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Tartrate dehydrogenase reductive decarboxylation: stereochemical generation of diastereotopically deuterated hydroxymethylenes

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Abstract

Tartrate dehydrogenase catalyzes the reductive decarboxylation of *meso*-tartrate to glycerate. Concomitant with the ketonization of the intermediate enolate the C3 hydroxymethylene of glycerate necessarily acquires a proton from solvent. In D_2O, the proton is shown to be added stereospecifically to form (2*R*,3*R*)-[3-^{2}H]glycerate. The ^{1}H-NMR assignments of the diastereotopic C3 protons of glycerate were confirmed by the enzymatic conversion of [1*R*-^{2}H]fructose-6-phosphate to (2*R*,3*R*)-[3-^{2}H]glycerate. The decarboxylation-protonation occurs with retention of configuration, implying that the general acid is positioned on the same face of the intermediate as the departing carboxylate. The stereochemically pure (2*R*,3*R*)-[3-^{2}H]glycerate is readily synthesized and serves as a chiral hydroxymethylene synthon as demonstrated by the synthesis of (2*S*,3*R*)-[3-^{2}H]serine.
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Keywords: Tartrate dehydrogenase; Stereochemistry; Chiral hydroxymethylene; Serine; Glycerate

1. Introduction

β-Hydroxyacid dehydrogenases produce β-keto acid products that may either be released or subsequently decarboxylated. The well-studied malate dehydrogenase [1]

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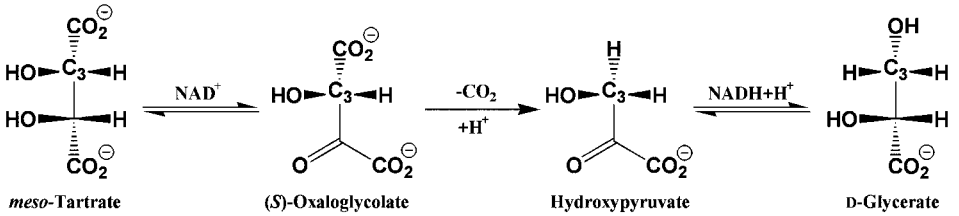
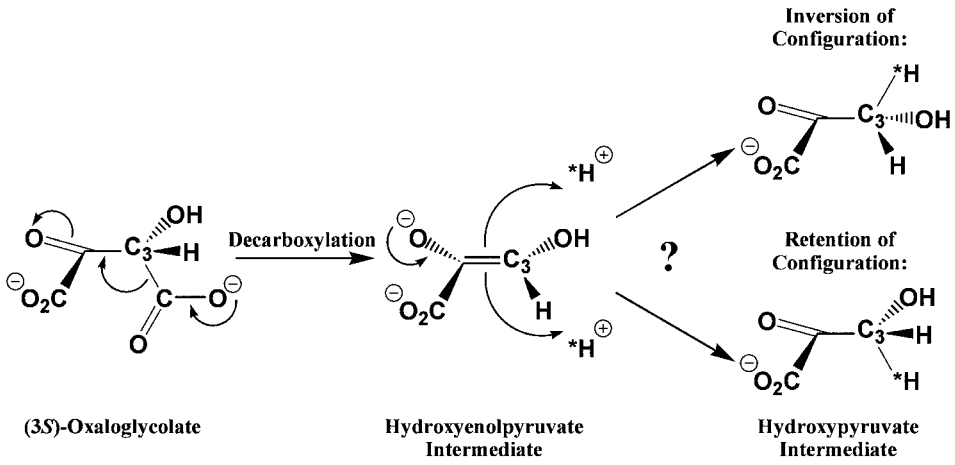
and malic enzymes [2] are paradigms for these reactions. Tartrate dehydrogenase is unusual in that whether the enzyme catalyzes the decarboxylation step depends on both the stereochemistry and nature of the substrate, e.g., (+)-tartrate is only dehydrogenated to oxaloglycolate, while D-malate is both dehydrogenated and subsequently decarboxylated. As in malic enzyme, the enolate formed by decarboxylation tautomerizes while bound at the active site [3] to generate a keto acid. Following the dehydrogenation, decarboxylation and ketonization, the keto acid intermediate of some substrates, defined by their stereochemical characteristics, is reduced by the NADH that is retained at the tartrate dehydrogenase active site [3,4]. By this process *meso*-tartrate is converted to D-glycerate, as shown in Scheme 1 , in a reaction reminiscent of UDP-glucose epimerase which contains a tightly bound nicotinamide dinucleotide that catalyzes both the oxidation and subsequent reduction of a single substrate [5]. A structural rationale for the variation in reaction course with substrate stereochemistry has been developed by Tipton and Beecher [6] who, on the basis of sequence comparisons, suggested that the enzyme has recently evolved from isopropylmalate dehydrogenase as a means for bacteria to grow on (+)-tartrate [7]. They propose that due to its fairly recent appearance, it has not yet been exposed to the selective pressure necessary for the development of rigorous substrate specificity.

