropropionyl CoA giving acrylyl-CoA and "CO_2" equally and at rates normal for the carboxylation of propionyl-CoA. A concerted mechanism should have resulted in (fluoromethyl)-malonyl-CoA which, though stable enough to have been detected was not observed.

Further support for the stepwise mechanism would be obtained if enzymes that carboxylate pyruvate were shown to convert enolpyruvate to pyruvate and oxalacetate. Enolpyruvate can be generated by action of a phosphatase on phosphoenolpyruvate (PEP).[6] The product of this reaction has sufficient stability ($t_{1/2} \simeq 5$ min at pD 6) to be studied as a substrate by both kinetic and stereochemical approaches as was done with pyruvate kinase.[6] Similar experiments are now reported with transcarboxylase.

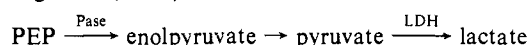
Transcarboxylase [methylmalonyl-CoA:pyruvate carboxytransferase (EC 2.1.3.1)] has been extensively studied both structurally and mechanistically.[7] It is a multimeric structure in which three kinds of subunits integrate their separate functions: (1) activating the methylmalonyl-CoA for carboxylation of biotin and formation of propionyl-CoA, (2) carrying the carboxylated biotin, and (3) activating the pyruvate for carboxylation, giving the β-keto acid oxalacetate. The enzyme is able to carry out pyruvate–oxalacetate exchange in the absence of either of the CoA substrates.[8]

Table I. Ketonization of Enolpyruvate by Transcarboxylase

additions[a]	1	2	3	4
(S)-MMCoA	–	+	+	–
(S)-prop-CoA	–	–	–	+
avidin	–	–	+	–
τ, min	6	0.9	5	5.7
(enolpyruvate, μM)$_{ss}$	15	4	20	25

[a] Each incubation of 1 mL in >95% D_2O (pD 6.4, 21 °C) contained Na maleate (50 mM), acid phosphatase (4×10^{-3} units), transcarboxylase (0.23 units), NADH (0.25 mM), LDH (4 units), and 2 mM PEP added last. The noted additions were (S)-methylmalonyl-CoA (250 μM), (S)-propionyl-CoA (500 μM), and avidin (1.5 μM). The reaction was followed in a 2-mm path length cuvette. The lag time, τ, was determined by extending the recorded trace of the rate in the steady state to the time axis.[6b] The concentration of enolpyruvate was determined as pyruvate present in the acid-quenched reaction mixture.[6b]

As done earlier,[6b] ketonization of enolpyruvate was followed by monitoring the reduction of pyruvate with NADH and lactate dehydrogenase (LDH).



$$\text{PEP} \xrightarrow{\text{Pase}} \text{enolpyruvate} \rightarrow \text{pyruvate} \xrightarrow{\text{LDH}} \text{lactate}$$

In the reaction begun with Pase, enolpyruvate accumulates until its ketonization rate equals the rate of its formation, which is fixed by the Pase rate. Any catalysis of ketonization should shorten the lag time necessary to reach this steady state and lower the concentration of enolpyruvate present in the steady state. As seen in Table I transcarboxylase shortened the lag time, τ, but only when methylmalonyl-CoA was also present. The concentration of enolpyruvate was then greatly decreased. Enolpyruvate was determined with LDH as pyruvate that arose after acid quenching. The LDH present during the incubation kept the pyruvate present prior to quenching to an undetectable level.[6b] The requirement for methylmalonyl-CoA is consistent with observations made with several biotin enzymes that activation of the C–H bond of the substrate appears to require carboxylating conditions. Malic dehydrogenase (MDH), added with the methylmalonyl-CoA and LDH, did not accelerate the oxidation of NADH, indicating that oxalacetate was at best a minor product. This was confirmed by analysis of labeled products by ion-exchange chromatography. In the presence of excess LDH the malate formed was <10% of the lactate. Avidin, known to interact specifically with biotin, blocked the action of transcarboxylase in the ketonization of enolpyruvate.

If enolpyruvate is indeed an intermediate, the observation that the overall reaction proceeds with retention[9] requires that the additions of proton or CO_2 occur from the same face of the plane of that molecule. With specifically labeled PEP-3-T to generate the enolpyruvate in D_2O, the specific formation of chiral methyl pyruvate would establish the direction of approach of D^+ (Scheme I). The 2.1-fold intramolecular H/D isotope effect of transcarboxylase in forming oxalacetate from pyruvate[9] yields, after reduction, (S)-malate that will be ~2-fold greater in tritium content at one poisition of its methylene group. Only the *pro-R* hydrogen at C-3 of the malate is activated by fumarase. A similar procedure has been used earlier for this purpose.[6a,9] *Direct* carboxylation of (Z)- or (E)-enolpyruvate-3-T by transcarboxylase would produce oxalacetate which becomes (S)-malate-3-T, from which fumarase would exchange either all or none of the tritium depending on the direction of carboxylation. Scheme I illustrates the consequences of D^+ or CO_2 addition by transcarboxylase to the face of enolpyruvate that is designated that *si* face by reference to C-2.

