Stereochemistry of Phosphoenolpyruvate Carboxylation Catalyzed by Phosphoenolpyruvate Carboxykinase†

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ABSTRACT: The stereochemistry of the carboxylation of phosphoenolpyruvate to yield oxalacetate, catalyzed by chicken liver phosphoenolpyruvate carboxykinase and by *Ascaris* muscle phosphoenolpyruvate carboxykinase, was determined. The substrate (*Z*)-3-fluorophosphoenolpyruvate was used for the stereochemical analysis. The carboxylation reaction was coupled to malate dehydrogenase to yield 3-fluoromalate, and the stereochemistry of the products was identified by ^{19}F NMR. In separate experiments, the enantiomeric tautomers of 3-fluorooxalacetate were shown to be utilized by malate dehydrogenase to yield (2*R*,3*R*)- and (2*R*,3*S*)-3-fluoromalate in nearly identical amounts. The products were identified by ^{19}F NMR. When (*Z*)-3-fluorophosphoenolpyruvate was used as a substrate for phosphoenolpyruvate carboxykinase from avian liver and from *Ascaris*, and malate dehydrogenase was used to trap the product, only a single diastereomer was observed. This product was shown to be (2*R*,3*R*)-3-fluoromalate in each case. The assignments were based on coupling constants taken from Keck et al. [Keck, R., Hess, H., & Rétey, J. (1980) *FEBS Lett. 114*, 287]. These results indicate that the stereochemistry of carboxylation, catalyzed by chicken phosphoenolpyruvate carboxykinase and by *Ascaris* phosphoenolpyruvate carboxykinase, is identical and takes place from the *si* side of the enzyme-bound phosphoenolpyruvate. The carboxylation reaction was run both in H_2O and in D_2O. No deuterium incorporation into fluoromalate was shown to occur. The product 3-fluorooxalacetate is thus released from phosphoenolpyruvate carboxykinase as the keto form and is reduced more rapidly by reduced nicotinamide adenine dinucleotide with malate dehydrogenase than by the occurrence of tautomerization.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) catalyzes an essential reaction in the formation of glucose from three- and four-carbon precursors. This reaction is the first committed step in gluconeogenesis in higher animals. P-enolpyruvate[1] carboxykinase has been reported to be present in a wide variety of animals, plants, and bacteria (Utter & Kolenbrander, 1972). The primary function of P-enolpyruvate carboxykinase from mammals appears to be the catalysis of the formation of P-enolpyruvate from oxalacetate. No other function has been described for this enzyme in mammals. P-enolpyruvate carboxykinase found in the muscle of the helminth *Ascaris*, and in several other anaerobic organisms, plays a different metabolic role. Its function is to catalyze the formation of oxalacetate from P-enolpyruvate and CO_2. This reaction plays a key role in glycolysis to produce energy in an anaerobic environment (Von Brand, 1950; Saz & Bueding, 1968; Saz, 1971). Thus, it is not clear that these enzymes, which have evolved to serve different physiological functions in these organisms, catalyze the reaction through the same mechanism. Information on the stereochemistry of the carboxylation reaction will give valuable clues to the relative spatial arrangement of the substrates at the catalytic site of the enzyme.

Initial methodology to examine the stereochemistry of such reactions had been developed by Cornforth et al. (1969), Arigoni (Lüthy et al., 1969), and Rose (1970), using isotopically chiral methyl or methylene groups. The ability of this isotopic substitution method to allow the quantitation of stereochemistry depends upon the intramolecular kinetic isotope effects. Recently, the use of fluorinated substrate analogues and inhibitors has received considerable attention in the investigation of mechanisms of biological reactions. The application of fluorinated substrate analogues to stereochemical problems allows a quantitative determination of the degree of stereospecificity in the reactions where fluoromethyl and fluoromethylene groups can serve as prochiral and chiral probes. Determination of the stereochemistry at a chiral center is much easier than at a prochiral center. Goldstein et al. (1978) used 3-fluoropyruvate to demonstrate that pyruvate kinase does not distinguish between the two prochiral hydrogen atoms at the fluoromethyl group of the substrate in the enzyme-catalyzed deprotonation reaction. In the same study, fluoropyruvate was converted to only the 3*R* isomer of 3-fluorooxalacetate by the biotin-dependent enzyme transcarboxylase. Walsh and co-workers have also investigated the enzymic processing of fluorinated substrates using ATP citrate lyase, malate synthase (Marletta et al., 1981), and fumarase (Marletta et al., 1982). ATP citrate lyase cleaved the C-3 bond of prochiral citrate at the *pro-S* position. Malate synthase does not distinguish between either of the two prochiral protons of 2-fluoroacetyl-CoA or the face of the aldehyde carbon of glyoxylate in the condensation reaction. The stereochemical course of hydration of monofluorofumarate by fumarase was shown to be trans with a constant orientation of the substrates. For instance, OH^- is always added from

