Stereochemical Course of the Maleate Hydratase Reaction*

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SUMMARY

Equilibration of maleate in D₂O with a partially purified preparation of maleate hydratase from rabbit kidney resulted in the formation of D-malate containing 1 atom of stably bound deuterium per molecule. The enzyme therefore catalyzes a stereospecific addition to the double bond of maleate.

The product of the enzymatic hydration in D₂O was examined by nuclear magnetic resonance spectroscopy. In addition, the monodeuteriosuccinate obtained by chemical reduction of the deuterated D-malate was analyzed for its optical rotatory properties. The absolute configuration of the enzymatically deuterated maleate was thus established and corresponded to the structure D-malate-3-d (3-R). It was concluded that the over-all reaction catalyzed by maleate hydratase is trans.

Maleate, 4-maleylacetoacetate, and 3-maleylpyruvate are intermediates in the degradation of mevalonate, homogentisic, and gentisic acids, respectively. The further metabolic utilization of these cis isomers involves a prior enzymatic isomerization to the corresponding trans isomers (1-3). Earlier reports on the direct conversion of maleate to malate in corn kernels and in a maleate-adapted organism (4, 5) could not be substantiated, and the observed interconversions appeared to proceed by a prior isomerization of maleate to fumarate followed by the action of fumarate hydratase (EC 4.2.1.2, fumarase) to yield L-malate (6). More recently, it has been shown that the initial intermediate in the conversion of maleate to CO₂ by mammalian kidney appears to be n-malate and that its formation is catalyzed by a maleate hydratase present in the mitochondria (7). The available information suggests that n-malate gains entry into the citric acid cycle through the action of n-2-hydroxyacid dehydrogenase (EC 1.1.2.4) reported to be present in rabbit kidney mitochondria (8).

The reversible enzymatic hydration and amination of fumarate have been shown to proceed by a trans mechanism of addition and elimination (9-13). Direct evidence has been provided that the succinate dehydrogenase-catalyzed oxidation of succinate to fumarate involves a stereospecific trans-directed elimination of hydrogen atoms (14, 15). Fumarate is a product of the enzymatic cleavage of L-argininosuccinate or adenylosuccinate, catalyzed, respectively, by L-argininosuccinate arginine-lyase (EC 4.3.2.1, argininosuccinase) and adenylosuccinate AMP-lyase (EC 4.3.2.2, adenylosuccinase). A trans mechanism of elimination of L-arginine and AMP in these reactions has also been shown (16, 17).

In view of the known stereochemical course of enzymatic addition and elimination reactions involving the trans double bond of fumarate, it was of interest to investigate the stereochemical consequences of analogous reactions with the corresponding cis isomer. The results presented in this paper show (a) that the enzymatic maleate hydratase catalyzes a stereospecific addition to the double bond of maleate and (b) that the absolute configuration of the product of the enzymatic hydration of maleate in D₂O is n-malate-3-d (3-R)† (three-n-malate-3-d). The over-all stereochemistry of the addition reaction is therefore trans.

EXPERIMENTAL PROCEDURE

Enzyme Preparation—Decapsulated rabbit kidneys (367 g) were homogenized for 2 min in a Waring Blender with 2 volumes of 0.15 M NaCl containing 5 × 10⁻⁴ M Fe(NH₄)₂(SO₄)₂ and 1.4 × 10⁻² M mercaptoethanol. The homogenate was centrifuged at 23,000 × g for 45 min. To the supernatant (750 ml) were added gradually 182.2 g of solid ammonium sulfate, and the mixture was stirred for 30 min. The suspension was centrifuged at 23,500 × g for 45 min, and the salt concentration of the supernatant was raised by the further gradual addition of 48 g of solid ammonium sulfate. The suspension was allowed to stand overnight in the cold and the precipitate was collected by centrifugation at 30,000 × g for 45 min. The residue was dissolved in 50 ml of D₂O containing 0.02 M Tris (pH 7.4), 1 × 10⁻⁷ M Fe(NH₄)₂(SO₄)₂, and 1.4 × 10⁻³ M mercaptoethanol. This protein solution, after standing at 0°C for 30 min, was brought to 52.5% saturation with ammonium sulfate by the addition of 16.6 g of the solid salt. After an adequate period of equilibration, the suspension was centrifuged at 30,000 × g for 45 min and the supernatant was discarded. The reprecipitation of the enzyme from D₂O was

† R and S according to the sequence rule of Cahn, Ingold, and Prelog (18), Cahn (19), and Hanson (20).

