Proton Magnetic Relaxation Studies of the Interaction of D-Xylose and Xylitol with D-Xylose Isomerase

CHARACTERIZATION OF METAL-ENZYME-SUBSTRATE INTERACTIONS*

(Received for publication, May 30, 1975)

J. MAITLAND YOUNG, KEITH J. SCHRAY,* and ALBERT S. MILYAVAN

From the Department of Chemistry, Bryn Mawr College, Bryn Mawr, Pennsylvania 19010, and The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

The interaction of D-xylose isomerase purified from two sources with Mn** and D-xylose or the competitive inhibitor xylitol has been examined by nuclear magnetic resonance. A greater paramagnetic effect of enzyme-bound Mn** on the a anomer of D-xylose than on the b anomer was observed, providing independent evidence for the specificity of D-xylose isomerase for the a anomic form of D-xylose. The exchange rate of a-D-xylose into the ternary complex, determined from the normalized paramagnetic contribution to the transverse relaxation rate (1/T2p) of the carbon 1 proton of a-D-xylose, exceeds Vmax for the enzymatic reaction by 3 orders of magnitude. The amount of xylitol necessary to displace a-D-xylose from the substrate-enzyme-Mn** complex is consistent with the Km value for a-D-xylose and the inhibitor constant Ki for xylitol previously determined by the methods of enzyme kinetics. These results suggest that the NMR experiments observe complexes of D-xylose isomerase which are kinetically and thermodynamically competent to participate in catalysis.

From the frequency dependence of the paramagnetic contribution to the longitudinal relaxation rate (1/T1p) of the carbon 1 proton of a-D-xylose, the correlation time (τc) which modulates the dipolar interaction between enzyme-bound Mn** and a-D-xylose has been determined (5.1 × 10-10 s). From these observations a range of calculated distances between enzyme-bound Mn** and the carbon 1 proton of a-D-xylose (9.1 ± 0.7 Å) has been found. The enzyme-bound Mn** has comparable effects on the carbon 1, carbon 2, and carbon 3 protons of a-D-xylose, suggesting that these protons of the enzyme-bound substrate are equidistant from the bound Mn**. A similar distance (9.4 ± 0.7 Å) between the enzyme-bound Mn** and the terminal methylene protons of xylitol, an analog of the open chain intermediate in the reaction, has been determined. The results of the present substrate relaxation and previous water relaxation studies suggest that two small ligands such as water molecules or a large portion of the protein intervene between the bound metal ion and the bound substrate in the active ternary complex.

D-Xylose isomerase (EC 5.3.1.5), an enzyme which catalyzes the aldose-ketose interconversion of D-xylose and D-xylulose, D-glucose and D-fructose, and D-ribose and D-ribulose, requires a divalent metal ion for activity1 (2). Manganese (Mn**) serves in this capacity for the xylose isomerases from Lactobacillus brevis (3, 4), and Streptomyces sp. (2), while magnesium (Mg***) serves only for the latter enzyme (2). The possibility of an enzyme-metal-substrate bridge complex has been suggested previously (5, 6) from magnetic resonance studies at a single frequency and from consideration of earlier kinetic data (3). The presence of substrates and inhibitors decreased the enhanced effect of the enzyme-Mn** complex on the relaxation rate of water protons (5). These observations are consistent with the replacement of water ligands on enzyme-bound Mn** and the formation of a bridge complex; alternative explanations have been presented, however (5).

Investigation of the effects of an enzyme-bound paramagnetic metal ion on the nuclear relaxation rates of substrates provides a direct and general method for examining enzyme-metal-substrate interactions (7, 8). Studies at more than one frequency permit the precise estimation of correlation times and metal-substrate distances (9, 10). In this paper the effects at two frequencies of the enzyme-Mn** complex on the protons of the substrate D-xylose and of xylitol, an analog of the open
chain intermediate in the reaction, are presented. The anomic specificity of d-xylose isomerase has been examined previously (4, 5, 11), and the results reported here provide further evidence for the specificity of this enzyme for the α anomeric form of d-xylose.