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[1] Abbreviations: P-enolpyruvate, phosphoenolpyruvate; F-oxalacetate, 3-fluorooxalacetate; D_2O, $[^2H]H_2O$; F-P-enolpyruvate, 3-fluorophosphoenolpyruvate; F-malate, (2*R*)-3-fluoromalate; NADH, reduced nicotinamide adenine dinucleotide; ATP, adenosine 5′-triphosphate; IDP, inosine 5′-diphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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the *si-si* face of fumarate while H^+ is always added from the *re-re* face of fumarate. More recently, Hoving et al. (1985) have studied the stereochemistry of the transcarboxylase-catalyzed carboxylation of 3-fluoropyruvate by using ^{19}F NMR analysis. Their results demonstrated that transcarboxylase is specific for one of the two prochiral hydrogens of fluoropyruvate and converts it to 3(*R*)-fluorooxalacetate with retention of configuration. These results confirm those previously reported by Walsh and co-workers (Goldstein et al., 1978). Hoving et al. have extended their studies to the investigation of the stereochemistry of the proton addition to enolpyruvate by enzyme I and by pyruvate kinase by coupling these reactions to transcarboxylase. Stereochemical experiments with enzyme I and with pyruvate kinase have shown that fluoroenolpyruvate is protonated from opposite faces by these two enzymes. Enzyme I protonates fluoroenolpyruvate from the 2-*re* face, and pyruvate kinase does so from the 2-*si* face.

Fluorine has been used as a replacement for hydrogen because of its small size and short C–F bond distance and because it is almost isosteric to hydrogen. The high electronegativity of carbon-bound fluorine introduces a polarity more like a C–OH substituent, however, and C–F has been used as a C–OH replacement in carbohydrate derivatives. Briley has used fluorine as an oxygen analogue and has obtained information about the geometric requirements of binding sites and reaction mechanisms involving carbohydrates (Briley et al., 1975).

(*Z*)-F-P-enolpyruvate, which is a good substrate for chicken liver P-enolpyruvate carboxykinase and for *Ascaris* muscle P-enolpyruvate carboxykinase (Duffy & Nowak, 1984), was used to determine the stereochemistry of carboxylation of P-enolpyruvate. The stereochemistry for the two enzymes is compared. The two possible diastereomers of fluoromalate can easily be identified by using ^{19}F NMR with little prior sample preparation.

Materials and Methods

Chicken liver P-enolpyruvate carboxykinase was purified as described previously (Lee & Nowak, 1984). The concentration of P-enolpyruvate carboxykinase was determined with an extinction coefficient of 16.5 ± 0.1 ($\epsilon_{280}^{1\%}$) and M_r 72 000 (Hebda & Nowak, 1982). P-enolpyruvate carboxykinase, purified from *Ascaris* muscle, was obtained from Dr. Susan Rohrer. Malate dehydrogenase (pig heart), pyruvate kinase (rabbit muscle), and lactate dehydrogenase (pig heart) were purchased from Boehringer Mannheim Corp.

(*Z*)-F-P-enolpyruvate was synthesized by the methods of Stubbe & Kenyon (1972) as modified by Duffy and Nowak (1984). This compound was characterized by high-resolution ^1H and ^{19}F NMR. The concentration of (*Z*)-F-P-enolpyruvate was measured by using the coupled pyruvate kinase–lactate dehydrogenase assay with a limiting amount of this substrate. 3-Fluorooxalacetate was synthesized by acid hydrolysis of diethyl fluorooxalacetate (Kun et al., 1958) and recrystallized from ether–chloroform (Dummel et al., 1971). Diethyl fluorooxalacetate was obtained by condensing diethyl oxalate and ethyl fluoroacetate in a suspension of sodium ethoxide in benzene, followed by washing with anhydrous ether (Stubbe & Kenyon, 1972). All other reagents were of the highest purity commercially available. All solutions were made by using distilled water that was passed through a mixed-bed deionizing column.

Reduction of (RS)-3-Fluorooxalacetate To Produce (2R)-3-Fluoromalate Using Malate Dehydrogenase. (*RS*)-3-Fluorooxalacetate was reduced by NADH with malate dehydrogenase. The incubation mixture (1 mL) contained (*RS*)-3-fluorooxalacetate (10 μmol) and NADH (15 μmol) in 200 μmol of Tris-HCl, pH 7.5 at 37 °C. The reaction was initiated by adding malate dehydrogenase (0.3 mg). Ten-microliter aliquots were periodically removed and diluted into 1 mL of buffer to check the formation of fluoromalate. The formation of fluoromalate was monitored at 340 nm on a Gilford 240 or 250 spectrophotometer by measuring NADH consumption. The reaction was stopped by heating, followed by lyophilization. The sample was redissolved in 0.5 mL of D_2O and centrifuged, and the ^{19}F NMR spectra were taken without further purification. The spectra were obtained both at 282 MHz on a Nicolet NTC-300 spectrometer equipped with a 239A pulse system and a Nicolet 1180E computer and at 94.1 MHz on a Varian XL-100-15 spectrometer interfaced to a TT1 pulse system and Nicolet 1080 computer.

