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Fluorine NMR Studies on Stereochemical Aspects of Reactions Catalyzed by Transcarboxylase, Pyruvate Kinase, and Enzyme I†

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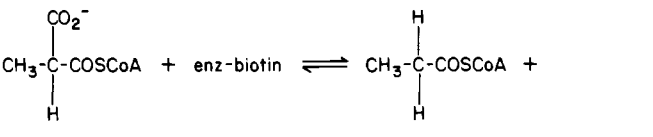
ABSTRACT: The stereochemistry of the transcarboxylase-catalyzed carboxylation of 3-fluoropyruvate has been studied by using fluorine NMR of unpurified reaction mixtures. When the product 3-fluorooxaloacetate was trapped by using malate dehydrogenase, only the 2*R*,3*R* diastereomer of 3-fluoromalate was formed. The fluoromethyl group of fluoropyruvate does not take up deuterium label from the solvent during the reaction. These results confirm and extend those obtained previously by Walsh and co-workers [Goldstein, J. A., Cheung, Y. F., Marletta, M. A., & Walsh, C. (1978) *Biochemistry 17*, 5567–5575] showing that transcarboxylase is specific for one of the two prochiral hydrogens in fluoropyruvate. Transcarboxylase, coupled to malate dehydrogenase, has been used to analyze samples of chiral fluoropyruvate obtained by dephosphorylation of (*Z*)-fluorophosphoenolpyruvate in D_2O in the presence of either pyruvate kinase or enzyme I from the *Escherichia coli* sugar transport systems. Analysis of the fluoromalate produced showed that fluoroenolpyruvate is deuterated from opposite faces by these two enzymes: enzyme I protonates (deuterates) fluoroenolpyruvate exclusively from the 2-*re* face and pyruvate kinase does so mainly from the 2-*si* face. Fluoropyruvate is carboxylated by transcarboxylase with absolute retention of configuration.

Transcarboxylase (methylmalonyl-CoA:pyruvate carboxyltransferase, EC 2.1.3.1) is a biotin-containing multimeric enzyme that has been purified from *Propionibacterium shermanii* (Wood et al., 1969). The transfer of CO_2 from methylmalonyl coenzyme A (methylmalonyl-CoA) to pyruvate involves two half-reactions 1 and 2, which take place on different subunits of the enzyme. Biotin, which is linked to the ε-amino group of a lysyl residue, carries the CO_2 from one subunit to another. The reaction sequence in eq 1 and 2 has been supported by results from kinetic studies (Northrop & Wood, 1969a,b) and by the isolation and subsequent utilization



(1)

$$CH_3\text{–}CO\text{–}CO_2^- + \text{enz–biotin–}CO_2^- \rightleftharpoons {}^-O_2C\text{–}CH_2\text{–}CO\text{–}CO_2^- + \text{enz–biotin} \quad (2)$$

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of a carboxybiotinyl enzyme intermediate (Wood et al., 1963). The stereochemistry of the reactions has been extensively studied by Walsh and co-workers; it has been shown that in both half-reactions carboxy and proton transfers take place with retention of configuration (Cheung et al., 1975). Goldstein et al. (1978) reported that the pure 3*R* enantiomer of fluorooxaloacetate is the product of carboxylation of fluoropyruvate.

As was pointed out by these authors, using a fluoromethyl group instead of a methyl group in such reactions greatly simplifies the stereochemical analysis. In the classical method developed by Cornforth et al. (1969) and Arigoni and coworkers (Lüthy et al., 1969), a chiral [^{1}H,^{2}H,^{3}H]methyl group is used, and the relative enrichment of tritium in the enantiotopic positions of the product is governed by an intramolecular primary kinetic ^{1}H/^{2}H isotope effect of uncertain size. However, when chiral [^{1}H,^{2}H]- or [^{1}H,^{3}H]fluoropyruvate is used, the outcome is no longer dependent on isotope effects, since, if the stereospecificity is absolute, the product (3*R*)-fluorooxaloacetate will contain either ^{1}H or ^{2}H and not a mixture of both in a ratio determined by isotope effects. In the event that both (3*R*)- and (3*S*)-fluorooxaloacetates were formed as products, absolute retention would yield only protonated (3*R*)- and only deuterated (3*S*)-fluorooxaloacetate, (or vice versa for inversion), again with no interference from kinetic isotope effects. As a consequence, the use of a fluorinated methyl group in the substance allows an accurate quantitative determination of the degree of stereospecificity in the carboxylation reaction.

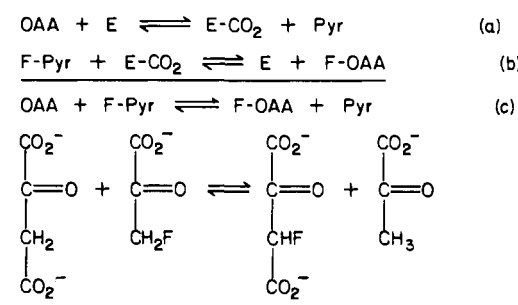
Since the stereochemical course of the transcarboxylase-catalyzed reaction, and of biotin-dependent carboxy transfer reactions in general, bears directly on the molecular mechanisms of the reaction (Rêtey & Lynen, 1965; Rose, 1970; Cheung et al., 1975), we decided to measure the degree of stereospecificity of the reaction by using chiral [^{1}H,^{2}H]-fluoropyruvate as a substrate. The stereochemical integrity and the deuterium content of the product fluorooxaloacetate can be determined at once from the ^{19}F NMR spectrum of the product after enzymatic reduction to fluoromalate. Not only are the two diastereomers of fluoromalate separated in ^{19}F NMR, but the protonated and deuterated species are also well separated, and each one is easily identified by its characteristic hyperfine pattern of F–^{1}H and F–^{2}H couplings.