EXPERIMENTAL PROCEDURE

Materials—d-Xylose, β-NADH (Grade III), and trishydroxymethylaminomethane (Trizma base) were purchased from Sigma Chemicals. The 2-N-morpholinoethanesulfonic acid was a product of Calbiochem. Deuterium oxide, a product of General Dynamics, was vacuum-distilled prior to use. All other chemicals were reagent grade.

Arabitol dehydrogenase was a gift of Dr. I. A. Rose of this institute, and xylitol was kindly provided by Dr. T. Asakura of the Department of Pediatrics, University of Pennsylvania.

Buffer, substrate, and enzyme solutions for magnetic resonance experiments were freed of metal ion contaminants by elution through Chelex 100 resin (Bio-Rad).

Methods—d-Xylose isomerase from Streptomyces sp was prepared by the method of Takasaki et al. (2). The procedure of Yamanaka (3) was followed in the preparation of d-xylose isomerase from Laetobacillus brevis, as previously described (12). Enzyme con centrations were determined by measuring the absorbance at 280 nm and using the value for the extinction coefficient of 0.888 (mg/ml)-1 cm-1 and a molecular weight of 172,000 (5). The enzyme from Streptomyces was homogeneous as indicated by the fact that it gave a single band on acrylamide gel electrophoresis under non-denaturing and sodium dodecyl sulfate-denaturing conditions (13, 14). Estimates of molecular weight from gel electrophoresis and data from x-ray crystallographic studies (14) indicate that the Streptomyces enzyme is a tetramer (13, 14). The enzyme from L. brevis was homogeneous as determined by ultracentrifugation (12).

A coupled enzymatic assay for the Streptomyces enzyme was employed. Components were 0.016 M Tris-maleate buffer, pH 7.0, 1.0 mM MnCl2, 0.2 mM EDTA, 0.1 mM β-NADH, and excess arabitol dehydrogenase. Xylose isomerase was added (final concentration ~1 mg/ml), and the decrease in absorbance at 340 nm was followed as a function of time.

The specific activity of xylose isomerase from Streptomyces sp. measured after elution from Sephadex G-25 (Pharmacia) equilibrated with 0.05 M Mes, p H 6.0, was found to be 2.1 µmol/min at 25° using the coupled assay, while that from L. brevis deter mined by the colorimetric method of Yamanaka was 13 µmol/min at 35° (3). These specific activities compare well with those reported previously for the homogeneous enzymes (2, 3).

The longitudinal (1/T1) and transverse (1/T2) relaxation rates of the anomeric (C-1) protons of d-xylose have been measured by pulsed FT-NMR at 100 MHz and 220 MHz as previously described (15, 16), using the Varian XL-100 and HR-220 FT-NMR spectrometers equipped with nitrogen flow temperature controls. At 100 MHz the signal to noise ratio of the carbon 1 protons was improved by decoupling procedures, namely, irradiating at the HDO resonance frequency. This procedure was used in all FT experiments and at all MnCl2 concentrations, and improved the signal to noise ratio by decreasing the amplitude of the HDO resonance. Measurements of 1/T1 and 1/T2, made in our earlier experiments employed the power saturation and line-broadening methods as previously described (7, 8) and utilized the Varian HA-60, HA-100, and XL-100 spectrometers. Under comparable conditions, no significant differences in the relaxation rate determined by the pulsed or continuous wave methods have been detected. The pulse methods were, however, much more convenient and precise. During the NMR experiments, some of which lasted 10 hours, no more than 5% of the enzymatic activity was lost.

In control experiments designed to check for the presence of extraneous paramagnetic ions the 1/T1 of water protons of the enzyme solutions was determined at 8 MHz and 22° using an NMR Specialities PS 60W pulsed NMR spectrometer. Upon addition of EDTA no change in the longitudinal relaxation rate was observed, indicating the presence of negligible amounts of paramagnetic cations.