The reduction of partially deuterated (*RS*)-3-fluorooxalacetate was performed by the solution of the reaction mixture in D_2O and preincubation for about 5 min prior to the addition of malate dehydrogenase. The reaction was treated as described above. The ^{19}F NMR spectrum was taken at 282 MHz. The product identification was based on the chemical shifts and coupling constants. Relative amounts of products were determined by digital integration of the NMR spectra.

Carboxylation of F-P-enolpyruvate by Chicken Liver P-enolpyruvate Carboxykinase. (*Z*)-F-P-enolpyruvate was used as a substrate for chicken liver P-enolpyruvate carboxykinase, and the reaction was coupled to malate dehydrogenase. The incubation mixture (5 mL) contained Tris-HCl (250 μmol, pH 7.4), KCl (500 μmol), (*Z*)-F-P-enolpyruvate (14.5 μmol), IDP (15 μmol), $MgCl_2$ (15 μmol), $MnCl_2$ (50 nmol), $KHCO_3$ (500 μmol), 2-mercaptoethanol (72.5 μmol), NADH (15 μmol), and malate dehydrogenase (0.3 mg) at 37 °C. The reaction was initiated by the addition of P-enolpyruvate carboxykinase (0.25 mg) and allowed to proceed to completion. The reaction was terminated by heating. EDTA (1 μmol) was added to chelate Mn(II) preferentially, and the sample was lyophilized. The lyophilized samples were redissolved in 0.5 mL of D_2O. The ^{19}F NMR spectra were taken at both 94.1 and 282 MHz. The product determinations were based on the chemical shifts and the coupling constants. The reaction was run both in H_2O and in D_2O.

Carboxylation of F-P-enolpyruvate by Ascaris Muscle P-enolpyruvate Carboxykinase. (*Z*)-F-P-enolpyruvate was used as a substrate for *Ascaris* muscle P-enolpyruvate carboxykinase. The experimental procedure was the same as that described with chicken liver enzyme. The ^{19}F NMR spectra were taken at both 94.1 and 282 MHz.

Results

Formation of (2R)-3-Fluoromalate from (RS)-3-Fluorooxalacetate. The specificity of malate dehydrogenase for the tautomeric forms of fluorooxalacetate was determined by a ^{19}F NMR analysis of the reduction products, (2*R*)-3(*RS*)-fluoromalates. A solution of chemically synthesized (*RS*)-3-fluorooxalacetate was reduced by malate dehydrogenase in the presence of an excess of NADH. Figure 1A shows the ^{19}F NMR spectrum of (2*R*)-3(*RS*)-fluoromalate, the product of the reaction carried out in H_2O. The downfield quartet is characterized by coupling constants of 49.1 ± 0.1 ($J_{H_3-F_3}$) and 25.0 ± 0.1 Hz ($J_{H_2-F_3}$), and the upfield quartet has coupling constants of 48.3 ± 0.1 ($J_{H_3-F_3}$) and 34.6 ± 0.1 Hz ($J_{H_2-F_3}$). The large coupling constants (49.1 ± 0.1 and 48.3 ± 0.1 Hz) are due to geminal coupling to the proton at the C-3 position. The smaller coupling constants (25.0 ± 0.1 and 34.6 ± 0.1 Hz) are from the vicinal proton at the C-2 position. The downfield quartet is assigned to (2*R*,3*R*)-3-fluoromalate and