In the formation of glycerate from *meso*-tartrate the C4 carboxylate is replaced by a proton, which must ultimately be derived from solvent, to generate a hydroxymethylene at C3. The two possible ways in which the proton may add to the intermediate enolate are depicted in Scheme 2. In D_2O the C3 hydroxymethylene will be chiral if the reprotonation is stereospecific. Protonation may be to the opposite face from which carbon dioxide leaves, resulting in an inversion of configuration. In this case the solvent-derived proton will occupy the pro-*S* position in the product. Conversely, the pro-*R* position would be occupied by the solvent proton if it were to add to the same face from which carbon dioxide leaves, leading to retention of configuration at the β-carbon. Determining the stereochemistry of this reaction will help to identify the operant general acid catalyst once a crystal structure has been elucidated. Therefore, this determination provides additional insight regarding the mechanism of this enzyme.

The reprotonation of the C3 carbon during enol–keto tautomerization has been used previously to generate C3-substituted pyruvate analogs from the reaction of 3-substituted malate analogs in D_2O [8]. If the tartrate dehydrogenase catalyzed



Scheme 1.



Scheme 2.

reprotonation of the enolate intermediates in these reactions occurs with the same stereochemical course, these 3-substituted pyruvate products could be formed with chirality at C3. Thus, the relaxed substrate specificity of tartrate dehydrogenase enables a variety of uses in the synthesis of C3 chiral methylene synthons.

As shown in Scheme 2 the product formed in D_2O will be D-glyceric acid that contains a chiral mono-deuterated hydroxymethylene. This synthetic intermediate would then provide a route to chirally deuterated compounds such as [3-^{2}H][1] hydroxypyruvate, L-[3-^{2}H]serine, L-[3-^{2}H]cysteine, [5-^{2}H]ribose, and [6-^{2}H]glucose. Currently reported methods for the synthesis of L-serine stereospecifically deuterated or tritiated at the β-carbon have a number of drawbacks. The use of phosphoglucoisomerase to set the stereochemistry and subsequent enzymatic conversion through the glycolytic pathway leads to only a 50% maximum incorporation of ^{2}H or ^{3}H, as only C1 of glucose or mannose receives the label, whereas both C1 and C6 become the β-carbon of serine due to the action of triosephosphate isomerase [10–13]. Purely chemical methods of introducing the stereochemistry have not lead to enantiomerically pure products [14,15]. Gani and Young [16] synthesized (2*S*,3*R*)-[3-^{2}H]serine using L-aspartase to introduce the desired chiral center; however, their method requires that intermediates be isolated throughout a series of specified chemical conversions. Following the determination of the stereochemistry of protonation of the hydroxyenolpyruvate intermediate of *meso*-tartrate, we have developed a "one-pot" synthesis of chiral L-serine-3-d as an example of the use of the product D-glycerate as a chiral hydroxymethylene synthon.

[1] The use of parentheses to enclose isotopic labels implies that the molecule is isotopically substituted, i.e., the mol fraction of isotope >90%. The use of the more common square brackets implies the molecule is incompletely labeled [9].