Reference:
1. A. Rose and E. L. O'Connell, unpublished data.
2. The abbreviations used are: Mes, 2(N-morpholino)ethanesulfonic acid; FT, Fourier transform; PRR, proton relaxation rates.
where \( l/T_1^*, \) and \( l/T_2^* \) are the relaxation rates of the paramagnetic and diamagnetic proton solutions, respectively. The values of the normalized relaxation rate \((1/T_{1p}),\) where \( f = [\text{Mn}^{2+}] / [\alpha-D-\text{xylose}] \), observed in these experiments are presented in Table I. From these data at varying enzyme concentrations a value of \( T_{1p} \) for the ternary xylose isomerase-Mn\(^{2+}\) complex is obtained by extrapolation of the data is justified (19). At infinite enzyme concentration the ternary complex will be the only species present in the absence of added paramagnetic ion: B, after addition of 33 \( \mu \)M Mn\(^{2+}\). Conditions: 50 mM Mes, measured pH 6.0 (99% D\(_2\)O), \( T = 31^\circ C \), 0.40 ml volume.

**Table I**

Longitudinal and transverse relaxation rates of \( \alpha-C_1 \) proton of \( \alpha-D-\text{xylose} \) in presence of \( \alpha-D-\text{xylose} \) isomerase (Streptomyces sp.) and Mn\(^{2+}\) at 31°

All values were obtained from pulsed Fourier transform NMR experiments (15). Enzyme solutions were prepared in 0.05 M Mes buffer, pH 6.0, lyophilized, and dissolved in D\(_2\)O. This procedure was repeated twice. \( \alpha-D-\text{xylose} \) solutions were prepared in a similar manner. Samples for NMR analysis were passed through Chelex resin (previously equilibrated with Mes buffer in D\(_2\)O) before use; aliquots of Mn\(^{2+}\) solutions were added subsequently. In Sample 5 the buffer concentration was 0.1 \( \mu \)M. As determined by power saturation and line width measurements the \( 1/T_1 \) and \( 1/T_2 \) values of the \( \alpha-C_1 \) protons of \( \alpha-D-\text{xylose} \) were \( 8.8 \pm 0.1 \) s\(^{-1}\) and \( 4.3 \pm 0.9 \) s\(^{-1}\), respectively, in the presence of xylose isomerase and \( 1.0 \pm 0.1 \) s\(^{-1}\) and \( 4.5 \pm 0.2 \) s\(^{-1}\) in the presence of 58 to 117 \( \mu \)M xylose isomerase. The corresponding values for the \( \beta-C_1 \) protons are \( 1.1 \pm 0.1 \) s\(^{-1}\) and \( 3.7 \pm 0.3 \) s\(^{-1}\) in the presence of xylose isomerase and 1.2 \pm 0.3 s\(^{-1}\) and 4.1 \pm 0.3 s\(^{-1}\) in the presence of 58 to 117 \( \mu \)M xylose isomerase.

In separate experiments, the longitudinal (\( 1/T_1 \)) and transverse (\( 1/T_2 \)) relaxation rates of the \( C_1 \) proton of \( \alpha-D-\text{xylose} \) have been examined by pulsed Fourier transform methods (9) in the presence of Mn\(^{2+}\) and both in the presence and absence of enzyme. The results indicate that the enzyme-bound Mn\(^{2+}\) is more effective than free Mn\(^{2+}\) in relaxing the \( C_1 \) proton of \( \alpha-D-\text{xylose} \). These studies were then extended using pulsed methods (Fig. 2) to a wide range of concentrations of xylose isomerase (Table I). Previous EPR studies (5) revealed tight binding of Mn\(^{2+}\) to the Streptomyces enzyme at 3.0 \±\, 0.1 sites characterized by \( K_B \) values (27 \pm\, 10 \( \mu \)M) which agreed with those obtained kinetically, as well as additional weak binding sites. By limiting our experimental conditions such that the total concentration of Mn\(^{2+}\) was \( \leq 0.5 \) times the enzyme concentration we could be certain that the concentration of Mn\(^{2+}\) bound at auxiliary sites (5) or free in solution would be negligible. Under these conditions the paramagnetic effect of enzyme-bound Mn\(^{2+}\) on the longitudinal relaxation rate of the \( C_1 \) proton of \( \alpha-D-\text{xylose} \) is given by:

