Stereochemistry of the Enzymatic Carboxylation of Phosphoenolpyruvate

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SUMMARY

Using specifically labeled 3-¹H-phosphoenolpyruvate, the stereochemistry of CO₂ (or HCO₃⁻) addition was determined for the reactions catalyzed by P-enolpyruvate carboxylase (from peanuts and Acetobacter xilinum), P-enolpyruvate carboxykinase (from pigeon liver), and P-enolpyruvate carboxytransphosphorylase (from Propionibacterium shermanii). In all cases the addition occurs from the same side of the plane of enzyme-bound P-enolpyruvate, the si side. These results relate the stereochemical course of the protons in these carboxylation reactions to that in the enolase, fumarase, and phosphoglucose isomerase reactions. Furthermore, the conservation of the addition in evolution implies that this stereochemistry may have significance to the reaction mechanism.

In the carboxylation of phosphoenolpyruvate to form oxalacetate, the CO₂ may approach the C-3 atom from the side of the plane in which the three substituents of C-2 (phosphate, carboxyl, and vinyl carbon) appear in a clockwise or counterclockwise sequence. These two sides of the plane of the carbon skeleton are designated re and si, respectively (1). To distinguish between these two directions of approach, a configurational comparison of the vinyl carbon of PEP specifically labeled with tritium with the methylene group of the oxalacetate formed in the carboxylase reaction is required. The former position can be stereospecifically labeled with ¹H by reaction of enolase with 3-¹H-glycerate-2-P that is prepared enzymatically either from 1-¹H-l[1R]fructose-6-P, obtained by glucose-6-P isomerase reaction with triH₂-OH or from 1-¹H-l[1S]fructose-6-P, obtained by isomerase reaction with 1-¹H-glucose-6-P (2). The stereochi in the carboxylation reaction has been determined by a proton magnetic resonance study of normal, (1-¹C) -, and (3-²H)-PEP and was found to occur by trans-elimination. Conversion of the formed oxalacetate to L-malate with malate dehydrogenase, and treatment of the latter with fumarase, should result in labilization of only the pro-R position at C-3 of malate (3, 4). The known and unknown relations are summarized in Scheme 1 where H₄ derives from the C 1 of glucose 6-P and H₄ from both C-2 of glucose-6-P and water in the P-glucose isomerase reaction (5).

Three carboxylation reactions were examined.

1 The abbreviations used are: PEP, phosphoenolpyruvate; OAA, oxalacetate.


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Methods

Preparation of Two Forms of (3-^H)PEP—(3^-H_A)PEP was prepared from (1^-H)glucose (New England Nuclear Corporation) as follows. The (1^-H)glucose was converted to fructose-1,6-di-P in normal water in a 10-min incubation containing (1^-H)glucose (2.4 ^mu moles, 10^6 cpm), Tris (50 ^mu moles, pH 7.5), ATP and MgCl_2 (5 ^mu moles each), and about 1 unit each of hexokinase, phosphoglucone isomerase, and P-fructokinase (Boehringer Mannheim Corporation) in 1 ml. The fructose-di-P was neutralized with NaOH, concentrated, and converted to glycerate-2-P as follows: 2 ml contained the fructose-di-P (1.8 ^mu moles, 9 x 10^6 cpm), Tris-chloride (0.1 M, pH 7.5), EDTA (5 ^mu moles), Na_2HAsO_4 (5 ^mu moles), DPN (2.5 ^mu moles), glyceraldehyde-P dehydrogenase (3.6 units), triose-P isomerase (240 units), aldolase (1 unit), and lactate dehydrogenase (1 unit). When the increase in absorbance at 340 mM began to slow, pyruvate (1 ^mu mole) was added stepwise in order to remove the DPNH. After 1 hour the glycerate-3-P was isolated on Dowex 1-CI^- by elution with 0.03 N HCl. The equilibrium between glycerate-3-P, glycylate-2-P, and PEP was established at 55°C as follows: 1 ml contained glycerate-3-P (7 x 10^6 cpm), Tris-chloride (0.1 M, pH 7.5), MgCl_2 (5 ^mu moles), EDTA (2 ^mu moles), glycylate-2-P (20 ^mu moles), 2 units each of enolase and phosphoglycerate mutase (Boehringer). After 1 hour the reaction was stopped with 200 ^mu moles of HCl. The neutralized and diluted solution was placed on Dowex 1-CI^- for separation of glycerate phosphates (5.6 x 10^4 cpm) and PEP (8.9 x 10^4 cpm) (6). (3^-H_A)PEP was prepared in much the same way except that glucose-6-P was equilibrated by P-glucose isomerase reaction with H_2O (200 ^mu moles of glucose-6-P, pH 8.0, with Tris, 400 units of P-glucose isomerase, and 0.5 ml of H_2O (0.5 Ci)). After 8 hours at 25°C the water was recovered by freeze-drying and the residue was dissolved in HCl. Only the fructose-6-P component (25%) of the isomerase equilibrium was used in the subsequent reaction with P-fructokinase, and fructose-di-P was isolated (18 ^mu moles, 2.5 x 10^6 cpm). The remaining steps for production of PEP were as before.