2. Materials and methods

The recombinant enzymes, tartrate dehydrogenase [6] and serine transacetylase, were both generous gifts from Paul F. Cook at the University of Oklahoma. All glycolytic enzymes and L-alanine dehydrogenase were obtained from Sigma Chemical Company. Calf intestinal alkaline phosphatase was from New England Biolabs. All enzyme preparations were used without further purification unless described otherwise below. All other reagents were purchased from commercial sources.

2.1. (2R,3R)-[3-^{2}H]-3-phosphoglycerate

The synthesis of (2*R*,3*R*)-[3-^{2}H]-3-phosphoglycerate was accomplished glycolytically in a manner similar to that of Floss et al. [17]. To 6 ml of 100 mM Tris buffer at pH 8.25 containing 20 mM $MgCl_2$, 10 mM KCl, and 0.2 mM EDTA in D_2O were added 54 μmol D-fructose-6-phosphate and 6 U phosphoglucose isomerase. The mixture was incubated 20 h at 7 °C to generate a mixture of glucose-6-phosphate and [1*R*-^{2}H]fructose-6-phosphate [18–20] and then frozen for later use. The solution was then thawed, and 80 μmol ATP was added along with 3 U phosphoglucose isomerase and 1.25 U phosphofructokinase to generate D-[1*R*-^{2}H]fructose-1,6-bisphosphate.

The extent of the reaction was monitored by assaying for ADP with phosphoenolpyruvate in the presence of pyruvate kinase and D-lactate dehydrogenase and observing NADH oxidation at 340 nm with $\varepsilon_{340} = 6220\,cm^{-1}\,M^{-1}$ [21]. The reaction was found to be 87% complete after 2.75 h incubation at room temperature. After 4 h the solvent was removed in a centrifugal vacuum concentrator. To the concentrate was added 75 μmol Na_2AsO_4 and the solids were redissolved in 1.2 ml of H_2O and concentrated again. Replacement of the D_2O with H_2O was necessary to prevent unintended deuteration in the subsequent steps.

The final concentrated solution was then dissolved in 5 ml H_2O and 10 U aldolase, 25 U triosephosphate isomerase, 80 U glyceraldehyde-3-phosphate dehydrogenase, 15 U lactate dehydrogenase, and 0.5 μmol NAD^+ were added. An absorbance increase at 340 nm was confirmed demonstrating the formation of 3-phosphoglycerate, and 100 μmol pyruvate in 100 μl H_2O is added to recycle the NADH. The reaction mixture was then incubated at room temperature for 19 h and in the process it generated (2*R*,3*R*)-[3-^{2}H]-3-phosphoglycerate. All enzymes were added in the form of ammonium sulfate suspensions.

The nucleotides were then removed from the solution by directly adding 250 mg Darco G-60 activated carbon and vigorously mixing for 5 min [22]. The suspension was then filtered and washed with water. The procedure was repeated with the resulting filtrate. The isolated 3-phosphoglycerate yield was determined by a coupled assay with 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase to be 45%. The 3-phosphoglycerate was purified by solid phase extraction using a 0.8×6 cm Dowex-1 anion exchange column. Unretained cations were eluted with 25 ml deionized water. Lactate and pyruvate was eluted with 45 ml 20 mM HCl, and (2*R*,3*R*)-[3-^{2}H]-3-phosphoglycerate was eluted with 25 ml 100 mM HCl. The (2*R*,3*R*)-[3-^{2}H]-3-phosphoglycerate solution was neutralized with LiOH, the solvent

removed in a speed vacuum concentrator, and the Li^+ salt formed by adding ethanol saturated with LiCl and washing the precipitate with 100% ethanol to remove the excess LiCl. A white powder resulted that probably also contained Li_2SO_4. Of the (2*R*,3*R*)-[3-^{2}H]-3-phosphoglycerate produced, approximately 32% was deuterated based on ^{1}H-NMR peak integrations. The 600 MHz ^{1}H-NMR spectrum was obtained in D_2O with the sodium salt of 3-(trimethylsilyl)proprionic-2,2,3,3-d_4 acid ($H_R = {}^2H$): δ 3.88 (ddd, $J_{RS} = 11$, $J_{R\alpha} = 6$, $J_{RP} = 7$), 4.01 (dd, $J_{SP} = 5$, $J_{S\alpha} = 3$), 4.03 (ddd, $J_{SR} = 11$, $J_{SP} = 6$, $J_{S\alpha} = 3$), 4.19 (d, $J_{\alpha S} = 3$), and 4.2 (dd, $J_{\alpha R} = 6$, $J_{\alpha S} = 3$).