As well as establishing the precise degree of stereoselectivity in the transcarboxylase-catalyzed reaction with fluoropyruvate, we also aimed to evaluate the potential of the coupled transcarboxylase and malate dehydrogenase reactions, combined with ^{19}F NMR of the products, as a method of examining the stereochemistry of other reactions (such as those discussed here involving pyruvate kinase and enzyme I) that can produce monodeuterated fluoropyruvate.

Materials and Methods

Enzymes. Transcarboxylase was purified from *P. shermanii* essentially as described by Wood and co-workers (Wood et al., 1977). The purified enzyme was stored in 200 mM sodium acetate, pH 5.5 at 9 °C. Enzyme concentrations were determined by incubating the enzyme with excess [1-^{14}C]oxaloacetate, trapping [1-^{14}C]pyruvate formed as lactate by using reduced nicotinamide adenine dinucleotide (NADH) and lactate dehydrogenase, and separating [1-^{14}C]lactate from [1-^{14}C]oxaloacetate by rapid anion-exchange chromatography on Zerolit-FFIP(SRA-66) (BDH): samples of the incubation mix in 0.3 M potassium phosphate buffer, pH 6.9, of a volume up to 500 L, were applied to 0.75-mL Zerolit columns in 1.5 mL of H_2O, lactate was washed off the columns with 6 mL of 0.125 M LiCl, and oxaloacetate was subsequently eluted with 7.5 mL of 1 M LiCl, at a rate of 0.5–1.0 mL/min; the two fractions were counted for radioactivity in Scintran cocktail T (BDH)/H_2O (1:1 v/v) just below room temperature. The [1-^{14}C]oxaloacetate used in these experiments was prepared from [1-^{14}C]pyruvate (20 mCi/mmol, Amersham) and nonradioactive oxaloacetate by using the transcarboxylase-catalyzed isotope exchange reactions. In a typical experiment, a mixture of 1 μM [1-^{14}C]pyruvate, 10 μM oxaloacetate, and 0.1 μM transcarboxylase in 400 μL of 0.3 M potassium phosphate buffer, pH 6.9, was used to generate [1-^{14}C]oxaloacetate. After 10 min at room temperature the sample was diluted 5-fold in the same buffer, and the [1-^{14}C]oxaloacetate/[1-^{14}C]lactate ratio was determined as described above, before and after the addition of 0.4 μM transcarboxylase.

Scheme I



The *Escherichia coli* PTS enzyme I was a kind gift of Prof. G. T. Robillard. Pyruvate kinase (rabbit muscle), lactate dehydrogenase (rabbit muscle), and malate dehydrogenase (pigeon breast muscle) were purchased from Sigma Chemical Co. and were used without further purification. Pyruvate kinase used in NMR experiments was dialyzed into 100 mM potassium phosphate buffer containing 100 mM KCl, pH 7.0 in H_2O, lyophilized, and taken up in ^2H_2O.

Chemicals. 3-Fluoropyruvate (sodium salt), sodium pyruvate, phosphoenolpyruvate (monopotassium salt), oxaloacetic acid, NADH (disodium salt), and malonyl-CoA (lithium salt) were all purchased from Sigma Chemical Co. and were used without any further purification. ADP (disodium salt) was from Boehringer.

Methods. Two carboxy donors were used in the transcarboxylase reaction. Malonyl-CoA was used as a carboxy donor in the overall reaction instead of the natural substrate (2*S*)-methylmalonyl-CoA. Spectrophotometric assays similar to the ones described by Wood and co-workers (Northrop & Wood, 1969) showed that malonyl-CoA gives the same V_{max} for the overall reaction as does (2*RS*)-methylmalonyl-CoA.

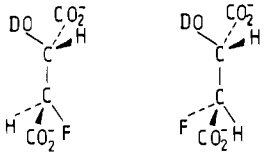
The second carboxy donor used in our studies was oxaloacetate. It is known from previous studies (Northrop & Wood, 1969a,b) that transcarboxylase catalyzes separate half-reactions, between pairs of CoA esters (eq 1) and between 2-oxo acids (eq 2). We used the 2-oxo acid half-reaction to carboxylate fluoropyruvate from oxaloacetate, as represented in Scheme I (OAA = oxaloacetate; Pyr = pyruvate). The second-order rate constant for the reaction of carboxylated enzyme with fluoropyruvate is 8 times slower than that with pyruvate. The overall equilibrium constant of reaction c of Scheme I is about 2.7 (methods to be published separately together with details on the kinetics of the reaction with protonated and deuterated 2-oxo acids in H_2O and D_2O).

All experimental details are given in the figure legends.

