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The Mechanism of the Stereospecific Enzymatic Hydration of Fumarate to L-Malate[1]

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RECEIVED FEBRUARY 7, 1957

It is found that the addition of water to the double bond of fumarate occurs in a *cis* manner when catalyzed by the enzyme fumarase from pig heart muscle. This is shown by the configuration of the monodeutero-L-malate formed when the hydration is carried out with deuterium oxide. The configuration of the monodeutero-L-malate was established by observing the dipolar broadening of the proton magnetic resonance absorption in solid DOOCCH(D)C(OD)HCOOD.

Introduction

When the hydration of fumarate to L-malate is catalyzed in deuterium oxide by the fumarase from pig heart muscle, the L-malate which is formed contains one deuterium atom per molecule which is not readily exchangeable with the medium.[2] Since only monodeutero-L-malate is formed in the over-all reaction and the fumarate does not incorporate deuterium, the addition of the deuterium is stereospecific, as is the addition of the OH or OD group. This observation has been confirmed.[3] Further work has shown that the deuterium atom is not exchanged with the medium by the action of the enzyme but is removed only in the over-all dehydration reaction.[4]

The question as to which of the two positions on the methylene group is deuterated in the monodeutero-L-malate is important because the answer will show whether water is added to the double bond of fumarate in a *cis* or *trans* manner. Whereas it frequently is assumed that substrates are bound on the surface of an enzyme, a *cis* addition by fumarase would prove that the entering groups came from the same side, and presumably from that of the protein since their addition is under stereochemical control. The ability of the enzyme to distinguish unerringly between the two hydrogen positions on the methylene group of L-malate may find practical application in the determination of the structure of partially deuterated compounds which may be transformed to malic acid.

The stereospecificity of the diphosphopyridine dehydrogenases already has been investigated.[5] It has been found that the hydrogen transfer catalyzed by the β-hydroxysteroid dehydrogenase[6] and glucose dehydrogenase[7] occurs at the opposite side of the nicotamide ring from that used by the alcohol, lactic and malic dehydrogenases. Therefore, it is not to be assumed that fumarase from other sources would necessarily cause deuterium to be added in the same position as pig heart fumarase.

The question of whether the fumarase catalyzed hydration occurs in a *cis* or *trans* manner can be answered by establishing the steric relation of the fumarate protons on carbons 2 and 3 in the monodeutero-L-malic acid. This is shown schematically in the right-hand portion of Fig. 1. If the hydration occurs in the *cis* manner as depicted in the figure, the protons are gauche to one another. On the other hand, if the hydration is *trans*, then the protons are *trans*. These particular relations hold only if the carboxyl groups are *trans* in L-malic acid, as shown; if the carboxyl groups are gauche, then the proton–proton relations are the reverse of those just given.

X-Ray diffraction studies of succinic and tartaric acids[8] have shown that in the crystalline solids the carboxyl groups are *trans*, and undoubtedly they are also *trans* in crystalline malic acid. Therefore, the only structural feature to be established is whether the protons are gauche or *trans* in the monodeutero-L-malic acid. This type of structural problem is suitable for proton magnetic resonance solid-state methods. The hydroxyl and acidic protons exchange readily with D_2O to give DOOCH(D)C(OD)HCOOD, in which the protons in question are the most important magnetic nuclei. The dipolar broadening of the proton magnetic resonance in the solid depends very largely on the intramolecular proton–proton distance. The broadening is very sensitive to the interproton distance so it is relatively easy to determine unequivocally whether the configuration is *trans* or gauche, as has been done for solid *sym*-tetrachloro- and tetrabromoethane.[9]

Experimental

Preparation of Deutero-L-malic Acid.—Crystalline fumarase obtained from pig heart extract[10] was added to a solution containing 0.2 *M* potassium fumarate and 0.0083 *M* dipotassium phosphate and 0.0017 *M* monopotassium phosphate in 99.5% D_2O[11] and the reaction was allowed to proceed to equilibrium, as determined spectrophotometrically. The reaction was stopped by heating to 90° for 3 minutes and the solution was then lyophilized.