2.2. (2R,3R)-[3-^{2}H]glycerate

(2*R*,3*R*)-[3-^{2}H]glycerate was produced from the (2*R*,3*R*)-[3-^{2}H]-3-phosphoglycerate product [23]. Approximately 9 μmol of the deuterated 3-phosphoglycerate product was dissolved in 750 μl 100 mM sodium phosphate pH 8.0 buffer in D_2O. Material that did not dissolve was removed by centrifugation. To this was added 0.1 μl of a 50% glycerol solution containing 1 U alkaline phosphatase. The mixture was incubated at room temperature for 21 h and frozen. The solution was then thawed and 0.5 μmol of the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid was added and a 600 MHz ^{1}H-NMR was obtained ($H_R = {}^2H$): δ 3.72 (dd, $J_{RS} = 12$, $J_{R\alpha} = 6$), 3.80 (bs), 3.82 (dd, $J_{SR} = 12$, $J_{S\alpha} = 3$), 4.08 (d, $J_{\alpha S} = 3$), and 4.09 (dd, $J_{\alpha R} = 6$, $J_{\alpha S} = 3$).

2.3. ^{1}H-NMR of the tartrate dehydrogenase catalyzed reaction

One hundred microliters of a 50% glycerol solution containing approximately 0.04 U of tartrate dehydrogenase was dialyzed 14 h at 7 °C into 10 ml of a D_2O buffer containing 100 mM potassium phosphate pH 8.0, 30 mM KCl, 20 mM $MgCl_2$, and 0.2 mM EDTA. The enzyme was then dialyzed again for 24 h at 7 °C against 9 ml of the same buffer. The D_2O buffer was centrifuged prior to dialysis due to precipitation of magnesium phosphate. Following dialysis the enzyme mixture was frozen for later use.

To 950 μl of the phosphate buffered D_2O in an NMR tube was added 24 μmol *meso*-tartaric acid, 0.05 μmol NAD^+, and 0.5 μmol of the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid. A ^{1}H-NMR was then taken of this starting reaction mixture. Approximately half, 150 μl, of the dialyzed preparation of tartrate dehydrogenase was then added, and ^{1}H-NMR spectra of the resulting solution were obtained periodically to monitor the time course of the reaction.

2.4. (2S,3R)-[3-^{2}H]serine

A procedure similar to the synthesis of L-serine from L-tartrate and D-glycerate by L-tartrate decarboxylase described by Furoyoshi et al. [24] and Soda et al. [25] was employed. An aqueous solution containing 72 μmol *meso*-tartaric acid was titrated to pH 7.5 with KOH, the solvent removed in a speed vacuum concentrator and redissolved in D_2O to 1 M. A buffer containing 100 mM TAPS, 30 mM KCl,

10 mM $MgCl_2$, and 0.5 mM EDTA at pH 8.0 was concentrated in a speed vacuum concentrator to replace the solvent with D_2O. To 690 μl of this D_2O buffer was then added 250 μl of 2 M ND_4Cl (D_2O), 5 μl of 2 M $MnCl_2$ (D_2O), and 50 μl of 1 M potassium tartrate (D_2O). The remaining dialyzed preparation of tartrate dehydrogenase, approximately 150 μl, was then added followed by approximately 2 U of L-alanine dehydrogenase in 10 μl 50% aqueous glycerol solution and 30 U of D-lactate dehydrogenase in 5 μl aqueous ammonium sulfate. Finally, 0.25 μmol NAD^+ and 0.25 μmol NADH was added in a total volume of 20 μl D_2O. The reaction was incubated at 30 °C.