PEP carboxykinase was prepared from pigeon liver according to the method of Utter and Kurashii (7). PEP carboxylase from peanut was prepared by the method of Maruyama et al. (8). PEP carboxylase of Acetobacter xylinum that is inhibited by ADP and ultrate cycle intermediates was prepared according to the method of Benzman (9). PEP carboxytransphosphorylase of propionibacteria was the preparation of Loechmiller, Wood, and Davis (10). The four preparations were essentially free of fumarase. Fumarase and malate dehydrogenase were obtained commercially.

Isolation of malate was by ion exchange on a Dowex 1-formate column (0.8 x 5 cm) with a gradient of formic acid, 0 to 4 n (11).

To determine the stereoisomerism of 3^-H-l-malate, the sample (<0.5 ^mu mole) was incubated in phosphate buffer (50 mM, pH 7.2) with 10 units of fumarase for an hour. The water of the sample was obtained by sublimation and counted by liquid scintillation spectrometry.

Radioactivity of malate that is not labilized by fumarase could be demonstrated to be present in malate since by the inclusion of malate dehydrogenase and excess acetylpyridine DPN^+ all the radioactivity was made volatile due to rapid enolization of oxalacetate at pH 9.5 of the incubation.

Results and Discussion

The stereochemistry of carboxylation of mononitritiated PEP, specifically labeled at H_2 or H_3, was examined as in Table I. With all of the carboxylations, it is evident that the H_A hydrogen derived from PEP is in the position in malate that is labilized in the fumarase reaction. This corresponds to addition of CO_2 in the case of PEP carboxykinase and PEP carboxytransphosphorylase (12) and HCO_3^- in the case of PEP carboxylase (8) to the same side of the plane in all cases. From the present evidence on the stereochemistry of the enolase reaction, this corresponds to addition from the si side. There is a degree of apparent nonstereospecificity especially in the case of the PEP carboxytransphosphorylase. It is noted that only in this case is there a significant loss of tritium in the net reaction, i.e. the specific activity of malate is much lower than that of PEP. There are two possible explanations for this loss. First, the formed oxalacetate may be incompletely trapped by malate dehydrogenase, thus allowing enolization and hence racemization of the methylene carbon of oxalacetate to occur. This explanation seems to be ruled out by the fact that increasing the malate dehydrogenase to carboxytransphosphorylase ratio 5-fold did not abolish the apparent racemization. It is noteworthy that in the absence of CO_2, this enzyme forms pyruvate and PP_i from PEP and P_i instead of oxalacetate (10). It is possible that under the conditions of the experiment a transient formation of bound pyruvate occurs. If the protonation to form the CH_2 group is not completely stereospecific, or, as seems more likely, the stereospecific protonation is followed by rotation at the C-2 to C-3 bond of the bound pyruvate, then the subsequent enolization-phosphorylation and carboxylation would account for the partial racemization of the isotope (13). It may be of importance to note that in both studies the degree of racemization was greater from the PEP H_A. This suggests incomplete and asymmetric randomization of the —CH_2 hydrogen atoms. Evidence for such a cycle of ketonization enolization ketonization prior to product formation is seen in the pyruvate kinase reaction in which 5 to 15% of the tritium of PEP is found in water during the net formation of pyruvate that is trapped by lactate dehydrogenase. In this case, no difference was observed between the two forms of tritiated PEP.