The deutero-L-malate was separated from the 0.18 mole fraction fumarate present at equilibrium by partition chro-

(1) The nuclear magnetic resonance experiments were supported by the U. S. Office of Naval Research and by a grant-in-aid from the Upjohn Co. The enzymatic experiments were supported by grants from the National Science Foundation and from the Research Committee of the Graduate School of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

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matography as described by Cleland and Johnson[12] with 0.5 N HCl replacing 0.5 N H_2SO_4 as the stationary phase. Treatment with $AgNO_3$ showed the deuteromalic acid recovered from the eluent to be free of chloride. Aqueous extractions of fractions collected midway between the fumaric acid and deuteromalic acid peaks showed no evidence of either substrate upon addition of concentrated solutions of fumarase, thus indicating that the separation was quantitative. The deuteromalic acid was redissolved three times in 1.5 ml. of 99.5% D_2O and each time lyophilized to dryness to replace exchangeable hydrogen with deuterium.

The resulting compound, DOOCCH(D)C(OD)HCOOD, had a melting point of 97–99° as compared with 103–104° for normal L-malic acid. The change in melting point upon deuteration is in the direction predicted by Schäfer[13] and compares favorably with the lowering of the melting point of succinic acid upon deuteration.[14] The deuteromalic acid contained 0.97 atom of deuterium per molecule in the specific methylene position and about 2.85 atoms of deuterium per molecule in the carboxyl and hydroxyl positions, as determined by mass spectrometric analysis.

Nuclear Magnetic Resonance Apparatus.—The broad line magnetic resonance spectrometer has been described elsewhere.[15] It consists of a self-detecting oscillator, a 30 c.p.s. phase sensitive amplifier and a strip chart recorder. The magnetic field is modulated at 30 c.p.s. with an amplitude a small fraction of the line width. The magnetic field is also swept linearly in time at an adjustable rate of about 3 gauss/min., while the frequency of the oscillator is constant. The system thus records the derivative of the magnetic resonance absorption as a function of applied magnetic field. A permanent magnet with a field strength of 6300 gauss was used in these experiments. The sample was 0.3 g. and was contained in a small, sealed test-tube. Measurements were made at liquid nitrogen temperature with a special cryostat, to improve signal to noise and also to ensure that motional narrowing[16] was unimportant.

Experimental Results and Discussion

The broadening of the magnetic resonance absorption is expressed quantitatively most conveniently by the second moment of the absorption line, *i.e.*, by the mean square deviation of the absorption intensity from the line center. For a crystal powder in which nuclear motions are unimportant, the second moment, ΔH_2^2, is given theoretically[17] by the equation

$$\Delta H_2^2 = \frac{6}{5}\frac{I(I+1)}{N} g^2\beta^2 \sum_{i>k} r_{ik}^{-6} + \frac{4}{15}\frac{\beta^2}{N}\sum_{i,f} I_f(I_f+1)\, g_f^2 r_{if}^{-6}$$

In this equation g is the g-value of the nuclear species observed, in this case the proton, and g_f is the value for other "foreign" magnetic species in the sample. I and I_f are the nuclear spins; r, the internuclear distances; and N is the number of protons considered in the calculation.

Because of the r^{-6} dependence of the broadening, the most important contributions arise from the dipolar interactions within the CHD–CH group. These intramolecular terms can be calculated quite accurately from the molecular structure.

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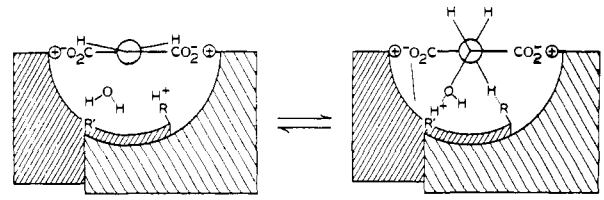

Fig. 1.—Schematic representations of the enzyme–fumarate and enzyme-L-malate complexes for the case of *cis* hydration (*cf.* Fig. 4 of the preceding article[4]).