The extent of the reaction was monitored using serine transacetylase [26]. To 900 μl of a buffer containing 140 mM Tris, 1.4 mM 5,5′-dithio-bis(2-nitrobenzoic acid), and 1.4 mM EDTA at pH 7.6 was added 100 μl 1.0 mM acetyl-CoA and between 1 and 10 μl of the reaction mixture. The reaction was initiated by the addition of serine transacetylase. The total change in absorbance at 412 nm was then recorded, and the total serine present calculated by taking ε_{412} for 5-thio-2-nitrobenzoic acid as $1.36 \times 10^4\ cm^{-1}\ M^{-1}$. After 17 days approximately 10 μmol serine had formed, and 2 U of L-alanine dehydrogenase and 60 U of D-lactate dehydrogenase were added in their respective solutions, along with 0.25 μmol more of both NAD^+ and NADH in 20 μl D_2O. After 27 days approximately 48 μmol (96% yield) had formed and the reaction was frozen prior to purification.

The solution was thawed and acidified to pH 2 with HCl. A precipitate results that was removed by centrifugation. The supernatant was loaded on a Dowex AG50-X8 cation exchange column (0.5×5 cm) equilibrated in the H^+ form with 10 mM HCl. The column was then washed with 10 ml of 10 mM HCl to remove unretained anions, and serine eluted with 1 M ammonium hydroxide. Fractions (1.5 ml) containing serine were identified by reaction with ninhydrin and combined. Chelex-100 (10 mg) was added to remove any remaining Mn^{2+} as was 50 mg Darco-G60 activated carbon at room temperature to remove any colored species eluted from the cation exchange resin. The suspension was then filtered and assayed for serine with serine transacetylase (27 μmol, 54% yield).

3. Results

3.1. Assignment of C3 ^{1}H-NMR resonances of (2R,3R)-[3-^{2}H]glycerate from D-fructose-6-phosphate

The (2*R*,3*R*)-[3-^{2}H]glycerate product of the glycolytic enzymes contains glycerol from the storage buffers which appears in the ^{1}H-NMR spectrum as a doublet of doublets at 3.66 and 3.56 ppm due to the two diastereotopic protons at C1 and C3. The C2 proton of glycerol appears as a triplet of triplets centered at 3.79 ppm. Multiplets are also seen at 4.19, 4.02, and 3.88 ppm arising from (2*R*,3*R*)-[3-^{2}H]-3-phosphoglycerate that was not hydrolyzed by the alkaline phosphatase. Because of the action of triosephosphate isomerase, the (2*R*,3*R*)-[3-^{2}H]glycerate is only partially deuterated and the α-proton resonance of the C3-$^1H_2$ isotopolog is a doublet

of doublets with coupling constants of 6 and 3 Hz at 4.09 ppm while that of [3*R*-^{2}H]glycerate is a doublet with a coupling constant of 3 Hz centered at 4.08 ppm. The known stereochemistry requires that the 3*R* β-proton produce a single resonance at 3.72 ppm as a doublet of doublets with coupling constants of 12 and 6 Hz. The 3*S* proton produces two resonances, one from non-deuterated glycerate at 3.82 ppm as a doublet of doublets with 12 and 3 Hz coupling constants, and the second from [3*R*-^{2}H]glycerate shifted up-field with $^2\Delta H_{DCH} = 10$ ppb to 3.81 ppm. The multiplicity of this latter resonance is difficult to discern on account of its overlap with the glycerol C2-H signal; nevertheless, it resembles a broad singlet which one might expect considering that it is coupled to the geminal deuterium.