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carried out in order to achieve equilibration of the exchangeable hydrogen atoms in the protein fraction with deuterium.

Enzymatic Conversion of Maleate to n-Malate in D2O—A mixture of 2.5 mmoles of Tris, 10 mmoles of maleic acid, 51 mmole of Fe(NH3)6(SO4)2, and 15.2 mmole of NaCl dissolved in water was adjusted to pH 7.4 and lyophilized. The dried residue was redissolved in 20 ml of 99.5% D2O and relyophilized. This procedure was repeated twice more to assure maximum exchange of hydrogen atoms by deuterium atoms. The D2O solution of the rabbit kidney enzyme was transferred to this reaction mixture; 0.05 ml (0.11 mmole) of mercaptoethanol was added, and the final volume was adjusted to 50 ml with D2O. The protein concentration was determined to be 62.4 mg per ml, and the final D2O content of the medium was 84%. Incubation was carried out at 30° and at various time intervals 0.1-ml aliquots were removed and analyzed for n-malate. It was thus established that 75, 87, 88, and 85% of the maleate had been converted to n-malate at 5, 25, 48, and 72 hours, respectively. The reaction was terminated after 72 hours by the addition of 50 ml of cold 10% perchloric acid, and the protein was removed by centrifugation at 30,000 X g for 10 min.

Isolation of n-Malic Acid-3-d—The clear deproteinized reaction mixture was adjusted to pH 8.6 with 6 n potassium hydroxide, and, after addition of 3 g of potassium chloride, was centrifuged to remove the precipitated potassium perchlorate. The supernatant was lyophilized, dissolved in 25 ml of water, and recenterfuged to remove insoluble material. The deep yellow solution was acidified with 4 n sulfuric acid to a final concentration of 0.8 n and continuously extracted with ether for 96 hours. A total of 7.02 mmole of n-malic acid was thus obtained. The ether-extracted acid, dissolved in approximately 25 ml of water, was adjusted to pH 2.0 by the addition of 1 n NaOH and placed in a water bath at 95°. Cinchonine (10 mmole) was added to the solution with stirring, and the cinchonine that failed to dissolve was removed by filtration. The filtrate was placed in the cold and within 24 hours a heavy crop of white needles was obtained. After one recrystallization from hot water, the cinchonine-n-malate melted at 127–130.5° (uncorrected). The cinchonine salt prepared under similar conditions from an authentic sample of n-malic acid melted at 127–131° (uncorrected). In order to recover free n-malic acid, the cinchonine salt was dissolved in H2O and the alkaloid was removed by precipitation with ammonia. The free acid was obtained by passage of the ammonium malate solution through a Dowex 50-X8 (H⁺ form) column.

The cinchonine salt of n-malate crystallizes with 2 water atoms of hydration which are retained with some tenacity even at elevated temperatures (21). For deuterium analyses, therefore, n-malic acid was isolated as the diphenacyl ester (22) and recrystallized from benzene-petroleum ether. The diphenacyl derivative of the product of the enzymatic hydration of maleate in D2O had 4.80 atom % excess of deuterium, equivalent to 0.86 atom of deuterium per mole (1 atom of deuterium per molecule of diphenacyl malate would correspond to a value of 5.6 atom % excess).

Conversion of n-Malic Acid-3-d to Succinic Acid-2-d—To 0.18 g (1.34 mmole) of the enzymatically prepared n-malic acid-3-d in an oven-dried flask was added dropwise 0.5 ml (6.9 mmole) of thionyl chloride previously distilled from quinoline, lincteed oil, and then over zinc dust. The reaction mixture was cooled to −10° and kept in the dark. A 0.21-ml (2.6 mmole) portion of purified pyridine was then added dropwise at a very slow rate so as to maintain the temperature below −5°. The reaction mixture was allowed to warm up to room temperature gradually and after 8 hours a saturated solution of potassium chloride was added. Extraction was carried out with several portions of ether and the combined etheral solution was evaporated under reduced pressure. The yellow residue was dissolved in water, treated with charcoal, and filtered. The aqueous solution was taken to dryness and the product was recrystallized from chloroform to yield 125 mg of L-chlorosuccinic acid (61% yield); m.p. 183° (uncorrected); [alpha]25 = −21°. The reaction thus proceeded with inversion of configuration (23).