Results

Formation of (3R)-Fluorooxaloacetate and (2R,3R)-

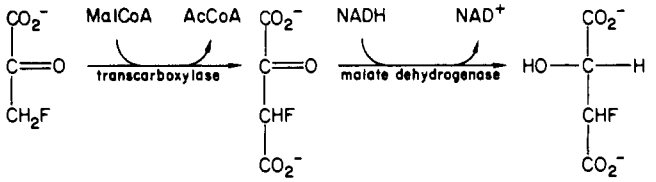
Chart I: Assignment of NMR Signals from Diastereomeric Fluoromalates



(2R,3R) fluoromalate (2R,3S) fluoromalate

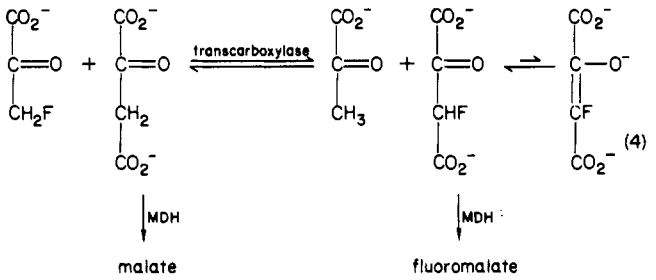
$J_{F-H(2)}$ = 25.4 Hz $J_{F-H(2)}$ = 35.7 Hz

Fluoromalate from Fluoropyruvate. When fluoropyruvate and malonyl-CoA are used as substrates for transcarboxylase, the fluorooxaloacetate formed can be trapped as fluoromalate by using malate dehydrogenase. This reaction is represented in eq 3. Figure 1A shows the ^{19}F NMR spectrum of fluoromalate as a product of this reaction carried out in D_2O. The four lines collapse into one upon proton decoupling, showing that they arise from only one fluorine coupled to two protons. This means that only one diastereomer of fluoromalate has been formed. It will be shown below that the resonance can be assigned to (2*R*,3*R*)-fluoromalate.



(3)

A mixture of (2*R*,3*R*)- and (2*R*,3*S*)-fluoromalates was generated by forming fluorooxaloacetate from fluoropyruvate with transcarboxylase in H_2O, allowing it to racemize by way of its keto–enol equilibrium, and then reducing it to fluoromalate with malate dehydrogenase. Oxaloacetate was used as the carboxy donor as shown in eq 4. The addition of malate dehydrogenase and excess NADH will cause reduction of both fluorooxaloacetate and the remaining oxaloacetate, so that at this point no net reaction from fluoropyruvate to fluorooxaloacetate and fluoromalate will occur: any fluoromalate formed originates from fluorooxaloacetate that was already present in the equilibrium mixture before the addition of NADH and malate dehydrogenase. The ^{19}F NMR spectra of the resulting fluoromalates are shown in Figure 1B. The downfield quartet contains F–H couplings of 49.4 and 25.4 Hz, and the upfield quartet is a fluorine resonance with F–H couplings of 49.0 and 35.4 Hz. The larger coupling constants in both (49.4 and 49.0 Hz) arise from the proton at the same carbon as fluorine. The smaller couplings (25.4 and 35.4 Hz) are due to the proton at C-2. Given the fact that malate dehydrogenase generates (2*R*)-fluoromalate (Goldstein et al., 1978), the latter coupling constants are consistent with J = 25.4 Hz in (2*R*,3*R*)-fluoromalate and J = 35.4 Hz in (2*R*,3*S*)-fluoromalate, since trans couplings are larger than cis couplings; see Chart I. Although the numbers are not exactly the same, this assignment is in agreement with proton NMR data reported by Kech et al. (1980): $J_{F-H(C-2)}$ = 23.7 and 32.2 Hz for (2*R*,3*R*)- and (2*R*,3*S*)-fluoromalates, respectively. Thus, the upfield quartet in Figure 1B belongs to (2*R*,3*S*)-fluoromalate, and the downfield quartet arises from the 2*R*,3*R* diastereomer.



(4)

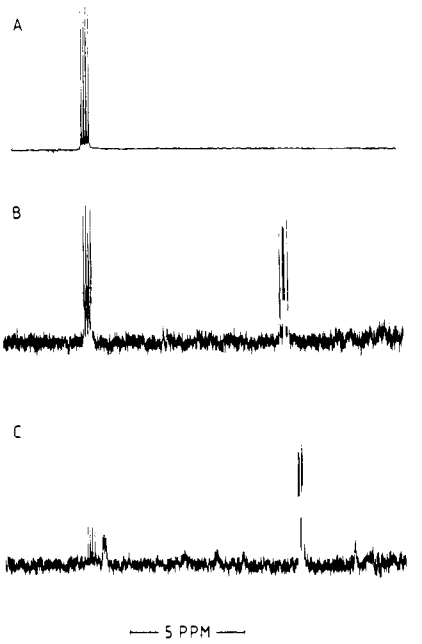


FIGURE 1: ^{19}F NMR spectra of fluoromalates as products of the following reactions: (A) 7 mM fluoropyruvate + 3.5 mM malonyl-CoA + 7 mM NADH + 20 units/mL malate dehydrogenase + 0.1 μM transcarboxylase (added last) were incubated in 300 mM potassium phosphate, pH(D) 6.9 in D_2O; the spectrum was recorded after 2 h at room temperature. (B) 3 mM oxaloacetate + 10 mM fluoropyruvate + 1.6 μM transcarboxylase were incubated in 200 mM potassium phosphate, pH 6.9 in H_2O; after 2 h at room temperature, 10 mM NADH + 30 units/mL malate dehydrogenase were added; after reduction the 1.2-mL reaction mixture was lyophilized and taken up in 400 μL D_2O and the NMR spectrum recorded. (C) The same reaction was carried out as for $(B)_2$ but in D_2O instead of H_2O; reaction volume was 400 μL; after reduction, the NMR spectrum was recorded directly. Spectra were recorded on a Bruker WM250 at 235 MHz in the quadrature detection mode, using a 5-μs 90° pulse. Other conditions: spectra width, 4000 Hz; acquisition time, 1.7 s; relaxation delay, 5 s; line broadening, 1 Hz; 600 scans (A) or 112 scans [(B) and (C)]; sample volume, 400 μL.