3.2. Stereospecificity of glycerate formed by the tartrate dehydrogenase reaction with meso-tartrate

Fig. 1A is the 600 MHz ^{1}H-NMR spectrum obtained after approximately 24 h of incubation of *meso*-tartrate in the presence of NAD^+ and tartrate dehydrogenase. Fig. 1B is the corresponding ^{1}H-NMR spectrum for standard D-glycerate. In Fig. 1A the glycerol C2H resonance is seen at 3.79 ppm. Peaks derived from D-[3-^{2}H]glycerate are seen at 4.08 ppm, $J_{HH} = 3$ Hz, and 3.81 ppm, the latter of which overlaps with the glycerol C2H signal. Notable by their absence is the ^{1}H resonance at 3.72 ppm and the associated vicinal 12 Hz J_{HH} of the resonance at 3.81 ppm. The residual resonance present at 3.72 ppm is attributed to the presence of HOD in the reaction mixture rather than to non-specific deuteration. These results indicate that the product glycerate incorporated over 95% ^{2}H. The stereochemistry of the product was determined to be (2*R*,3*R*)-[3-^{2}H]glycerate by comparison of the ^{1}H-NMR with that obtained from glycerate produced by the action of the enzymes of glycolysis.

3.3. Synthesis of [3-^{2}H]serine from meso-tartrate

The ^{1}H-NMR spectra in pH 7 phosphate buffered D_2O solutions of the product from *meso*-tartrate and standard L-serine are shown in Figs. 2A and B, respectively. Prior syntheses of deuterated serine [15,16], along with the stereochemistry of tartrate dehydrogenase determined here permit assignment of the two β-protons. The pro-*R* proton is identified at 4.00 ppm, $J_{RS} = 12$ Hz and $J_{R\alpha} = 4$ Hz, and the pro-*S* at 3.95 ppm, $J_{S\alpha} = 6$ Hz. A doublet of doublets is observed for each proton signal in the standard sample. In the product spectrum, the pro-*S* signal has shifted up-field with $^2\Delta H_{DCH} = 16$ ppb and along with the α-proton has undergone the expected collapse in multiplicity to a doublet, $J_{S\alpha} = 6$ Hz. Non-deuterated L-serine is also seen in the product spectrum and is attributable to HOD in the reaction mixture. The ratio of the pro-*R* to the pro-*S* peak integrations is 0.06, which implies approximately 94% deuteration. There is no evidence of a doublet originating from the pro-*R* β-proton expected from (2*S*,3*S*)-[3-^{2}H]serine, or a singlet originating from the α-proton expected from (2*S*)-[3,3-$^2H_2$]serine. For these reasons the pro-3*R* signal is not believed to be the result of racemization of the hydroxyenolpyruvate intermediate or non-stereospecific protonation.

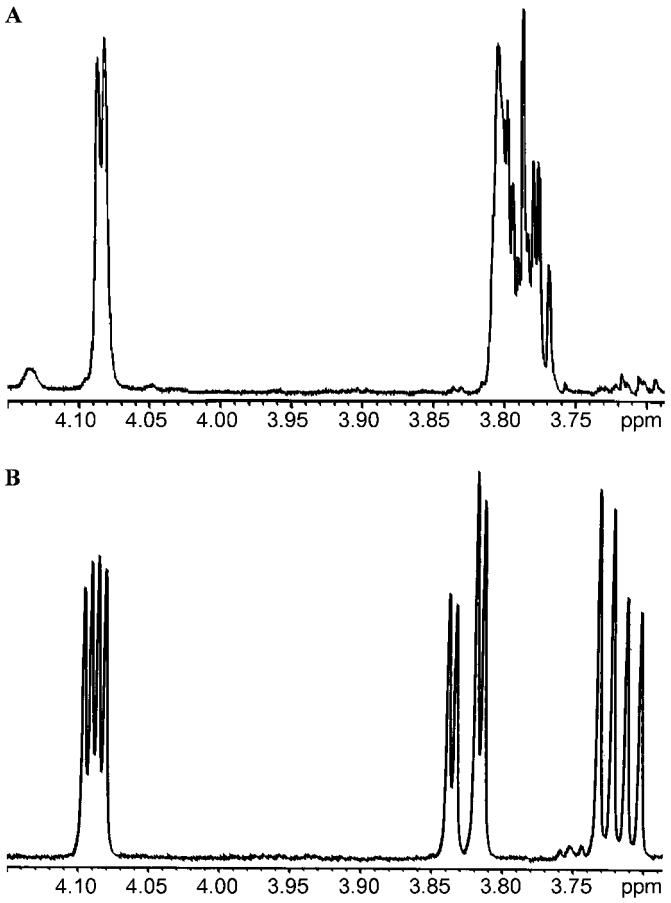


Fig. 1. 600 MHz ^{1}H-NMR spectra of D-glycerate. (A) From the actions of tartrate dehydrogenase on *meso*-tartrate in a buffered D_2O solution after approximately 24 h. (B) Standard D-glycerate in pH 7 phosphate buffered D_2O.