To 0.000 g (0.6 mmole) of the above prepared L-chlorosuccinic acid-3-d dissolved in water were added 70 mg of palladium catalyst on charcoal, and the reaction flask was evacuated. Hydrogen gas was bubbled in and the reaction mixture was allowed to stand in an hydrogen atmosphere at 10 pounds of pressure. The catalyst flocculated after taking up hydrogen for 10 min, and the reaction was permitted to proceed for 4 hours. The solution was then filtered to remove the catalyst and evaporated to dryness under reduced pressure. The white solid was purified by repeated sublimations, yielding 58 mg (82% yield) of succinic acid-2-d; m.p. 192°. The product had 13.0 atom % excess of deuterium, equivalent to 0.78 atom of deuterium per molecule and represents an isotope recovery of 91% from the starting n-malic acid-3-d.

Analytical Procedures—Protein was estimated by the biuret method (24). n-Malate was determined by its quantitative enzymatic conversion to oxaloacetate with n-2-hydroxyacid oxidoreductase with the use of ferricyanide as an electron acceptor. The assay was completed by quantitatively decarboxylating the oxaloacetate to pyruvate (25) and determining the pyruvate by the method of Friedemann and Haugen (26). Samples of deuterated diphenacylmaleate and succinic acid were diluted with their protonated counterparts to contain between 0.6 and 1.0 atom % of excess deuterium. For deuterium analysis, the samples were oxidized in liquid air oxygen and the water was converted to hydrogen by passage through a uranium converter connected in series with a mass spectrometer (27). The proton magnetic resonance absorption measurements were performed on a Varian HR-60 NMR spectrometer at a frequency of 60 megacycles. For this purpose, all exchangeable hydrogen atoms of the molecule were replaced with deuterium by dissolving approximately 117 mg of the enzymatically synthesized n-malic acid-3-d in 4 ml of D2O and flash evaporating the solution to dryness. This was repeated three times and for analysis the dried sample was dissolved in 1 ml of D2O. The optical rotatory dispersion measurements were performed on the Cary model 60 recording spectropolarimeter. A cell of 1-cm light path length was used, and the temperature within the cell compartment was maintained at 27°. The slit widths of the polarimeter were programmed to give a half band width of 1.5 mrad or less over the entire spectral region under examination. A 10-sec response time was used, and the recorder speed was adjusted accordingly for accurate results near the extremes of the dispersion curves.

RESULTS AND DISCUSSION

When maleate was incubated with a partially purified preparation of maleate hydratase in a medium containing 84% D2O, the n-malate formed contained 0.86 atom of deuterium per molecule.

* J. S. Britten, manuscript in preparation.
On full isotopic equilibration, a nonstereospecific reaction should lead maximally to 1.68 and 2.52 atoms of deuterium per molecule of maleate and n-malate, respectively, fractionation due to isotope effects being neglected. Since the reaction proceeded for at least 48 hours after chemical equilibrium had been reached (see "Experimental Procedure"), ample opportunity had been allowed for the maleate to become labeled and for the n-malate to contain more than 1 atom of deuterium per molecule if the reaction proceeded nonstereospecifically. Although the deuterium content of the residual maleate was not determined in the present study, the isotope incorporated into the n-malate corresponded quantitatively to the deuterium content of the medium. It is therefore evident that the maleate hydratase-catalyzed equilibration of maleate in D₂O leads to the exclusive formation of monodeuterated n-malate. This establishes conclusively that the hydrogen atom is added stereospecifically to carbon atom 3 in the formation of the methylene group of n-malate and that a hydrogen atom from the identical position is removed in the reverse direction.

The two possible isomeric forms of n-malate-3-d, depending on the mechanism of the enzymatically catalyzed addition reaction determined, by analogy to other systems the reaction should be freely reversible.
to maleate, are shown in Scheme 1, Reaction Sequence A. The isomers differ only with respect to the C-3 configuration, the assignment of the indicated absolute configuration for C-2 being dictated by the known specificity of the enzyme for the n isomer (7). With the C-2 and C-3 carboxyl groups of maleate in a trans configuration, as shown, a trans mechanism of D,O addition to maleate results in a product in which the two protons are gauche to one another, whereas the same two protons are trans to each other in the isomer arising from a cis mechanism of D,O addition. The relative proximity of these two protons can be determined by measuring the spin-spin interactions of the protons by nuclear magnetic resonance (29). Also shown in Reaction Sequence A are the corresponding monodeuterated isomers of succinate which would be obtained by chemical reduction of each of the two n-maleate-3-d isomers. The two isomeric forms of monodeuteriumsuccinate can be distinguished by application of ultraviolet optical rotatory dispersion data (30).