From this it is clear that the product in reaction 3 is (2*R*,3*R*)-fluoromalate (Figure 1A), in agreement with the results from chemical separation experiments reported earlier by Walsh and co-workers (Goldstein et al., 1978). The result in Figure 1B shows that malate dehydrogenase can reduce both (3*R*)- and (3*S*)-fluorooxaloacetates and therefore supports the conclusion by Walsh and co-workers that no (3*S*)-fluorooxaloacetate is formed as an intermediate in reaction 3. The possibility that some (3*S*)-fluorooxaloacetate *was* formed by transcarboxylase, but that its conversion to the 3*R* enantiomer *via* the enol and its subsequent reduction to (2*R*,3*R*)-fluoromalate is much faster than direct reduction to (2*R*,3*S*)-fluoromalate, can be excluded because in that case a deuteron label would have been incorporated from the solvent during the ketonization yielding (ultimately) (2*R*,3*R*)-[3-^{2}H]fluoromalate. As can be seen, no deuterated product is present in Figure 1A [compare Figure 3C for deuterated (2*R*,3*R*)-fluoromalate].

Proton Exchange Studies. The ^{19}F NMR spectra of the two protonated diastereomers of (2*R*)-fluoromalate having

been characterized, the deuterated species were prepared by repeating the experiment in Figure 1B in D_2O instead of H_2O. It is to be expected that (3*R*)-[3-^{1}H]fluorooxaloacetate is formed initially and that the C-3-deuterated 3*R* and 3*S* enantiomers will be formed via the keto–enol equilibrium in D_2O (see eq 4). Subsequent reduction by malate dehydrogenase should then give the C-3-deuterated (2*R*,3*R*)- and (2*R*,3*S*)-fluoromalates. Note that the C-2 proton in fluoromalate is derived from NADH, not from solvent. The result of this experiment is shown in Figure 1C. The upfield signal can be assigned to (2*R*,3*S*)-[3-^{2}H]fluoromalate: $J_{F-H(C-2)}$ = 5.4 Hz, as in the protonated diastereomer, and $J_{F-D(C-3)}$ = 7.5 Hz, which is consistent with $(1/6.5)J_{F-H(C-3)}$ in the protonated diastereomer (^{2}H has a 6.5-fold smaller gyromagnetic ratio than ^{1}H; ^{2}H has spin 1 and hence three equally intense lines in the triplet). Comparison of the upfield signals in spectra B and C of Figure 1 reveals that the fluorine resonance of the deuterated species is shifted upfield by 0.5 ppm with respect to the protonated species. The two downfield signals in Figure 1C arise from protonated and deuterated (2*R*,3*R*)-fluoromalates (compare Figures 1A and 3C).

The result in Figure 1C seems to indicate that (3*S*)-fluorooxaloacetate was formed in excess over the 3*R* enantiomer during the keto–enol tautomerization. The (3*R*)- and (3*S*)-[3-^{2}H]fluorooxaloacetates are however initially generated in equal amounts, although the deuterium label in the 3*R* and not the 3*S* enantiomer is being continuously washed out by transcarboxylase-catalyzed equilibration with excess unlabeled fluoropyruvate (see reaction C of Scheme I). This explains why the (2*R*,3*R*)-fluoromalate in Figure 1C is partly protonated whereas the 2*R*,3*S* diastereomer is fully deuterated; however, it does not explain why at chemical equilibrium the total amount of (3*S*)-fluorooxaloacetate should have been larger than the total amount of 3*R* isomer. This difference can be ascribed to a primary deuterium kinetic isotope effect in the enolization of fluorooxaloacetate: (3*S*)-fluorooxaloacetate is fully deuterated and is converted to 3*R* and 3*S* more slowly than 3*R*, which is partially protonated. When the experiment in Figure 1C was repeated, except that transcarboxylase was inhibited (with either 1 mg/mL avidin or 10 mM oxalate) after some (3*R*)-[3-^{1}H]fluorooxaloacetate had been formed, equal amounts of (3*R*)- and (3*S*)-[3-^{2}H]-fluorooxaloacetates were formed. This result confirms that the asymmetry in Figure 1C is brought about by the transcarboxylase-catalyzed equilibration between fluorooxaloacetate and fluoropyruvate.

When fluoropyruvate and fluorooxaloacetate are present at equilibrium with transcarboxylase in D_2O, the fluoropyruvate will become deuterated as well. This is illustrated by the data in Figure 2. The downfield triplet in Figure 2 arises from the fluorine coupled to two protons in [3,3-$^1H_2$]fluoropyruvate, J_{F-H} = 46.6 Hz; the next upfield two triplets are from [3,3-^{1}H,^{2}H]fluoropyruvate, J_{F-H} = 46.6 Hz, J_{F-^2H} = 7.1 Hz; the upfield signal is the quintet from [3,3-$^2H_2$]fluoropyruvate. The fluorine resonance is shifted upfield by 0.6 ppm for every deuteron in the fluoromethyl group of fluoropyruvate. Given absolute stereoselectivity of transcarboxylase, we should expect an enzyme-dependent rate of formation of [3,3-^{1}H,^{2}H]-fluoropyruvate plus the formation of [3,3-$^2H_2$]fluoropyruvate (due to nonenzymatic keto–enol tautomerization of fluorooxaloacetate), at a rate independent of the enzyme concentration.