4. Discussion

The reactions of tartrate dehydrogenase provide a valuable means for the stereospecific introduction of hydrogen isotopes into biologically relevant small molecules. When *meso*-tartrate is provided as its substrate, the product (2*R*,3*R*)-[3-^{2}H]glycerate was formed stereospecifically as a single proton from the solvent was incorporated at C3 during the enzyme catalyzed enol–keto tautomerization of the hydroxyenolpyruvate intermediate [3]. As shown in Scheme 2, this result indicates that the decarboxylation protonation sequence occurs with the retention of stereochemistry. Since the intermediate is reprotonated on the same face of the enolate double bond that the CO_2 departs from, the proton donor must be in close proximity to the C4 carboxylate of the substrate. This is consistent with the postulation that a protonated group

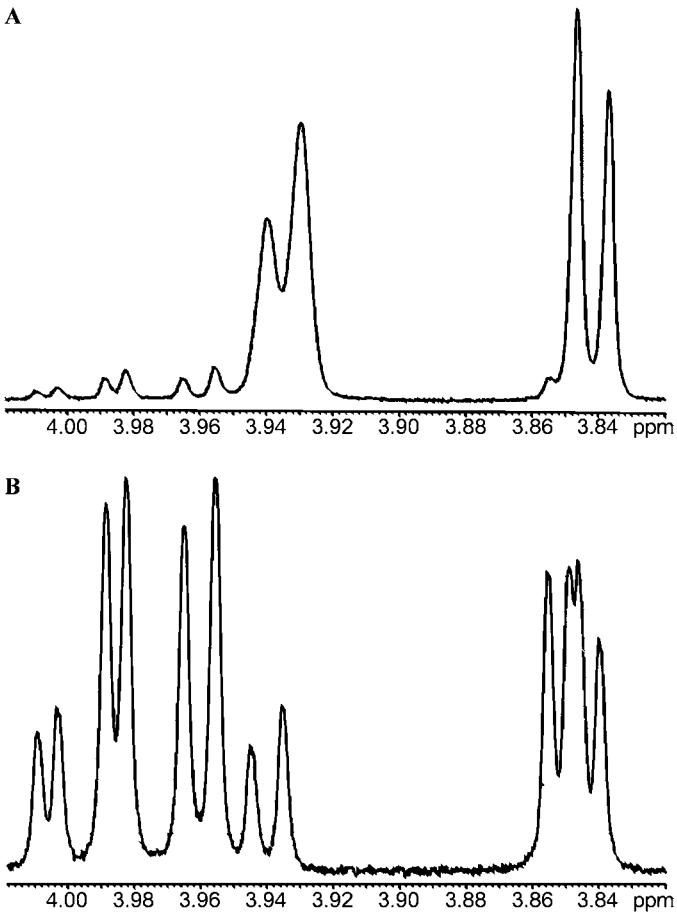
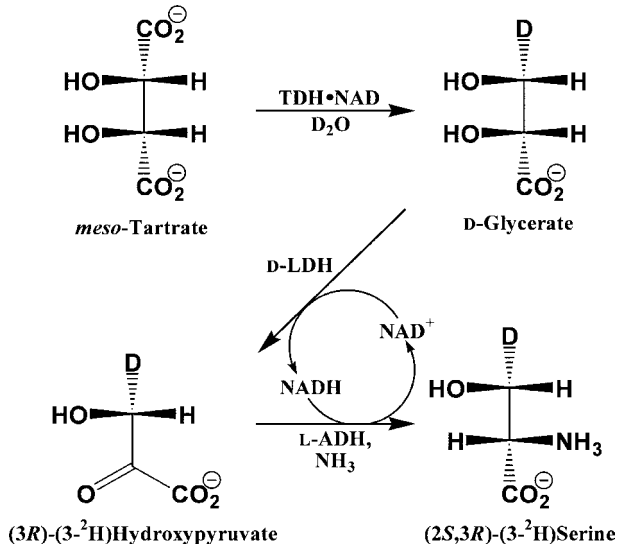


