Stereochemistry of Pyruvate Kinase, Pyruvate Carboxylase, and Malate Enzyme Reactions*

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SUMMARY

Using isotopically asymmetric phosphoenolpyruvate-3-d,t it was established that pyruvate kinase causes the addition of a proton to C-3 from the face designated by a counter-clockwise sequence of phosphate, carboxyl, and vinyl groups, the 5i face. Having shown that the pyruvate carboxylase reaction has a normal primary isotope effect, it was possible to determine its stereochemistry with enantiomorphic pyruvate prepared in the pyruvate kinase reaction. Carboxylation occurs with retention of configuration at carbon-3 of pyruvate. With this information, it was possible to analyze the enantiomorphic pyruvates formed by reaction of (3S)-pyruvate and (3R)L-malate-3-d,t with malate enzyme. The decarboxylation occurred with retention.

An approach to the stereochemistry of reactions leading to creation of the methyl group of acetate has recently been found (1, 2). With the use of the kinetic isotope effect of the malate synthase reaction, it was shown with asymmetrically labeled acetate-2-H, d,t that preferential displacement of the proton by glyoxylate gave rise to malate that was asymmetrically labeled with tritium at C-3, as shown by subsequent reaction with fumarase. The analysis of these results makes it possible to distinguish isotopic enantiomers of acetate.

In addition, recent studies (3) resulting in specific isotopic synthesis and the nuclear magnetic resonance assignment of the vinyl protons of phosphoenolpyruvate have made it possible to determine the stereochemistry of ketonizing addition to the enolic carbon (4, 5).

In the present study, the stereochemistry of the pyruvate kinase reaction was solved by conversion of PEP labeled specifically with deuterium and tritium to pyruvate in H2O. The pyruvate was converted to acetate which was analyzed by the route to malate with malate synthase. This work results in the preparation of isotopically enantiomeric forms of pyruvate. With these forms, and applying the observation that the pyruvate carboxylase reaction discriminates against the heavier hydrogen isotopes, the stereochemistry of this reaction was directly determined by examination with fumarase of the malate formed in trapping the oxalacetate produced. Finally, with specific 1-malate-d,t, the stereochemistry of the malate dehydrogenase-decarboxylating (here referred to as malate enzyme) reaction in H2O was determined by analysis of the pyruvate with the pyruvate carboxylase-fumarase sequence.

The results of these studies are considered in terms of structural and mechanistic aspects of the three reactions.

MATERIALS AND METHODS

Preparation of Compounds—A mixture of 2 and 3 d-phosphoglycerates (PGA), containing both deuterium and tritium stereospecifically located at C-3, were prepared with purified glycolytic enzymes as previously described (3) in such a way as to produce (3S)PGA-3d,t by starting with glucose-1-t and using D2O in the phosphoglucone isomerase step, and to produce (3R)PGA by starting with glucose-1-d and using 1HOH in the phosphoglucone isomerase step. The glucose-1-d was synthesized from d-glucono-δ-lactone by Na-Hg reduction (7) in D2O. Both compounds should contain greater than 95% deuterium in their specific positions and tracer amounts of tritium mixed with predominant hydrogen in the position geminal with the deuterium. The specific activities of tritium were: 107 cpm per pmole for the (3S)PGA and 5.7 x 104 cpm per pmole for the (3R)PGA.

1-Malate, containing both deuterium and a tracer of tritium, both stereospecifically located at C-3, were prepared as follows: (3R,l-malate-2d-3d,t was prepared by reaction in tritiated water of fumarase with fumarate-2,3-dt (89 atom %), which was kindly provided by Dr. Sasha Englard. The malate was recovered from a Dowex 1-formate column with 1 N formic acid. (3S,l-Malate-2t-3d,t was prepared by reaction in D2O of fumarase with fumarate-2,3-t3. The latter compound was prepared from d,l-malate-2-t (New England Nuclear) by extracting

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1 The abbreviations used are: PEP, phosphoenolpyruvate; PGA, d-glycerate-2-P and/or -3-P.

2 The R/S system of Cahn, Ingold, and Prelog is used with the priority rule that higher mass number precedes lower (6). Since tritium is present only in tracer concentration, the chirality symbol R or S is understood to refer to that component of the mixture of isotopic species that contains all three hydrogen isotopes in the case when a methyl group is named and to the specified two hydrogen isotopes when a methylene group is named.
the fumarase-treated solution with ether at acid pH to extract fumaric acid preferentially, followed by elution on a Dowex-1-formate column with 4 N formic acid.