A comparison of spectra A and B of Figure 2 shows that doubling the enzyme concentration results in a faster deuteration at only one position in the fluoromethyl group, while

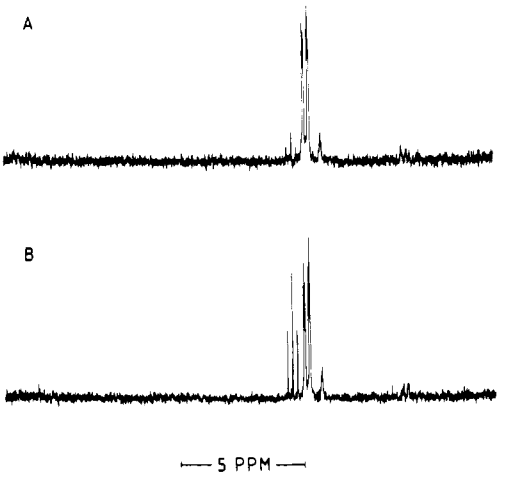


FIGURE 2: ^{19}F NMR spectra of fluoropyruvate in equilibrium with fluorooxaloacetate in D_2O with catalysis by transcarboxylase: 20 mM fluoropyruvate + 6 mM oxalate were incubated with 6.8 (A) and 3.4 μM (B) transcarboxylase for 55 min at room temperature in 300 mM potassium phosphate, pH(D) 6.9 in D_2O. Spectra were recorded as in Figure 1. Spectral width was 4717 Hz, with 96 scans at a different offset: the fluoropyruvate resonance is 41.1 ppm upfield from that of (2*R*,3*R*)-fluoromalate in Figure 1.

the second deuteration step to yield [3,3-$^2H_2$]fluoropyruvate is not affected. It can therefore be concluded that transcarboxylase is specific for one of the two enantiotopic protons in the prochiral fluoromethyl group. This implies that (3*R*)-fluorooxaloacetate (see above) must be formed either with absolute retention or with absolute inversion of configuration. Since the stereochemistry with pyruvate is known to be retention (Cheung et al., 1975), we anticipate that the carboxylation of fluoropyruvate will also proceed with retention of configuration: transcarboxylase replaces the *pro-S* proton in fluoropyruvate to yield (3*R*)-fluorooxaloacetate.

Deuteration of fluoropyruvate in the transcarboxylase reaction involves the decarboxylation of fluorooxaloacetate to fluoropyruvate. When this reverse reaction was prevented, by trapping fluorooxaloacetate as fluoromalate in an experiment described in eq 3 and Figure 1A, the ^{19}F NMR spectra of the remaining fluoropyruvate revealed no deuteration of the fluoromethyl group faster than the nonenzymatic background rate ($t_{1/2}$ approximately 48 h at pH 6.9). Similar results have been reported by Prescott & Rabinowitz (1968), who found no uptake of solvent tritium into propionyl-CoA during the propionyl-CoA carboxylase reaction.

Carboxylation of (3R)-[3,3-^{1}H,^{2}H]Fluoropyruvate Generated with PTS Enzyme I. Enzyme I, an enzyme that functions in bacterial phosphoenolpyruvate-dependent sugar transport, transfers a phosphoryl group from phosphoenolpyruvate to a phosphocarrier protein, HPr [for a review, see Robillard (1982)]. In a first reaction step the enzyme is phosphorylated from phosphoenolpyruvate, yielding pyruvate as a product. It has been demonstrated that the enzyme transfers a proton to the C-3 atom of enolpyruvate to yield ketopyruvate as the product (Hoving et al., 1981). In a recent study using (*Z*)-phosphoenolbutyrate as a substrate analogue it was shown that this proton transfer takes place at the 2-*re*,3-*si* face of the enolate. In the reverse reaction, starting with phosphoenzyme I and 2-oxobutyrate, (*Z*)-phosphoenolbutyrate was found to be the sole product (Hoving et al., 1983). Similar experiments were carried out by using fluoropyruvate as a phosphoryl group acceptor and proton NMR to analyze the product, and it was found that again the pure *Z* isomer of fluorophosphoenolpyruvate is the product (Hoving, Nowak, and Robillard, unpublished data).

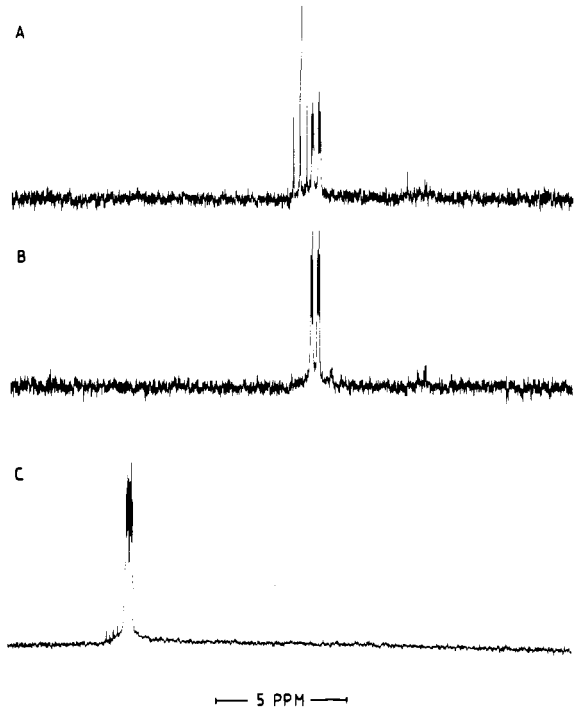
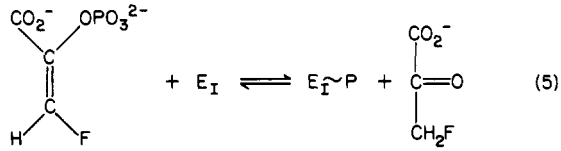