The results for the *trans* and gauche configurations are summarized in Table I. In the calculations,

TABLE I

VALUES FOR THE SECOND MOMENT OF THE PROTON MAGNETIC RESONANCE CALCULATED FOR THE *trans* AND GAUCHE CONFIGURATIONS OF THE PROTONS IN DOOCCH(D)C(OD)HCOOD

	trans	gauche
CHD–CH: H–H	0.46 gauss^2	1.63 gauss^2
D–H	0.18	0.16
CH_2–CH: H–H	8.90	8.90
Intramolecular	1.02	2.14
Intermolecular	1.4 ± 0.4	1.8 ± 0.5
Effect of CO_2H and OH	−0.16 ± 0.03	−0.3 ± 0.05
Net ΔH_2^2 predicted	2.25 ± 0.5	3.65 ± 0.6

tetrahedral bond angles were assumed and the bond distances used were C–C,[8] 1.50 Å. and C–H, 1.09 Å. The D–H distance in the CHD group is sufficiently short that the resultant broadening of the proton resonance is significant, even though the magnetic moment of deuterium is small. For the same reason, the contribution to the proton second moment from the CH_2 group interactions cannot be neglected even though only 3 mole % of the molecules are CH_2–CH. In calculating this correction, it must be remembered that the relative contributions to ΔH_2^2 of protons in different molecular species depend upon the proton fraction rather than the mole fraction. Thus the contribution of the 3 mole % CH_2–CH is 0.045 × 8.9 gauss.^{2}

An accurate calculation of the other contributions to the broadening requires a knowledge of the crystal structure of malic acid, which does not appear to have been determined. However, an estimate sufficiently good for our purpose can be made by referring to the calculations made[9] for *trans* and gauche $CHCl_2CHCl_2$. The main additional contributions are from the proton–proton interactions between neighboring molecules. Consideration of the crystal structures of succinic and tartaric acids suggests that, as in the *sym*-tetrachloroethane, the intermolecular broadening will be greater for the gauche configuration than for the *trans*, but not by as large a factor. The molar volume of the *sym*-tetrachloroethane is 1.25 that of malic acid, so if all intermolecular distances were reduced proportionally, the intermolecular contribution to the second moment in malic acid would be $(1.25)^2 = 1.5$ times that in the tetrachloroethane. In the latter, the *trans* and gauche intermolecular contributions were calculated to be 0.7 ± 0.2 and 1.4 ± 0.3 gauss.^{2} Using the arguments just outlined, the values for the monodeutero-L-malic acid are estimated to be 1.4 ± 0.4 and 1.8 ± 0.5 gauss.^{2}

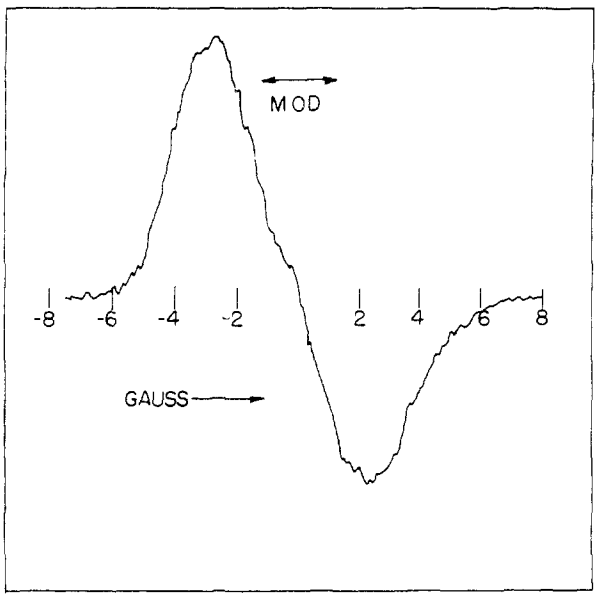

Fig. 2.—The first derivative of the proton magnetic resonance absorption in DOOCCH(D)C(OD)HCOOD at −196°, plotted as a function of applied magnetic field at a fixed frequency of 25.27 Mc. The arrow indicates the amplitude of the 30 c.p.s. modulation of the magnetic field.