The enzymes used were obtained as follows: pyruvate carboxylase (EC 6.4.1.1) of liver (17 units per mg of protein) was a generous gift of Dr. Michael Scrutton. Malate enzyme (EC 1.1.1.40) of Escherichia coli was generously provided by Dr. Joseph Spina of the Department of Biochemistry, University of Pennsylvania. The enzyme, which had a specific activity of 5 units per mg, was not yet homogeneous (8). The highly purified malate enzyme of pigeon liver was a kind gift of Dr. Robert H. Hau. Malate synthase (EC 4.1.3.2) from yeast was prepared from yeast by the method of Dixon and Kornberg (9). The other enzymes were obtained commercially from Boehringer Mannheim.

Acetate was converted to malate by the sequence acetate → acetyl-P → acetyl CoA → malate with the enzymes acetate kinase (EC 2.7.2.1), phosphotransacetylase (EC 2.3.1.8), and pyruvate kinase, and the PGA-3-d, t samples (0.5 pmole, including the radioactive material and carrier), sodium acetate (1 µ mole, including the radioactive material and carrier), sodium glyoxylate (20 µ mole), acetate kinase (1 unit), phosphotransacetylase (1 unit), and malate synthase (0.1 unit). Care was taken to limit the amount of these enzymes as much as possible to limit the introduction of significant fumarase activity. The reaction mixture was dried from dilute acid to remove unreacted acetic acid and tritiated water. Malate, 0.5 to 0.8 µ mole, was isolated from a Dowex 1-Cl− column by elution with 5 mM HCl.

To determine the tritium distribution in the C-3 positions of malate, part of the malate was reacted with fumarase (3.5 units) in 40 mM KPO4 buffer, pH 7.0, for 30 min at 25°C. The counts that are recovered in the distillate of such a reaction mixture are attributed to the C-3 pro-R (10) position of malate (11, 12). A second sample of malate was converted to oxalacetate within 10 min at pH 6.5 with malate dehydrogenase (70 units) and 3-acetylpyridine-DPN and hence served as the assay for malate (13). As predicted from the data of Banks (14), both methylene hydrogens of oxalacetate fully exchange with the alkaline medium within 60 min and could be determined by distillable radioactivity. The difference in counts between the two determinations is attributed to the C-3 pro-S position of malate.

RESULTS AND DISCUSSION

Pyruvate Kinase—The two kinds of PGA, (3S)PGA-3-d, t and (3R)PGA-3-d, t, were converted to pyruvate by the reactions of phosphoglycerate mutase, enolase, and pyruvate kinase incubated in H2O with the following components: imidazole buffer (50 mM, pH 6.5), KCl (50 mM), MgCl2 (5 mM), ATP (0.2 mM), 2,3-dPG (5 µM), EDTA (1 mM), glucose (5 mM), and 0.2 unit per ml each of phosphoglycerate mutase, enolase, hexokinase, and pyruvate kinase, and the PGA-3-d, t samples (0.5 mM to 1.5 mM). The glucose and hexokinase were present to regenerate ADP in the reaction. Pyruvate formation was monitored on small samples with lactate dehydrogenase. When the reaction was complete in about 10 min, H2O2 (100 µmoles) was added to convert the pyruvate to acetate, which was eluted from Dowex-1-Cl− with 5 mM HCl. The neutralized acetate was concentrated in a vacuum and converted to malate by the acetate kinase-transacetylase-malate synthase system. The isolated malate was treated to determine the distribution of tritium, with the results shown in Table I.

The malate is observed to have 80% of the trition-specific activity of the starting PGA. The decrease in specific activity may be attributed to several sources: in the pyruvate kinase step at pH 6.5, about 3 to 5% of the counts of PEP-3-t are diverted into water during the conversion to pyruvate-3-t. In the conversion of acetate-3-t to malate, about 16% of the counts are diverted into water as a result of the malate synthase step and any fumarase present. Finally, any contaminating acetate that is picked up from the reagents of the incubation or the ion exchange step will contribute to the decreased specific activity of the malates.

The results of this study, taken together with the known stereochemical course of enolase and malate synthase, lead to the conclusion that the proton adds to the si face of C-3 of PEP in the pyruvate kinase reaction (see Scheme 1). Thus, the anti-elimination of water from (3S)PGA-3-d, t in the enolase reaction leads to the indicated form of PEP (3), and in the malate synthase reaction the proton of acetyl-CoA is displaced with inversion (1, 2). To the extent that proton is displaced in this reaction in preference to deuterium, tritium will be enriched in the C-3 pro-R position of malate, as indicated by the transfer of radioactivity to water in the fumarase reaction. The degree of enrichments seen are comparable with those previously reported (15), and the more extreme results with (3S)phosphoglycerate suggest an isotope effect of $k_R/k_D = 5$.