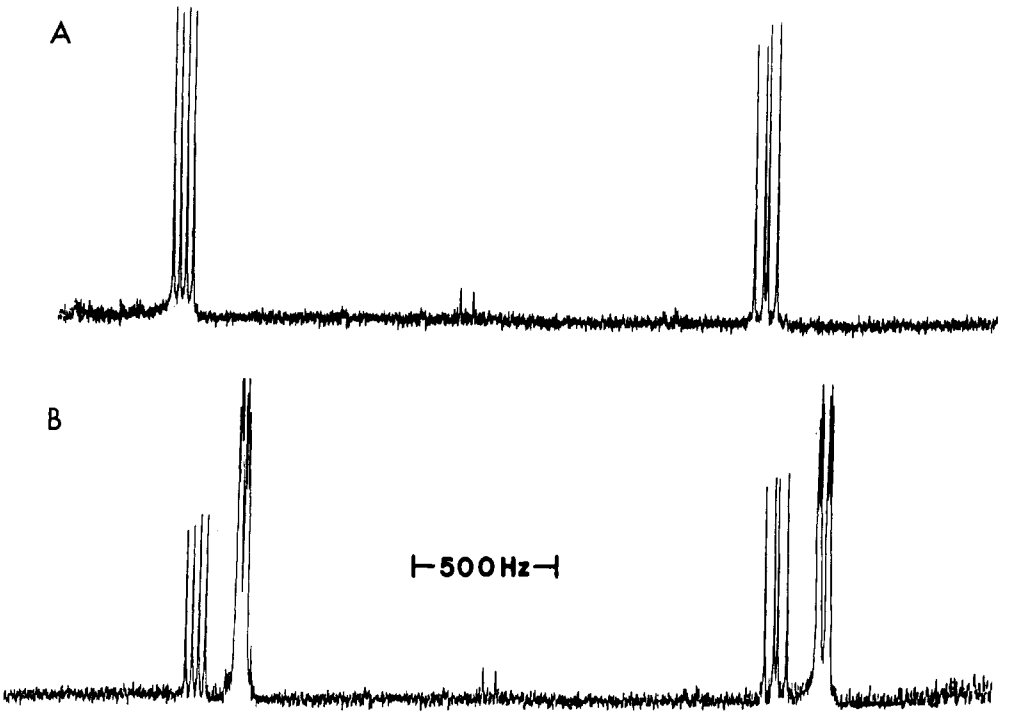
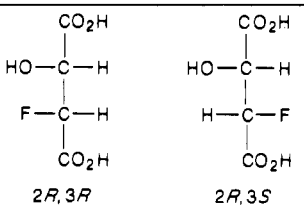


FIGURE 1: ^{19}F NMR spectra of products, (2*R*)-3-fluoromalates, from the reduction of (*RS*)-3-fluorooxalacetate by malate dehydrogenase (pig heart) in the presence of an excess of NADH. (A) The reduction of (*RS*)-3-fluorooxalacetate was performed by the solution of the reaction mixture containing H_2O. (B) The reduction of partially deuterated 3-fluorooxalacetate was performed by the solution of the reaction mixture containing D_2O. The reaction mixture was preincubated for 5 min prior to the addition of malate dehydrogenase. The preparation of each sample was described under Materials and Methods. The ^{19}F NMR spectra were taken at 282 MHz in the quadrature phase detection mode, with a 7-μs, 90° pulse, spectral width 2000 Hz, number of scans 100 (A) or 1000 (B), data size 16K, line broadening 0.5 Hz, and a recycle delay of 500 ms.

the upfield quartet is assigned to (2*R*,3*S*)-3-fluoromalate. These assignments are taken from the proton NMR data of the diastereomers of (2*R*)-3-fluoromalate reported by Keck et al. (1980). Since trans coupling is usually larger than cis coupling and the product of malate dehydrogenase is (2*R*)-3-fluoromalate, the 25-Hz coupling is from the 2*R*,3*R* diastereomer and the 34.6-Hz coupling is from the 2*R*,3*S* diastereomer. From these data, it is clear that both enantiomeric tautomers of fluorooxalacetate were utilized by malate dehydrogenase. Integration of the respective resonances shows the distribution of (2*R*,3*S*)- and (2*R*,3*R*)-3-fluoromalate in a 9:11 ratio, respectively. This slight preference for the 2*R*,3*R* diastereomer can occur either if malate dehydrogenase has a kinetic preference for the 3*R* isomer of fluorooxalacetate or if the 3*R* isomer results from the more stable tautomeric form of fluorooxalacetate at equilibrium.

The effect of partial deuterium substitution at the C-3 position of (2*R*)-3-fluoromalate on the ^{19}F spectra was examined. (*RS*)-3-Fluorooxalacetate was reduced by NADH with malate dehydrogenase in buffer containing D_2O. Figure 1B shows the ^{19}F spectra of the products obtained. A mixture of (2*R*)-3-fluoromalates containing ^1H and ^2H at C-3 was observed. The ^2H caused a small (0.62 ppm) upfield shift of the ^{19}F resonance and a change in the geminal coupling constants (7.2 ± 0.1 and 7.1 ± 0.1 Hz). Table I summarizes the coupling constants for the various diastereomers of (2*R*)-3-fluoromalate. The coupling of deuterium to fluorine is approximately 6.5-fold smaller than coupling by proton as expected since ^2H has a 6.5-fold smaller gyromagnetic ratio than ^1H. Deuterium has a spin state of 1; therefore, coupling by deuterium results in a triplet with three equally intense lines. These changes in the ^{19}F spectra indicate that tautomerization of fluorooxalacetate in solution can easily be determined from the ^{19}F NMR spectra.