The observed stereospecificity of the PEP carboxykinase reaction is significant in terms of the tritium- and deuterium-labeling patterns of glycogen derived from labeled precursors in the whole rat. In 1955 Hoberman (14) showed that deuterium from (2^-H)lactate, fed to a fasted rat, was incorporated into the C-4 and C-6 positions of the glycogen units. The asymmetry of labeling at C-4 relative to C-3 was explained by the known stereoisomerism of the triose-P isomerase and aldolase reactions. The explanation of the labeling at C-6, but not at C-1, depended on the assumed stereoisomerism of the PEP carboxykinase reaction and its relation to that of the fumarase and phosphoglucone isomerase reactions. It was proposed that the coupling of lactate and malate dehydrogenases in the liver resulted in (2^-H)_m-malate which upon equilibration in the fumarase reaction gives (2,3^-H)malate. Oxidation with malate dehydrogenase would give (3^-H)oxalacetate which, if it entered gluconeogenesis by the route of PEP carboxykinase, might produce a particular isotopic

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3 I. A. Rose, unpublished data.
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The conditions for the four enzyme experiments were as follows: Experiment 1, PEP (0.5 mM), IDP (1.4 mM), MnCl₂ (0.7 mM), Tris-acetate (65 mM, pH 7.4), NaHCO₃ (25 mM), DPNI (2 mM), PEP carboxykinase (0.2 unit per ml), and malate dehydrogenase (2 units per ml); Experiment 2, PEP (0.25 mM), potassium orthophosphate (10 mM, pH 6.8), MgCl₂ (10 mM), NaHCO₃ (10 mM), DPNH (2 mM), PEP carboxylase from peanut (1.1 units per ml), and malate dehydrogenase (150 units per ml); Experiment 4, PEP (0.45 mM), Tris-acetate (65 mM, pH 7.4), NaHCO₃ (25 mM), DPNH (1.2 mM), PEP carboxytransphosphorylase (0.2 or 1 unit per ml), and malate dehydrogenase (8.6 units per ml); Experiment 3, PEP (0.8 mM), Tris-chloride (0.01 M, pH 7), KHCO₃ (5 mM), MgCl₂ (5 mM), glutathione (3 mM), PEP carboxylase from peanut (1.1 units per ml), and malate dehydrogenase (150 units per ml).

<table>
<thead>
<tr>
<th>Experiment and enzyme</th>
<th>P-enolpyruvate</th>
<th>Malate</th>
<th>H⁺ released</th>
<th>Ratio of malate dehydrogenase to carboxylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. P-enolpyruvate-GDP carboxykinase</td>
<td>+H₄</td>
<td>7,100</td>
<td>7,740</td>
<td>98</td>
</tr>
<tr>
<td>2. P-enolpyruvate carboxyphosphotransferase</td>
<td>+H₄</td>
<td>18,300</td>
<td>14,400</td>
<td>83, 90</td>
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<tr>
<td>3. P-enolpyruvate carboxylase (peanut)</td>
<td>+H₄</td>
<td>50,700</td>
<td>41,200</td>
<td>25, 34</td>
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<td>4. P-enolpyruvate carboxylase (Acetobacter)</td>
<td>+H₄</td>
<td>35,000</td>
<td>36,500</td>
<td>92.5</td>
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<td></td>
<td>+H₄</td>
<td>91,600</td>
<td>91,800</td>
<td>254</td>
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<tr>
<td></td>
<td>+H₄</td>
<td>48,400</td>
<td>47,800</td>
<td>4.0</td>
</tr>
</tbody>
</table>

**Scheme 1**

Sommer of (3-²H)PEP. The path from PEP to fructose-6-P would then result in labeling at C-1 and C-6. It was then postulated that the deuterium at C-1 was lost in the P-glucose isomerase reaction. The results of the present investigation are entirely consistent with the stereochemical requirements of this pathway. It may be of evolutionary significance that the three known PEP carboxylation reactions proceed with the same stereochemistry. In addition, this constancy in the stereochemistry suggests a structural dependence between the phosphoryl transfers and carboxylation steps. Stereochemical variability has been noted within the class of reductive carboxylations represented by isocitrate dehydrogenase and 6-P-gluconate dehydrogenase (15) in which the replacement of hydrogen by carboxyl occurs in independent steps with the formation of an enol intermediate. It will be of additional interest in these respects to record the stereochemistries of reactions of PEP with different adducts. Onderka and Floss (16) have shown that the addition of erythrose-4-P to PEP by the enzyme of the shikimic acid pathway of *Escherichia coli* occurs with a dominant stereospecificity that is the same as that for the carboxylation reactions reported here.

**References**

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