The fumarase-catalyzed trans-hydration of fumarate in D,O (9-11) resulting in the formation of l-maleate-3-d (3-R) (erythro-L-maleate-3-d) is shown in sequence B. This isomer has been reported to have a coupling constant (referring to the spin-spin interaction of the 2 hydrogen atoms on the adjacent C-2 and C-3) of 6.3, 6, and 7.1 cps (9, 13, 31). l-Maleate-3-d (3-R) on reduction yields succinate-2-d-(2-R) which has a plain negative rotatory dispersion curve in the 350 to 250 mp spectral region (30). As shown in Reaction Sequence C, the enzymatic oxidative decarboxylation of threo-D,-isocitrate proceeds with retention of configuration (32, 33) and provides a means for the synthesis of dextrorotatory succinate-2-d-(2-S). These reference standards of known absolute configuration were used in the present study to determine the steric course of the enzymatic hydration of maleate.

Nuclear magnetic resonance spectroscopic examination of the n-malic acid-3-d obtained by the maleate hydratase-catalyzed hydration of maleate in D,O gave coupling constants of 4.1, 4.3, and 4.2 on repeated analyses. Under similar conditions of analysis, a sample of L-malic acid-3-d-(3-R) obtained by reaction of fumarate in D,O with fumarase (11) was found by us to have a coupling constant of 6.7 cps, a value in close agreement with those reported for this isomer (9, 13, 31). The coupling constants obtained for the product of the enzymatic hydration of maleate in D,O correspond, however, to the values of 4 and 4.2 ± 0.2 cps reported for (a) a sample of n-malic acid-3-d-(3-R) obtained by chemical inversion at C-2 of 1-aspartate-3-d-(3-R) (erythro-L-aspartate-3-d) prepared in turn by (trans-) enzymatic amination of fumarate in D,O (13) and (b) a synthetic racemic mixture of n-malic acid-3-d-(3-R) and L-malic acid-3-d-(3-S) (threo-L-malic acid-3-d) obtained by the trans opening of the oxide ring of 3,4-epoxy-2,5-dimethoxymethylenhydrofuran with lithium aluminum deuteride, followed by acid hydrolysis and subsequent oxidation of the dialdehyde to the corresponding hydroxydecarboxylic acid (9, 10).

The monodeuterated succinic acid obtained by chemical reduction of the n-maleate-3-d prepared in turn by enzymatic hydration of maleate in D,O was examined in the spectropolarimeter. Curve A in Fig. 1 represents the optical rotatory dispersion properties of this succinic acid-2-d in the spectral range from 300 to 200 mp. The low negative rotations, which extend into the visible region, decrease to a minimum near 223 mp and subsequently increase toward the positive direction crossing zero near 210 mp. The trough is the first extreme of a Cotton effect which is associated with the absorption of the carboxyl group in this region. The curve for succinic acid-2-d-(2-S) (Fig. 1, Curve B) obtained through the sequence of reactions initiated by the TPX-specific isocitric acid dehydrogenase-catalyzed oxidative decarboxylation of threo-D,-isocitrate (32) (Scheme 1, Reaction Sequence C) is almost an exact mirror image of Curve A. It is therefore evident that the two samples of succinic acid-2-d are optical isomers of each other and establishes the configuration of the deuterated succinate derived from the product of the maleate hydratase reaction as succinate-2-d-(2-R).

n-Malate-3-d-(3-R), which on chemical reduction yields succinate-2-d-(2-R), is configurationally compatible only with a trans mechanism of hydration (Scheme 1A). Maleate hydratase thus belongs to that group of enzymes which includes fumarase, aspartase, succinic dehydrogenase, argininosuccinase, adenylosuccinase, and succinatase catalyzing a trans addition and elimination (9-17, 37, 38). Although the conversion of stearic acid to oleic acid in Corynebacterium diphtheriae apparently involves the cis removal of hydrogen atoms from carbon atoms 9 and 10 (39), the hydration of 5-dehydroshikimate to 5-dehydroquininate catalyzed by 5-dehydroquininate dehydratase (EC 4.2.1.10) is to date the only known example of a reversible enzymatic cis addition to a double bond (38).

* Previously published rotations for succinic acid-2-d-(2-R) (90, 34, 35) and succinic acid-2-d-(2-S) (82, 34, 35) were restricted to the region of the plain dispersion curve and the values are similar to the rotations reported here. In the present study, the measurements have been extended to include the region of the Cotton effect.
REFERENCES