Table I: Coupling Constants for the Diastereomers of (2*R*)-3-Fluoromalate



2*R*,3*R* 2*R*,3*S*

(2*R*)-3-fluoromalates

F-malate	$^3J_{H_2-F_3}$ (Hz)	$^2J_{H_3-F_3}$ (Hz)	$^2J_{D_3-F_3}$ (Hz)
2*R*,3*S*[a]	32.2	47	
2*R*,3*R*[a]	23.7	47.5	
2*R*,3*S*[b]	34.6 ± 0.1	48.3 ± 0.1	
2*R*,3*R*[b]	25.0 ± 0.1	49.1 ± 0.1	
2*R*,3*S*[c]	35.0 ± 0.1		7.1 ± 0.1
	(34.9 ± 0.1)	(48.5 ± 1)	
2*R*,3*R*[c]	25.4 ± 0.1		7.2 ± 0.1
	(25.3 ± 0.1)	(49.9 ± 1)	

[a] ^1H spectra, taken from Keck et al. (1980). [b] ^{19}F spectra of the sample run in H_2O, taken at 282.3 MHz. [c] ^{19}F spectra of the sample run in D_2O, taken at 282.3 MHz. The values in parentheses are from minor amounts of 3-F-malate that contain ^1H at C-3. The samples that contain ^2H at C-3 are 0.62 ppm upfield from the resonances that contain ^1H at C-3.

Carboxylation of (Z)-F-P-enolpyruvate by Chicken Liver P-enolpyruvate Carboxykinase. The carboxylation of (*Z*)-F-P-enolpyruvate by chicken liver P-enolpyruvate carboxykinase to yield fluorooxalacetate was coupled to malate dehydrogenase to yield (2*R*)-3-fluoromalate. The stereochemistry of the process was examined by the ^{19}F NMR analysis of the product. The reaction was carried out both in H_2O and in D_2O to determine if any possible tautomerized product is generated in solution prior to being trapped by malate de-

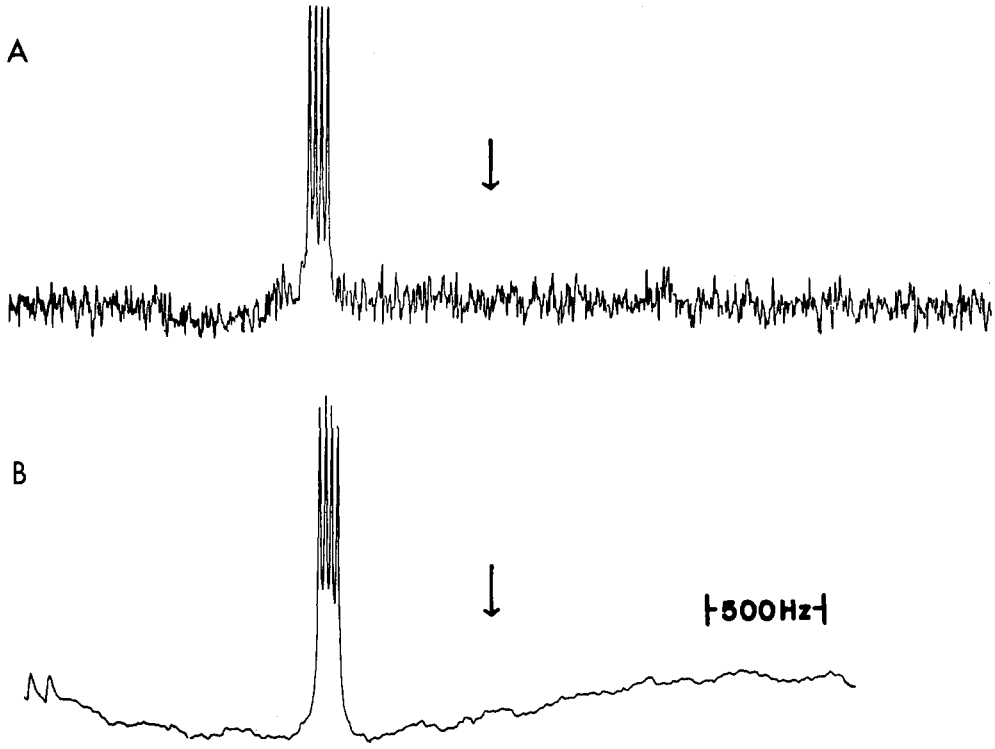


FIGURE 2: ^{19}F NMR spectra of the product obtained from the chicken liver P-enolpyruvate carboxykinase catalyzed reaction. (*Z*)-F-P-enolpyruvate (2.9 mM) was used as a substrate for the chicken liver P-enolpyruvate carboxykinase, which was coupled to malate dehydrogenase. This substrate was in limiting amounts, and the reaction was allowed to proceed to completion. The reaction was carried out both in (A) H_2O and in (B) D_2O. The arrow upfield from the observed resonance designates the expected resonance of (2*R*,3*S*)-fluoromalate. The ^{19}F NMR spectra were obtained at 94.1 MHz with a 50-μs, 90° pulse, spectral width 5000 Hz, number of scans 800 (A) or 10000 (B), data size 16K, line broadening 0.5 Hz, and a recycle delay of 500 ms.