\[
1/T_{1p} = 1/T_1^* - 1/T_1^p
\]

where \( 1/T_1^* \) and \( 1/T_1^p \) are the relaxation rates of the paramagnetic and diamagnetic proton solutions, respectively. The values of the normalized relaxation rate \((1/T_{1p}),\) where \( f = [\text{Mn}^{2+}] / [\alpha-D-\text{xylose}] \), observed in these experiments are presented in Table I. From these data at varying enzyme concentrations a value of \( T_{1p} \) for the ternary xylose isomerase-Mn\(^{2+}\) complex is obtained by extrapolation (Fig. 3) to infinite enzyme concentration using the procedure of Mildvan and Cohn (19). Since the concentration of free Mn\(^{2+}\) was negligible in these experiments, a linear extrapolation of the data is justified (19). At infinite enzyme concentration the ternary complex will be the only species contributing measurably to \( T_{1p} \) which under these conditions is equal to 4.45 \times 10^{-5} \text{ s}. The lack of a strong dependence of \( T_{1p} \) on enzyme concentration (Fig. 3) is consistent with the high affinity of the enzyme and the low affinity of \( \alpha-D-\text{xylose} \) for Mn\(^{2+}\) (5). The more limited data available on the enzyme from Lactobacillus brevis (Table II) indicate comparable paramagnetic effects on \( T_{1p} \) and a greater effect on \( T_{2p} \) of the bound substrate.

**Fig. 2.** Magnetic relaxation (100 MHz) of the \( \alpha-C_1 \) proton of \( \alpha-D-\text{xylose} \) (100 \( \mu \)M) in the presence of \( \alpha-D-\text{xylose} \) isomerase (XI) (37 \( \mu \)M). Measurement of \( T_{1p} \) by the partially relaxed Fourier transform method (15, 16). A, in the absence of added paramagnetic ion; B, after addition of 33 \( \mu \)M Mn\(^{2+}\). Conditions: 50 mM Mes, measured pH 6.0 (99% D\(_2\)O), \( T = 31^\circ C \), 0.40 ml volume.

**Fig. 3 (left).** Normalized paramagnetic contribution to the longitudinal relaxation time \((1/T_{1p})\) of the \( C_1 \) proton of \( \alpha-D-\text{xylose} \) is plotted against \( 1/[\text{xylose isomerase}] \). The data were fit by a least squares analysis and extrapolated to infinite enzyme concentration to provide the value of \( T_{1p} \) (4.45 \times 10^{-5} \text{ s}) for the xylose isomerase-Mn\(^{2+}\)-\( \alpha-D-\text{xylose} \) complex. Conditions were as described in Table I.

**Fig. 4 (right).** The effect of xylitol concentration on the line width of the \( \alpha-C_1 \) protons of \( \alpha-D-\text{xylose} \) in the presence of xylose isomerase (55 \( \mu \)M), Mn\(^{2+}\) (170 \( \mu \)M), and 50 mM K\(^+\)-Mes, measured pH 6.0 (99% D\(_2\)O), and \( T = 31^\circ C \).
frequency power at saturation indicating decreases in \(1/T_{1p}\) and \(1/T_{2p}\) respectively. The paramagnetic contribution to the longitudinal relaxation rate \(1/T_{1p}\) determined by pulsed methods also decreases upon addition of xylitol (Table I). These observations indicate that the competitive inhibitor has displaced the substrate from the ternary complex.