Fig. 2. 600 MHz ^{1}H-NMR of L-serine obtained in pH 7 phosphate buffered D_2O. (A) Product synthesized from *meso*-tartrate using tartrate dehydrogenase. (B) Standard L-serine.

with a pK_a of ~8.6 is required for the binding of tartrate and protonation of the enolate [27]. Like *meso*-tartrate, D-malate also does not dissociate from the enzyme prior to protonation of the enolpyruvate intermediate [8]. Because all of the tartrate dehydrogenase catalyzed reactions occur at the same active site [3], the solvent-derived proton is likewise expected to add with retention of configuration following oxidative decarboxylation of D-malate and its halogenated derivatives [8]. Thus (2*R*,3*S*)-3-halomalates and (2*R*,3*R*)-3-halomalates will be converted to (2*R*,3*R*)-3-halopyruvate and (2*R*,3*S*)-3-halopyruvate, respectively.

The ability of tartrate dehydrogenase to stereospecifically add a deuteron from the surrounding solvent to the pro-*R* position of C3 in the product provides a route to a number of stereospecifically deuterated compounds in addition to the immediate D-glycerate and 3-halopyruvate products. An example of this is the synthesis of (2*S*,3*R*)-[3-^{2}H]serine from *meso*-tartrate. With D_2O as the solvent, this conversion



Scheme 3.

can be performed in a "single-pot" as depicted in Scheme 3 with the ^{1}H-NMR of the product (2*S*,3*R*)-[3-^{2}H]serine (54% isolated yield) shown in Fig. 2. These assignments of the diastereotopic resonances correspond to those obtained from previous work on deuterated and non-deuterated L-serine [15,16,28], further confirming the stereochemical analysis of the product glycerate.

The (2*R*,3*R*)-[3-^{2}H]glycerate can serve as a chiral hydroxymethylene synthon. Reversal of glycolysis in the presence of a triosephosphate isomerase inhibitor and dihydroxyacetone phosphate will make available (in sequence), (2*R*,3*R*)-[3-^{2}H]1, 3-diphosphoglycerate, (2*R*,3*R*)-[3-^{2}H]glyceraldehyde-3-phosphate, (6*R*)-[6-^{2}H]fructose-1,6-bisphosphate, (6*R*)-[6-^{2}H]fructose-6-phosphate, and (6*R*)-[6-^{2}H]glucose-6-phosphate. This use of the readily available gluconeogenic enzymes is well precedented [29–31]. The (6*R*)-[6-^{2}H]glucose-6-phosphate can be readily converted to (6*R*)-[6-^{2}H]glucose by the action of alkaline phosphatase and to (6*R*)-[6-^{2}H]6-phosphogluconate and (5*R*)-[5-^{2}H]ribulose-5-phosphate by the enzymes of the glucose-6-phosphate shunt [29]. The (2*S*,3*R*)-[3-^{2}H]serine can be converted to *O*-acetyl-(2*S*,3*R*)-[3-^{2}H]serine and then to (2*S*,3*R*)-[3-^{2}H]cysteine by the action of serine *O*-acetyl transferase and *O*-acetylserine sulfhydrylase, respectively [12,32]. *O*-acetylserine sulfhydrylase has a low specificity for the second nucleophile so that numerous additional products could be readily synthesized [33].

5. Conclusions

Tartrate dehydrogenase converts *meso*-tartrate to D-glycerate with retention of configuration at C3. The (2*R*,3*R*)-[3-^{2}H]glycerate product formed by performing

the reaction in D_2O may be readily converted by additional enzymatic reactions to a large number of other metabolites where the stereochemistry of the ^2H-labeled hydroxymethylene is retained.

Acknowledgments

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