hydrogenase. The reaction pathways are illustrated in Scheme I. Figure 2 shows the ^{19}F NMR spectra of the products obtained from the P-enolpyruvate carboxykinase catalyzed reaction run in H_2O and in D_2O. Only one diastereomer corresponding to the downfield quartet is found in both ^{19}F NMR spectra. The product is identified as (2*R*,3*R*)-3-fluoromalate. These data indicate that CO_2 addition must take place stereospecifically from the 3-*si* face of (*Z*)-F-P-enolpyruvate as catalyzed by chicken liver P-enolpyruvate carboxykinase. No deuterium-incorporated product was formed as seen in Figure 2B. The rate of reduction of fluorooxalacetate by malate dehydrogenase under experimental conditions is faster than tautomerization of fluorooxalacetate. The keto form of fluorooxalacetate and not the enolate form is released from the enzyme.

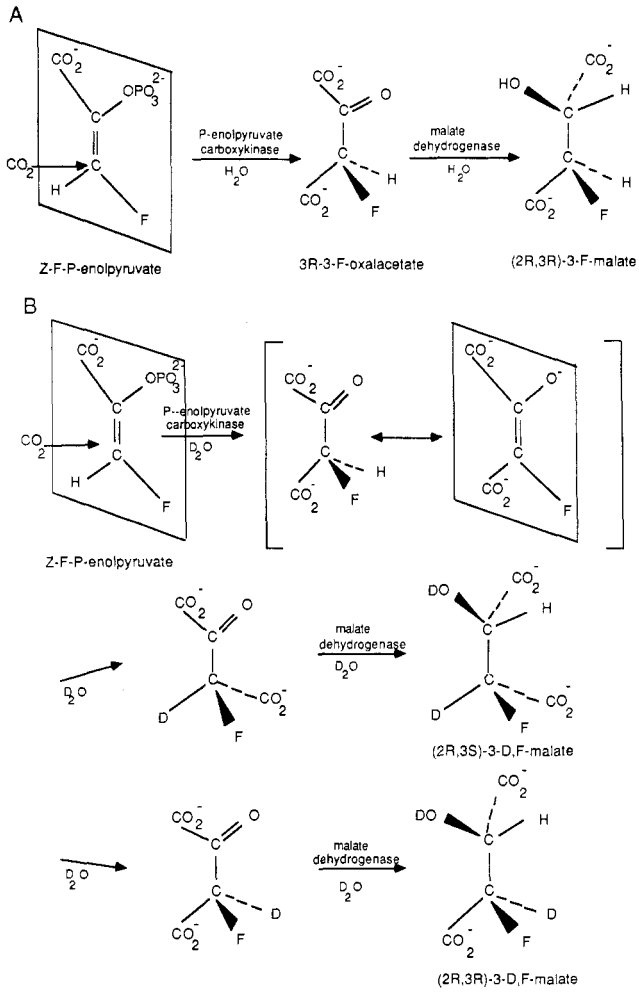
Carboxylation of (Z)-F-P-enolpyruvate by Ascaris Muscle P-enolpyruvate Carboxykinase. The identical experiment was performed with *Ascaris* muscle P-enolpyruvate carboxykinase. The spectra taken of the products of this reaction are identical with those obtained with the chicken enzyme and indicate that the P-enolpyruvate carboxykinase from *Ascaris* muscle catalyzes CO_2 addition to F-P-enolpyruvate with the same stereochemistry as the chicken liver enzyme.

Discussion

The use of fluorinated substrate analogues to determine the stereochemical course of an enzyme-catalyzed reaction has the potential advantage in that the stereochemical analysis of a chiral center is easier than the analysis of a prochiral center. This method can only be applied to enzymes that utilize the fluorinated analogues as a substrate. (*Z*)-F-P-enolpyruvate is a good substrate for chicken liver P-enolpyruvate carboxykinase and for *Ascaris* muscle P-enolpyruvate carboxykinase with V_{max} values of 42% and 50%, respectively, with respect to P-enolpyruvate (Duffy & Nowak, 1984). Analogous studies to determine the stereochemistry of the carboxylation of pyruvate catalyzed by pyruvate carboxylase using fluoropyruvate as a substrate, as an example, cannot be performed since fluoropyruvate is not used as a substrate by this enzyme.