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Scheme I

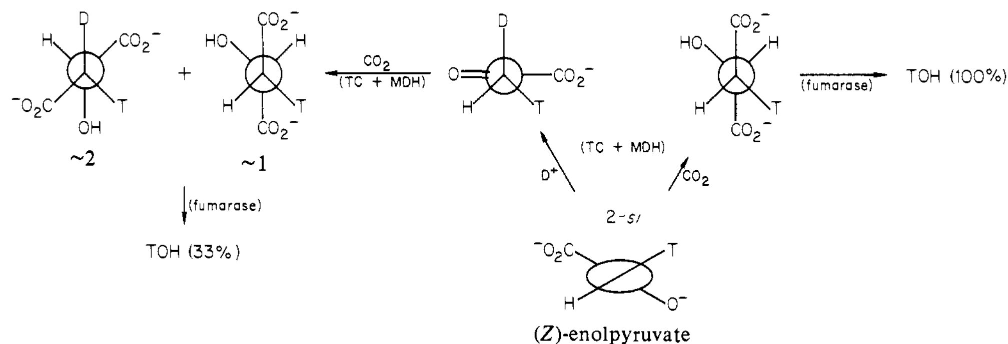

Table II. Stereochemistry of Transcarboxylase Reaction with Enolpyruvate

reaction conditions[a] (form of PEP-3-T)	tritium removed by fumarase (%)	
	no LDH	+LDH[b]
Z	37.1 ± 0.8 (6)	36.0 ± 0.3 (6)
Z[c]	49.0 ± 0.5 (7)	50.6 ± 0.2 (6)
E	64.3 ± 0.5 (6)	63.6 ± 0.3 (4)
E = Z	49.7 ± 0.3 (3)	50.6 ± 0.2 (6)

[a] Each incubation was contained in 0.5 mL of D_2O (at pD 6.0, 20 °C): Na maleate (40 mM), NADH (2 mM), malate dehydrogenase (20 units), transcarboxylase (0.23 units), PEP-3-T (Z, E, or E = Z, as noted, 0.8 mM), (RS)-methylmalonyl-CoA (4 mM), acid phosphatase (0.016 units) and lactate dehydrogenase, LDH (13 units or none as noted). The reactions were monitored at 340 nm and were followed to completion. (E)- and (Z)-PEP-3-T were prepared as before,[10] and malate was isolated and analyzed for purity and position of tritium as before.[6a] In all cases the malate was radiochemically >95% pure as shown by its ability to give up its tritium to water upon prolonged incubation with malate dehydrogenase.[6a] The number of determinations of counts made volatile by fumarase is shown in parentheses. [b] With LDH only 5-10% of the reaction yielded oxalacetate (or malate). [c] Avidin present at 20 μM.

Because protonation is much more rapid than carboxylation, the results shown in Table II when lactate dehydrogenase was omitted are those expected for chiral pyruvate. In fact, they agree with values obtained for pyruvate that was formed by action of pyruvate kinase and ADP on PEP-3-T in D_2O.[6a,9] D^+ addition is made to the 2-*si* face of enolpyruvate: (3*S*)-pyruvate is formed from (*Z*)-PEP-3T and (3*R*)-pyruvate from (*E*)-PEP-3-T. With avidin present no distinction was made between the two faces of enolpyruvate, as is consistent with the loss of enzyme function. In the presence of lactate dehydrogenase (13 units compared with ~0.23 units of transcarboxylase) all free pyruvate would be trapped immediately as lactate, and any malate formed would have come from direct carboxylation of enolpyruvate *and* carboxylation of enolpyruvate derived from pyruvate that had not yet dissociated from the enzyme. The similarity of results in which malate dehydrogenase was present with and without lactate dehydrogenase seems to show that *direct* carboxylation of enolpyruvate is negligible. Were it to occur it would raise the counts exchanged by fumarase when (*Z*)-PEP-3-T was used and lower them in the case of (*E*)-PEP-3-T relative to the values obtained without lactate dehydrogenase.

Observations almost identical with these have been made by using rat liver pyruvate carboxylase. In that case stereospecific ketonization of enolpyruvate occurred only in the presence of the reagents necessary to carboxylate the biotin: ATP, HCO_3^-, and acetyl-CoA. As with the transcarboxylase, direct carboxylation of enolpyruvate could not be observed by stereochemical means.

Although the evidence given here in support of a carbanion mechanism would undoubtedly be more complete if direct carboxylation of enolpyruvate had been demonstrated, simple kinetic parameters of the reaction might prevent this step from being observed by a stereochemical approach. Slow release of oxalacetate relative to ketonization and liberation of pyruvate would readily explain the observations. It would be surprising to discover that the avidin-sensitive ketonization was a side reaction unrelated to the carboxylation of pyruvate: ketonization requires that the enzyme be carboxylated, is stereospecific, and forms pyruvate at a site from which its carboxylation can be observed without prior dissociation from the enzyme.

It may be of interest to note that in all reactions of PEP that may be expected to use enolpyruvate as an intermediate, addition to C-3 occurs from the 2-*si* face.[10,11] The pyruvate-activating subunits of transcarboxylase and pyruvate carboxylase acting on enolpyruvate directly are now known to continue this generalization.

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Registry No. E.C.2.1.3.1, 9029-86-1; enolpyruvate, 19071-34-2; methylmalonyl-CoA, 1264-45-5.

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Evidence for Two Types of Binding Sites in Cadmium Metallothionein Determined by Perturbed Angular Correlation of γ Rays[1]

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Metallothionein is a widely distributed metal- and sulfur-rich low molecular weight (~6800) protein which is of considerable interest in view of its presumed involvement in metal metabolism, homeostasis, and detoxification.[2] All mammalian forms (including man) characterized to date contain a single polypeptide chain of a total of 61 amino acids, out of which 20 are cysteine residues and serve as binding ligands for all metal ions.[3] Recent ^{113}Cd

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