It may be argued that the face of enolpyruvate, to which the proton is added in the pyruvate kinase reaction, is turned toward the medium, whereas the opposite face is bound to the surface of the enzyme. It has been demonstrated in this laboratory that under certain conditions PEP-3-t can undergo loss of as much as 70% of its tritium to the medium during the net reaction in which pyruvate is trapped by rapid reduction with lactate dehydrogenase. Since all elements of the reaction, i.e. ATP, enolpyruvate, K+, and a divalent cation, particularly Co2+, are present in the complex at the time of proton release, the extensive reorganization of the complex that would be required if proton release occurred from the direction of the binding face of the enolpyruvate kinase.

Table I

| Source of PGA | Specific activity of malate | H in C-3 of malate | H in C-3 of malate pro-R | H in C-3 of malate pro-S |
|--------------|---------------------------|------------------|-------------------------|-------------------------|
| (3S)Phosphoglycerate (165,000) | 87,000 | 97 | 84 | 16 |
| (3R)Phosphoglycerate (67,500) | 54,200 | 98 | 32 | 68 |

The conversion of acetate-3-t to malate, about 16% of the counts are diverted into water as a result of the malate synthase step and any fumarase present. Finally, any contaminating acetate that is picked up from the reagents of the incubation or the ion exchange step will contribute to the decreased specific activity of the malates.

REFERENCES

1. J. L. Robinson and I. A. Rose, unpublished results.

2. In the re/si system of Hanson (10), the face of a trigonal carbon such as C-2 of PEP is designated by determining whether the substituents of that carbon, when ordered according to the sequence rules of Cahn et al. (6), are seen as a clockwise, re, or counterclockwise, si, sequence. In designating C-3 of PEP where identical ligands prevent the application of this rule, the faces at C-3 are named according to those of the adjoining trigonal carbon (10), C-2 in this case.
Scheme 1

Pyruvate would be difficult to envisage. Strong evidence in support of this concept comes from the study of Phillips and Kosicki, in which it was shown that pyruvate reduced by NaBH₄ in the presence of pyruvate kinase formed lactate that was primarily (74%) the d-enantiomer (16). Assuming that BH₄⁻ approaches the enzyme-polarized carbonyl carbon from the medium, one would predict the formation of d-lactate if the si face were turned to the medium.

The direction of the allylic phosphoryl addition to the presumed enolpyruvate anion intermediate in the reverse reaction remains to be determined.

It is of interest that with the three PEP carboxylation reactions, in which phosphoryl transfer is to H₂O, P₄, or GDP, CO₂ addition is invariably to the si face (5) and that this is also the case in the allylic replacement by erythrose-4-P to produce 2-keto-3-deoxy-7-P heptulonic acid (4). These correlations suggest that a unique arrangement of amino acids responsible for PEP binding specificity may be a conserved structural unit in evolution.

Pyruvate Carboxylase—In a preliminary experiment carried out with Dr. Michael Scrutton, it was determined that in the carboxylation of pyruvate-3-t coupled to malate dehydrogenase, only 8% of the tritium was found in water. This indicates a discrimination factor of 4.2-fold in favor of protium relative to tritium, and hence about 2.7-fold (17) relative to deuterium. From this it follows that carboxylation of pyruvate of known chirality, prepared as in the pyruvate kinase study, would yield malate-3-t that would be stereoselectively tritiated in a manner
dependent on the stereochemistry of the proton displacement (Scheme 2).

The pyruvate used in these experiments were prepared by reaction of phosphoglycerate mutase, enolase, and pyruvate kinase with the two forms of PGA-3-d, in H2O as above. They were isolated on Dowex 1-Cl- columns by elution with 10 mM HCl. Their stereochemical assignments are derived from the conclusion that pyruvate kinase adds a proton to the si face of PEP. The incubations contained, in 1 ml: Tris-Cl (0.2 M, pH 7.8); ATP (0.8 mM); MgCl2 (5 mM); KHCO3 (20 mM); K2SO4 (2 mM); acetyl-CoA (60 μM); DPNH (0.2 mM); malate dehydrogenase (7 units); pyruvate carboxylase (0.4 unit); and about 0.1 μmole of (3S)- or (3R)pyruvate. Within about 10 min, the absorbance decrease at 340 nm was complete and the malates were recovered from Dowex 1-Cl- columns by elution with 5 mM HCl. The malate was examined for tritium distribution as before.