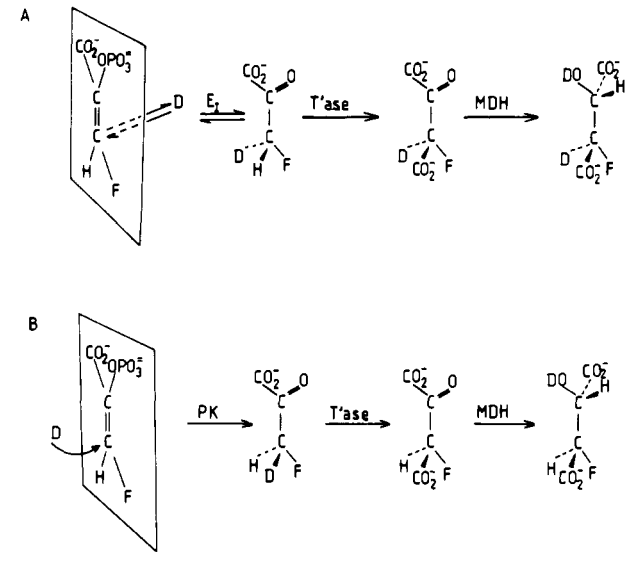
FIGURE 3: ^{19}F NMR spectra of fluoropyruvate [(A) and (B)] at equilibrium with (*Z*)-fluorophosphoenolpyruvate in D_2O with catalysis by enzyme I and of fluoromalate (C) as the product of the coupled transcarboxylase and malate dehydrogenase reactions with the fluoropyruvate of (B) as substrate. Experimental conditions: 4 mM fluoropyruvate and 2 mM phosphoenolpyruvate with 8 μM enzyme I in 100 mM potassium phosphate buffer containing 100 mM KCl and 12.5 mM $MgCl_2$, pH(D) 6.9 in D_2O, after 75 (A) and 210 min (B) at room temperature. After 230 min, 5 mM malonyl-CoA, 7 mM NADH, 40 units/mL malate dehydrogenase, and 0.25 μM transcarboxylase were added, and the spectrum of the fluoromalate formed was recorded (C). Spectra were run on a Bruker WM200 at 188 MHz in the quadrature detection mode, using a 13-μs 90° pulse. Other conditions: spectral width, 4000 Hz; acquisition time, 2 s; relaxation delay, 5 s; line broadening, 1 Hz; 96 scans [(A) and (B)] or 2400 scans (C); sample volume, 2 mL. The spectral region in (C) is 34 ppm downfield from those in (A) and (B).

Figure 3A,B shows the ^{19}F NMR spectra of fluoropyruvate at equilibrium with (*Z*)-fluorophosphoenolpyruvate via the enzyme I (de)phosphorylation reaction in D_2O (see eq 5).



Deuteration is seen to take place at only one of the two enantiotopic positions in the fluoromethyl group. This fluoropyruvate was used as a substrate for transcarboxylase in reaction 3, and the ^{19}F NMR spectrum of the product fluoromalate is shown in Figure 3C. It is clear that the main product is C-3-deuterated (2*R*,3*R*)-fluoromalate: $J_{F-H(C-2)} = 25.3$ Hz as in protonated (2*R*,3*R*)-fluoromalate, and $J_{F-H(C-3)} = 7.6$ Hz, which is equal to $(1/6.5)J_{F-H(C-3)}$ in protonated (2*R*,3*R*)-fluoromalate. There is also a small amount of unlabeled (2*R*,3*R*)-fluoromalate present in Figure 3C; the fluorine resonance of the deuterated material is shifted upfield by 0.6 ppm. This small amount of protonated fluoromalate could have arisen from some remaining [3,3-$^1H_2$]fluoropyruvate in Figure 3B, or from some (3*S*)-[3,3-^1H,^2H]fluoropyruvate in the 3*R* enantiomer. Note that a little [3,3-$^2H_2$]fluoropyruvate is also present in Figure 3B, presumably as a result of nonenzymatic enolization, indicating that the [3,3-^1H,^2H]-fluoropyruvate will not be totally enantiomerically pure.

Scheme II



On the basis of the earlier results with 2-oxobutyrate and (*Z*)-phosphoenolbutyrate mentioned above, we expect that fluoropyruvate is deuterated at the *pro-R* position. Therefore, the result in Figure 3C demonstrates that the transcarboxylases reaction proceeds with absolute retention of configuration, as it has been reported to do with the natural substrate pyruvate (Cheung et al., 1975). The stereochemical course of the reactions in Figure 3 is summarized in Scheme IIA.