The determination of the specificity of malate dehydrogenase for the reduction of both enantiomeric forms of fluorooxalacetate by NADH to yield two possible diastereomers of 3-fluoromalate is essential to the design of the stereochemical analysis of the carboxylation of F-P-enolpyruvate by P-enolpyruvate carboxykinase. Goldstein et al. (1978) reported that both enantiomers of 3-fluorooxalacetate were reduced by radiolabeled NADH with high concentrations of malate dehydrogenase. The products were detected by column chromatography. This result appeared to be contrary to the observation of Skilleter et al. (1972). This latter group reported that only (2*R*,3*R*)-3-fluoromalate was obtained from the reduction of (*RS*)-3-fluorooxalacetate by NADH using L-malate dehydrogenase. The product was characterized by infrared and ^1H NMR spectroscopy in the latter study. In our initial studies using ^1H NMR, only (2*R*,3*R*)-3-fluoromalate was detected. The reason for these observations has not been determined. By use of ^{19}F NMR analysis (Figure 1A), our results show that malate dehydrogenase has little or no selectivity and uses both enantiomers of 3-fluorooxalacetate to produce both diastereomers of (2*R*)-3-fluoromalate. This observation is consistent with the results of Goldstein et al. (1978) and has been confirmed by Hoving et al. (1985). The ^{19}F NMR analysis provides a simple, easy, and direct identification and characterization of the products. The two diastereomers of (2*R*)-3-fluoromalate formed by the reduction of (*RS*)-3-fluorooxalacetate catalyzed by malate dehydrogenase can be identified by ^{19}F NMR spectra on the basis of chemical shifts and relative coupling constants. The as-

Scheme I: Reaction Pathway of Carboxylation by P-enolpruvate Carboxykinase (A) and Possible Tautomerization (B) prior to Being Trapped by Malate Dehydrogenase



signments of the spectra were taken from the proton NMR data of the two diastereomeric forms of (2*R*)-3-fluoromalate, which were synthesized from the condensation of fluoroacetyl-CoA and glyoxylate by malate synthase (Keck et al., 1980).

The partially deuterated 3*R* and 3*S* enantiomers of fluorooxalacetate were formed via keto–enol equilibrium in D_2O. Subsequent reduction by malate dehydrogenase produces the C-3 deuterated (2*R*,3*R*)- and (2*R*,3*S*)-fluoromalate. The C-2 proton in fluoromalate is derived from NADH and not from the solvent. The ^{19}F resonances of the protonated and deuterated diastereomers of 3-fluoromalate are well separated by their chemical shifts and have characteristic hyperfine patterns. The nonenzymatic enolization of fluorooxalacetate in solution causes scrambling of stereochemical information in the product of the carboxylation of P-enolpyruvate catalyzed by P-enolpyruvate carboxykinase. This possibility of scrambling can be differentiated by running this reaction in D_2O.

The stereochemistry of carboxylation of F-P-enolpyruvate to yield fluorooxalacetate by chicken liver and by *Ascaris* muscle P-enolpyruvate carboxykinase is identical. These two enzymes play different physiological roles in gluconeogenesis and in glycolysis. These enzymes from different sources may have an active site homology and may catalyze the reactions through the same mechanism. The addition of CO_2 by both enzymes was shown to occur from the 2-*si* face of the enzyme-bound P-enolpyruvate. This is the same stereochemistry of carboxylation as shown by Rose et al. (1969) with pigeon liver P-enolpyruvate carboxykinase. In the experiments reported by Rose, specifically labeled [3-^{1}H,^{3}H]P-enolpyruvate was used as a substrate. The stereospecificity of the product was determined by an analysis of malate. The ^3H release from malate upon treatment with fumarase was utilized to study the stereochemistry. Rose et al. (1969) also determined the stereochemistry of CO_2 addition using the P-enolpyruvate-utilizing enzymes P-enolpyruvate carboxylase (from peanut and from *Acetobacter xylinum*) and P-enolpyruvate carboxytransphosphorylase (from *Propionibacterium shermanii*). In each case the addition of CO_2 also occurs from the same (2-*si*) side of the plane of enzyme-bound P-enolpyruvate. The stereochemical course of either proton or carboxyl group addition to enolpyruvate by P-enolpyruvate-utilizing enzymes occurs at the *si* face of the plane of the enzyme-bound P-enolpyruvate (Rose, 1970; Cohn et al., 1970; Hoving et al., 1985) with one exception. The P-enolpyruvate-dependent enzyme involved in sugar transport (enzyme I from *Escherichia coli*) protonates enolpyruvate from the 2-*re* face to yield pyruvate (Hoving et al., 1983, 1985). These latter analyses were performed by using P-enolpyruvate analogues. As pointed out in the introduction to this paper, this method of using F-P-enolpyruvate instead of radiolabeled P-enolpyruvate allows the quantitative determination of the stereochemistry of CO_2 addition and provides for easy sample preparation. The determination is not based on isotope discrimination and requires only that the analogue act as a substrate. The simple preparation of the samples from the unpurified reaction mixture avoids possible artifacts caused by any potential exchange during the purification. This method has recently been reported by Hoving et al. (1985) to determine the stereochemistry of carboxylation of 3-fluoropyruvate catalyzed by transcarboxylase. The observation that no deuterium-incorporated product was found in our experiments does not imply that phosphoryl group transfer and CO_2 addition take place in a concerted manner. It is clear that P-enolpyruvate carboxykinase does not release the enolate form of oxalacetate into solution. The keto form of oxalacetate is the substrate for P-enolpyruvate carboxykinase. This conclusion is consistent with the early observation of Utter (Graves et al., 1956) and with the suggestion made by Duffy and Nowak (1984).