The final correction is for the incomplete exchange of protons in the CO_2H and OH groups. These protons have no near magnetic neighbors and thus they have a much smaller second moment than protons in the CHD–CH group. A value of 0.3 ± 0.1 gauss^2 is estimated. About 5% of the protons in the CO_2H and OH groups were not exchanged. This reduces the net second moment by 7.5% of the difference between 0.3 ± 0.1 gauss^2 and the value computed assuming complete exchange. The various terms are summarized in Table I. The net values predicted for ΔH_2^2 are 2.25 ± 0.5 gauss^2 for the *trans* and 3.65 ± 0.6 gauss^2 for the gauche configuration.

A typical recording of the first derivative of the proton absorption in the monodeutero-L-malic acid sample at −196° is reproduced in Fig. 2. Numerical integration of this curve gave a value of 3.43 gauss^2 for one side and 3.63 gauss^2 for the other. A second curve gave values of 3.28 and 3.71 gauss.^{2} The resulting best experimental ΔH_2^2 is 3.5 ± 0.2 gauss.^{2} Line shapes plotted at room temperature had the same widths as those at low temperature so motional narrowing does not appear to be important. Comparison of the experimental value for the second moment with the calculated values shows conclusively that the protons are in the gauche configuration.

In addition, line shapes were plotted at −196° for a sample of $DOOCH_2CH(OD)COOD$. The second moment for this sample was 11.2 ± 0.6 gauss.^{2} A detailed comparison of this value with the intramolecular value of 8.9 gauss^2 computed for CH_2–CH confirms the estimates of the intermolecular contributions to the broadening. The experimental second moments were corrected for the modulation broadening[18] produced by the relatively large modulation used.

In the preceding article,[4] the deuterium labelling experiments and the effect of *p*H on the kinetics of the enzymatic hydration of fumarate indicated that the reaction occurs in the *cis* manner represented by the structures of the enzyme–fumarate and enzyme–L-malate complexes given in Fig. 1. The finding here that the protons in crystalline DOOCCH(D)C(OD)HCOOD are in the gauche configuration proves that the addition is *cis*. In view of this mechanism it is perhaps not surprising that *meso*-tartrate is a much more powerful competitive inhibitor of pig heart fumarase than *d*- or *l*-tartrate.[19]

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[CONTRIBUTION FROM THE MOORE LABORATORY OF CHEMISTRY, AMHERST COLLEGE]

Heats of Adsorption of Water Vapor on Bovine Serum Albumin[1]

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RECEIVED FEBRUARY 21, 1957

Heats of adsorption of water vapor on bovine serum albumin were measured calorimetrically at 20° and are presented together with the isotherms. The heat curves are discussed and critically compared with calculated values from the literature. Possible mechanisms for the adsorption process are considered, with special regard to the high initial heats and the appearance of a maximum in the heat-coverage curve near the B.E.T. value for a complete monolayer.

Introduction

The interaction of proteins and water is a subject of considerable interest to the biochemist and biologist. An extensive amount of data has been accumulated over the past two or three decades, the bulk of the systems studied having been aqueous solutions of various proteins. While this has yielded important information regarding the extent of hydration as well as the size and shape of the hydrated molecules,[3] solution techniques tend to be insensitive to non-uniformity in properties over various regions of the interacting polymer molecule. For this reason, a number of workers

(1) Supported by the Office of Naval Research under contract N8-onr-66902. This paper was presented before the Colloid Division of the 128th National Meeting of the American Chemical Society in Minneapolis, Minn., September, 1955.

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