The results of such an experiment using enantiomorphous forms of pyruvate are given in Table II. They indicate that the reaction occurs with retention of configuration. The degrees of tritium asymmetry correspond to intramolecular isotope effects, kH/kD of 3.5 and 1.8 for the two substrates. The disagreement is in the same direction as found in the previous experiment (Table I) for which the same sources of pyruvate were used. One might reasonably attribute this discrepancy to a lack of isotopic homogeneity in the glucose-1-d in the preparation of (3R)PGA-3-d, and hence (3R)pyruvate.

### Table II

| Substrate (specific activity) | Malate isolated |
|-----------------------------|-----------------|
|                            | Specific activity | %  |
| (3S)Pyruvate (~104)         | 780,000          | 94 |
| (3R)Pyruvate (57,000)       | 56,000           | 93.5 |

The stereochemistry of only one other biotin-containing carboxylase has been determined, namely propionyl-CoA carboxylase, which was shown to proceed by retention in the formation of (3S)methylmalonyl-CoA (18, 19). Additional examples should be analyzed before any suggestion of a stereochemical rule can be made. It is noteworthy, however, that enzymes of this class undergo proton activation of the substrate only when the biotin-protein is in the carboxylated state (19, 20). This result alone might be interpreted in terms of a concerted mechanism, such as suggested by Mildvan and Scrutton (21):

![Scheme 3](https://example.com/scheme3.png)

This mechanism restricts the stereochemistry of proton replacement to that of retention, and hence the present results are consistent with this mechanism.

**Malate Enzyme**—To study the stereochemistry of the decarboxylation of malate by this enzyme, the pyruvate formed from specifically labeled L-malate-3-d, in H2O was converted back to malate with the pyruvate carboxylase-malate dehydrogenase sequence, and the malate was analyzed for its C-3 tritium distribution as before (Scheme 3).

In the conversion of malate to pyruvate, care was taken to limit the effect of a contaminating fumarase activity that was present in the enzyme from *E. coli* by carrying out the reaction to only about 28% conversion in a 0.3-ml incubation containing Tris-Cl (50 mM, pH 7.4); EDTA (1 mM); dithiothreitol (1 mM); KCl (0.1 M); TPN (15 mM); MnCl2 (5 mM); *E. coli*-malate enzyme (0.03 unit); and 2 μmoles of (3R)- or (3S)malate-3-d, . The reaction was monitored at 340 nm and pyruvate was recovered on Dowex-1Cl- by elution with 10 mM HCl. In the case of the (3R)malate, about 24% of the radioactivity applied to the column was present as water in the column breakthrough, presumably the result of action by the fumarase contaminant in
exchanging the C-3 \textit{pro-R} tritium for a proton from the medium. In (3S)malate, the exchange is between protons; hence the tritium in the \textit{pro-S} position remains fixed. The \textit{pyruvate} was converted to malate as before, and the distribution of radioactivity in C-3 of the synthesized malate was determined with the \textit{malate} enzyme.

The results shown in Table III indicate clearly that the decarboxylation occurs with retention of configuration. The isotope discrimination seen with (3S)malate agrees with the larger variability in the stereochemical course of the reactions of this class than has been considered reasonable in view of evidence that decarboxylation and proton exchange have been found to be independent steps (23–25). Definitive experiments on the dependence of proton exchange on the pressure of CO₂ with malate enzyme, however, have not been published to date. It may be significant that a correlation between stereochemical course and metal requirement is provided by the fact that only the malate enzyme and isocitrate dehydrogenase require metal ion cofactors.