Carboxylation of (3*S*)-[3,3-^1H,^2H]*Fluoropyruvate Generated with Pyruvate Kinase.* From previous studies it is known that pyruvate kinase and enzyme I transfer a proton to opposite faces of phosphoenolpyruvate (and analogues) during the conversion to pyruvate (Hoving et al., 1983). Stubbe & Kenyon (1972) and Duffy & Nowak (1985) have shown that (*Z*)- and (*E*)-fluorophosphoenolpyruvates are substrates for pyruvate kinase. We chose to couple the pyruvate kinase reaction, using (*Z*)-fluorophosphoenolpyruvate as a substrate, to transcarboxylase and malate dehydrogenase in order to look at the degree of stereospecificity in the pyruvate kinase reaction and to complement our results with the enzyme I reaction presented above. The pyruvate kinase reaction in D_2O will yield [3,3-^1H,^2H]fluoropyruvate, and if the deuteron transfer takes place with a certain degree of stereospecificity, we should now expect to see an excess of C-3-protonated over C-3-deuterated (2*R*,3*R*)-fluoromalate as the product of the coupled transcarboxylase and malate dehydrogenase reactions (see Scheme IIB).

The course of the experiment is depicted in Figure 4. Figure 4A shows the ^{19}F NMR spectrum of (*Z*)-fluorophosphoenolpyruvate, which was used as the substrate for pyruvate kinase. The coupling constants are $J_{F-H} = 76.7$ Hz and $J_{F-P} = 4.8$ Hz. At the time that the experiment was done we did not know where the ^{19}F resonances of the *E* isomer would have appeared; we now know that they appear 10.7 ppm downfield from the *Z* isomer (Duffy and Nowak, personal communication) and, in fact, only the upfield peak of the F–H doublet would have been visible in the spectrum of Figure 4A. The absence of this peak shows that the product as formed from fluoropyruvate via the enzyme I transphosphorylation reaction is the pure *Z* isomer (see previous paragraph). The fluoropyruvate region of the spectrum showed no detectable signal.[1]

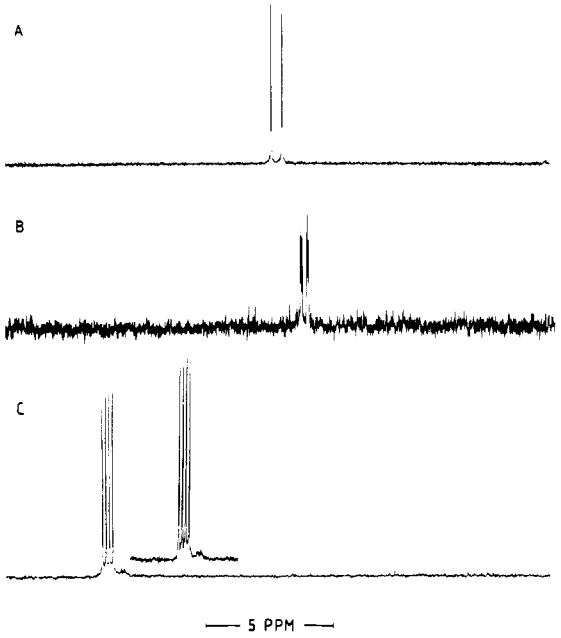


FIGURE 4: ^{19}F NMR spectra of substrate (A), intermediate (B), and final product (C) of reaction B of Scheme II. Experimental conditions: (A) 10 mM (*Z*)-fluorophosphoenolpyruvate in 100 mM potassium phosphate buffer containing 300 mM KCl and 12.5 mM $MgCl_2$, pH 6.9 in D_2O; (B) same sample plus 12 mM ADP, 6 mM malonyl-CoA, 12 mM NADH, 40 units/mL malate dehydrogenase, 0.25 μM transcarboxylase, and 1 mg/mL pyruvate kinase (added last); fluoropyruvate spectrum run after 35 min at room temperature; (C) same sample as for (B), but fluoromalate spectrum run after 90 min at room temperature; the inset shows the result of a separate experiment. The spectral regions are different: (B) and (C) are 87.2 and 53.2 ppm upfield from (A), respectively. Spectra were run on a Bruker WM200 at 188 MHz in the quadrature detection mode, using a 13-μs 90° pulse. Other conditions: spectral width, 4000 Hz; acquisition time, 2 s; relaxation delay, 5 s; line broadening, 0.2 (A) or 1 Hz [(B) and (C)]; 96 scans [(A) and (B)] or 400 scans (C); sample volume, 2 mL.

Figure 4B shows the ^{19}F NMR spectrum of [3,3-^1H,^2H]-fluoropyruvate as the intermediate in reaction B of Scheme II, and the spectrum of the final product, (2*R*,3*R*)-fluoromalate, is shown in Figure 4C. It is clear that the main product is (2*R*,3*R*)-fluoromalate bearing a proton at C-3, although a small amount of deuterated material is reproducibly present. In contrast to the experiment with enzyme I in Figure 3, nonenzymatic enolization of fluoropyruvate cannot have taken place here since fluoropyruvate is formed and immediately trapped by transcarboxylase and malate dehydrogenase. Since the transcarboxylase reaction apparently proceeds with absolute retention of configuration, these results show that the pyruvate kinase reaction takes place with somewhat less than 100% stereospecificity. Similar results with pyruvate kinase have been reported when phosphoenolbutyrate was used as a substrate (Bondisell & Sprinson, 1970; Stubbe & Kenyon, 1971), whereas Rose (1970) has shown that the reaction with the natural substrate phosphoenolpyruvate does proceed with absolute stereospecificity.