The structural studies of the ternary enzyme–Mn–P-enolpyruvate complex at the catalytic site of P-enolpyruvate carboxykinase suggest that P-enolpyruvate interacts with the enzyme-bound Mn(II) as a second-sphere coordination complex (Duffy & Nowak, 1985). The Mn(II) may be situated either to the 2-*si* or the 2-*re* face of P-enolpyruvate. Steady-state kinetic studies also indicate that the CO_2 interaction with P-enolpyruvate carboxykinase is independent of Mn(II) (Hebda & Nowak, 1982). On the basis of those kinetic observations and the stereochemistry of carboxylation, the spatial orientation of CO_2 and of Mn(II) with respect to P-enolpyruvate at the catalytic site of P-enolpyruvate carboxykinase can be proposed. These results suggest that the enzyme-bound Mn(II) and the CO_2 are located on opposite sides of the substrate. The CO_2 approaches P-enolpyruvate from "above" the plane (2-*si* face) of the C-3 atom of P-enolpyruvate while the Mn(II) interacts with P-enolpyruvate from "below" (2-*re* face) the plane.

ACKNOWLEDGMENTS

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Registry No. EC 4.1.1.32, 9013-08-5; (*Z*)-$CO_2HC(OPO_3H_2)$=CFH, 44976-98-9; (*RS*)-$CO_2HCHFCOCO_2H$, 103729-95-9; (2*R*,3*R*)-$CO_2HCH(OH)CHFCO_2H$, 74806-82-9; (2*R*,3*S*)-

$CO_2HCH(OH)CHFCO_2H$, 74806-81-8; malate dehydrogenase, 9001-64-3.

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Reactivity of Small Thiolate Anions and Cysteine-25 in Papain toward Methyl Methanethiosulfonate†

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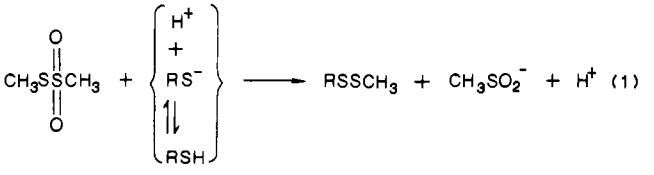
ABSTRACT: The dependence on thiol pK of the second-order rate constant (k_S) for reaction of thiolate anions with MMTS was shown to follow the Brønsted equation $\log k_S = \log G + \beta pK$ with $\log G = 1.44$ and 3.54 and $\beta = 0.635$ and 0.309 for aryl and alkyl thiols, respectively. The reactivity toward MMTS of the protonated thiol group was found to be negligible in comparison to that of the thiolate anion. For 2-mercaptoethanol the reactivity toward MMTS of the protonated form of the thiol group was shown to be at least 5×10^9 smaller than that of the thiolate anion. The pH dependence of the second-order rate constant for reaction of the thiolate group of Cys-25 at the active site of papain was determined and shown to be consistent with the previously determined low pK for Cys-25 and its electrostatic interaction with His-159. The small dependence of the reactivity of Cys-25 on thiol pK ($\beta \sim 0.09$) suggested that the charge–charge interactions that act through space to perturb the pK of the nucleophile at the active site of papain and perhaps other enzymes may serve to increase the fraction of nucleophile present in the reactive basic form without introducing the decrease in nucleophilic reactivity seen in model systems where pK's are lowered primarily by charge–dipole interactions.

In 1975 Kenyon and his co-workers (Smith et al., 1975; Nishimura et al., 1975) demonstrated the utility of methyl methanethiosulfonate (MMTS)[1] for selective modification of thiol groups in proteins, via the reaction depicted in eq 1.[2]



Since then MMTS has gained wide use as a probe of the function of thiol groups in proteins [e.g., see Bruice & Kenyon (1977), Lewis et al. (1978), Bloxam et al. (1979), Marshall & Cohen (1980), Gavalanes et al. (1982), Claiborne et al. (1982), and Kopczynski & Babior (1984)]. A major advantage of this reagent is that it blocks a thiol sulfur atom with

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[1] Abbreviations: bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; BzArgPNA, N^{α}-benzoyl-L-arginine *p*-nitroanilide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium salt; MCA, 7-amino-4-methylcoumarin; MMTS, methyl methanethiosulfonate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); PDT, potentiometric difference titration; ZArg–MCA, N^{α}-benzyloxycarbonyl-L-arginine–7-amido-4-methylcoumarin.

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