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REFERENCES

1. CORNFORTH, J. W., REDMOND, J. W., EGGERER, H., BUCKEL, W., AND GUTSCHEW, C., Nature, 221, 1212 (1969).
2. LUDTH, J., RÉTÉY, J., AND ABIGONI, D., Nature, 221, 1213 (1969).
3. COHN, M., PHARSON, J. F., O'CONNEL, E. L., AND ROSE, I. A., J. Amer. Chem. Soc., 92, 4065 (1970).
4. ONDERKB, D. K., AND FLOSS, H. G., Biochem. Biophys. Res. Commun., 35, 501 (1969).
5. ROSE, I. A., O'CONNEL, E. L., NOCE, P., UETER, M. F., WOOD, H. G., WILLARD, M. J., COOPER, T. G., AND BENZIMAN, M., J. Biol. Chem., 244, 6120 (1969).
6. CARN, R. S., INGOLD, C. K., AND PRELOG, V., Angev. Chem. Int. Ed. Engl., 78, 413 (1966).
7. ABRAM, S., AND Harris, W. Z., in S. P. COLOWICIC AND N. O. KAPLAN (Editors), Methods in enzymology, Vol. 4, Academic Press, New York, 1965, p. 663.
8. CARN, R. S., INGOLD, C. K., AND PRELOG, V., Angev. Chem. Int. Ed. Engl., 78, 413 (1966).
9. DIXON, G. H., AND KORNBERG, H. L., in S. P. COLOWICIC AND N. O. KAPLAN (Editors), Methods in enzymology, Vol. 5, Academic Press, New York, 1966, p. 663.
10. HANSON, K. R., J. Amer. Chem. Soc., 83, 3634 (1961).
11. ABIGONI, D., CORNFORTH, J. W., DONNINGER, C., MALLABY, R., REDMOND, J. W., AND CARN, R. S., J. Biol. Chem., 221, 1212 (1969).
12. SALZMAN, H., AND SBLK, G., CORNFORTH, J. W., DONNINGER, C., MALLABY, R., REDMOND, J. W., Nature, 228, 517 (1970).
13. PHILLIPS, T. M., AND KOSICHI, G. W., Fed. Proc., 29, 462 (1970).
14. BANKS, B. E. C., J. Chem. Soc., 63 (1962).
15. EGGERER, H., BUCKEL, W., LENZ, H., WUNDWALD, P., GOTTSHALK, G., CORNFORTH, J. W., DONNINGER, C., MALLABY, R., AND REDMOND, J. W., Nature, 228, 517 (1970).
16. EGGERER, H., BUCKEL, W., LENZ, H., WUNDWALD, P., GOTTSHALK, G., CORNFORTH, J. W., DONNINGER, C., MALLABY, R., AND REDMOND, J. W., Nature, 228, 517 (1970).
17. SWAIN, C. G., STIVERS, E. C., REUWERE, J. F., AND SCHAD, L. J., J. Amer. Chem. Soc., 80, 3885 (1958).
18. RÉTÉY, J., AND LYNNEF, F., Biochem. Z., 343, 256 (1965).
19. PRESCOTT, D. J., AND RABINOWITZ, J. L., J. Biol. Chem., 243, 1551 (1968).
20. MILDVA, A. S., AND COHN, M., J. Biol. Chem., 244, 1178 (1969).
21. MILDVA, A. S., AND SCRUTTON, M. C., Biochemistry, 6, 2978 (1967).
22. ENGLAND, S., AND LITZOWSKY, I., Biochem. Biophys. Res. Com- mun., 12, 356 (1963).
23. LIENHARD, G. E., AND ROSE, I. A., Biochemistry, 3, 185 (1964).
24. ROSE, I. A., J. Biol. Chem., 241, 3511 (1966).
25. LIENHARD, G. E., AND ROSE, I. A., Biochemistry, 3, 190 (1964).
26. SCHUTZBACH, J. S., AND FEINGOLD, D. S., J. Biol. Chem., 245, 2470 (1970).

**TABLE III**

Steroselectivity of the E. coli-malate enzyme reaction

| Substrate (specific activity) | Formed pyruvate | Malate isolated |
|-----------------------------|-----------------|-----------------|
| cpm/μmol                      | Specific activity | H in C-3 | H in C-3 pro-R | H in C-3 pro-S |
| (3R)Malate-2d, t (130,000)   | 104,000 | 90,000 | 99 | 59 | 41 |
| (3S)Malate-2d, t (73,200)     | 36,000 | 28,200 | 99 | 24 | 76 |

**TABLE IV**

Steroechemistry of the pigeon liver malate enzyme reaction

| Substrate (specific activity) | Product malate |
|-----------------------------|----------------|
| cpm/μmol            | Specific activity | H in C-3 | H in C-3 pro-R | H in C-3 pro-S |
| (3R)Malate-3d, t (130,000) | 104,000 | 99 | 77 | 23 |
| (3S)Malate-3d, t (130,000) | 63,800 | 95 | 96 | 74 |

The stereochemistries of several other enzymes that carry out pyridine nucleotide-dependent oxidative dehydrogenations of β-hydroxyacid have been reported. Both the TPN-specific (22, 23) and DPN-specific (24) isocitrate dehydrogenases also show complete retention in the decarboxylation step. However, 6-P-glucuronate dehydrogenase (25) brings about inversion in the replacement of carboxylic, and a related reaction catalyzed by UDP glucuronate carboxylase leads to inversion (26).
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