Discussion

The two main results on the transcarboxylase reaction from this study are the following: (i) carboxylation of fluoropyruvate takes place without any uptake of solvent protons/deuterons into the fluoromethyl group, and (ii) carboxylation of fluoropyruvate proceeds with absolute retention of configuration. All previous studies on these aspects of biotin-dependent carboxy group transfer reactions have led to similar results: no exchange of substrate protons with solvent due to a preenolization step and retention of configuration in the overall reaction.

Various proton exchange studies have been reported in the literature: pyruvate carboxylase does not release any tritium from tritiated pyruvate in the absence of bicarbonate or of Mg^{2+}–ATP (Mildvan et al., 1966). Cheung et al. (1975) found that propionyl-CoA carboxylase and transcarboxylase release tritium from (*RS*)-[2-^3H]propionyl-CoA in complete reaction mixture at exactly 50% of the rate of (2*S*)-methylmalonyl-CoA formation, indicating that there is no intermolecular tritium isotope effect and no exchange of tritium into solvent apart from the tritium released during carboxylation. Alternatively, exchange may be balanced by the kinetic isotope effect. The absence of uptake of tritium (or deuterium) from solvent into substrate is a better criterion for no exchange: such experiments have been reported by Prescott & Rabinowitz (1968), who found no uptake of solvent tritium into propionyl-CoA either during net propionyl-CoA carboxylase reaction or in reaction mixture (lacking either HCO_3^- or Mg^{2+}–ATP). To this we can add our observation that no uptake of solvent deuterium into fluoropyruvate takes place during net transcarboxylase reaction.

The stereochemical course of all biotin-dependent CO_2 transfer reactions studied appears to be retention. Using (2*S*)-[2-^3H]propionyl-CoA as a substrate for propionyl-CoA carboxylase, Retey & Lynen (1965) demonstrated formation of (2*S*)-methylmalonyl-CoA that retained all the tritium. In similar experiments Arigoni et al. (1966) and Prescott & Rabinowitz (1968) showed 100% release of tritium during carboxylation of (2*R*)-2-tritiopropionyl-CoA by propionyl-CoA carboxylase. Carboxylation of [2-^3H]propionyl-CoA by transcarboxylase gives similar results: Cheung et al. (1975) used a preparation of [2-^3H]propionyl-CoA that lost 80% of its tritium during carboxylation by propionyl-CoA carboxylase (i.e., comprised of 80% 2*R* and 20% 2*S*) and found that the transcarboxylase reaction also led to 80% loss of tritium. A similar experiment with 23% 2*R* and 77% 2*S* showed, however, somewhat less than 23% abstraction of tritium with transcarboxylase. The stereochemical mode of pyruvate carboxylation has also been shown to be retention both with pyruvate carboxylase (Rose, 1970) and with transcarboxylase (Cheung et al., 1975). As we point out in the introduction to this paper, the experiments with pyruvate do not allow the conclusion that the stereospecificity is absolute. Our experiments with fluoropyruvate now show that, at least with transcarboxylase, carboxylation does proceed with 100% retention.

The observation that the stereospecificity is absolute is of some mechanistic significance because the concerted mechanism for biotin (de)carboxylation as proposed by Retey & Lynen (1965) requires 100% retention. Less than absolute stereospecificity would have been inconsistent with such a mechanism. As it is, the combined proton exchange and stereochemical data now available can be explained by either a concerted or a nonconcerted mechanism.

Our results with enzyme I and pyruvate kinase demonstrate again that, although almost all enzymes that add a proton or a carboxy group to enolpyruvate do so from the 2-*si* face (Rose, 1970; Kuo & Rose, 1982), this is not a mechanistic imperative: *E. coli* enzyme I protonates enolpyruvate analogues from the 2-*re* face, as we have shown in a previous study using (*Z*)-

[1] The primary deuterium kinetic isotope effect (k_H/k_D) in the transcarboxylase reaction is only 1.2 (Hoving and Leadlay, unpublished results), so that the enzymatic carboxylation of fluoropyruvate in Figure 4 is even faster than in Figure 1A.

phosphoenolbutyrate (Hoving et al., 1982) and in this paper using (*Z*)-fluorophosphoenolpyruvate. Also, the degree of stereospecificity in the protonation by enzyme I is very high if not absolute, whereas pyruvate kinase has a lower degree of specificity both with phosphoenolbutyrate (Bondisell & Sprinson, 1970; Stubbe & Kenyon, 1971; Hoving et al., 1983) and with fluorophosphoenolpyruvate (this paper).

The results in Figure 3 and 4 also demonstrate that transcarboxylase can be very conveniently used to distinguish the two enantiomers of singly deuterated fluoropyruvate, as anticipated by Walsh and co-workers (Goldstein et al., 1978). The ^{19}F NMR detection method offers excellent sensitivity and accuracy for determination of diastereomeric purity and degree of deuteration of the reaction product fluoromalate. A quantitative analysis of the amounts of protonated and deuterated substrates, reaction intermediates, and products is greatly facilitated by the fact that the ^{19}F resonances of protonated and deuterated species are well separated. The analyses do not involve any chemical manipulation of the samples and can be done on unpurified reaction mixtures, thereby avoiding artifacts due inter alia to exchange during purification and the different chemical behavior of diastereomers.

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