A titration of the effect of xylitol on the line width of the \(\alpha\)-CD proton resonance (Fig. 4) may be fitted by assuming simple competition between D-xylose (dissociation constant for the ternary complex, \(K_s = 5 \text{ mM}\)) and xylitol (\(K_s = 0.51 \text{ mM}\)). The \(K_s\) value for xylitol reported here is in reasonable agreement with the kinetically determined inhibitor constant (\(K_s = 1.5 \text{ mM}\), determined in maleate buffer) and with the \(K_s\) value obtained by PRR titrations (0.59 mM, determined in Mes buffer) (5). The \(K_s\) value for \(\alpha\)-D-xylose used to fit the titration data was equal to that obtained by PRR titrations and is in reasonable agreement with the \(K_w\) of the substrate (10 mM) (5). Thus, the dissociation constants of the ternary complexes of substrate or inhibitor determined by NMR agree with those found by kinetics and are consistent with both compounds binding to the active site of D-xylose isomerase. At saturating levels of xylitol (Figs. 1 and 4) at least 83% of the paramagnetic effects of the enzyme-bound Mn\(^{2+}\) on the relaxation rates of \(\alpha\)-D-xylose are removed, indicating negligible outer sphere contributions to the relaxation rates (20).

**Effect of D-xylose Isomerase-Mn\(^{2+}\) on Relaxation Rates of Protons of Xylitol**—The proton NMR spectrum of xylitol at 100 MHz includes a multiplet structure 4.1 ppm downfield from tetramethylsilane which can be assigned to the terminal \((\alpha, \beta)\) methylene protons. This assignment is based upon the chemical shift, the integrated area, and simplification from tetramethylsilane which can be assigned to the terminal \((\alpha, \beta)\) methylene protons. This assignment is based upon the chemical shift, the integrated area, and simplification of this signal using the shift reagent EuCl\(_3\) or by deuteration at \(C_2\) by the reduction of xylulose with NaBD\(_4\).

The longitudinal \(1/T_1\) and transverse \(1/T_2\) relaxation rates of the protons of xylitol have been examined by pulsed Fourier transform methods (9) in the presence of Mn\(^{2+}\) and by guest on March 22, 2020http://www.jbc.org/Downloaded from

### Table II

| Sample | \([\text{Enzyme}]\) | \([\text{Mn}^{2+}]\) | \(1/T_{1p}\) | \(1/T_{2p}\) | \(1/T_{1p}^*\) | \(1/T_{2p}^*\) |
|--------|----------------|----------------|-----------|-----------|-----------|-----------|
| \(\mu\text{M}\) | \(\text{mM}\) | \(\text{s}^{-1}\) | \(\text{s}^{-1}\) | \(\text{s}^{-1}\) |
| 1 | 0 | 29.1 | 0.045 | 100 | 0.28 | 620 |
| 2 | 39.1 | 4.7 | 0.028 | 345 | 1.01 | 13,900 |
| 3 | 38.0 | 29.1 | 0.025 | 655 | 6.55 | 14,500 |

*The normalization factor \(1/f\) equaled \([\alpha\text{-D-xylose}]^[\text{Mn}^{2+}]\).*

### Table III

| Sample | \([\text{Enzyme}]\) | \([\text{Xylitol}]\) | \([\text{Mn}^{2+}]\) | \(1/T_{1p}^*\) | \(1/T_{2p}^*\) |
|--------|----------------|----------------|----------------|-----------|-----------|
| \(\mu\text{M}\) | \(\text{mM}\) | \(\text{s}^{-1}\) | \(\text{s}^{-1}\) |
| 1 | 0 | 29.0 | 37-133 | 64 |
| 2 | 15^4 | 33.8 | 43 | 520 |
| 3 | 150 | 32.9 | 173 | 452 |
| 4 | 146 | 32.1 | 276 | 520 |
| 5 | 95 | 31.7 | 274 | 434 |
| 6 | 75 | 31.4 | 272 | 512 |
| 7 | 42 | 18-31 | 73-270 | 713 |
| 8 | 33 | 20-31 | 133-270 | 572 |
| 9 | 27 | 22.0 | 133 | 475 |

*Conditions and sample preparation as described in Table I.*

*The normalization factor \(1/f\) equaled \([\text{xylitol}]^[\text{Mn}^{2+}]\).*

*Determined from the width of the signal at half-height.*

*Based on pulsed F\(1\)-NMR measurements.*

### Table IV

| Complex | \(1/T_{1p}^*\) | \(1/T_{2p}^*\) | \(f(r_s)\) (frequency-independent) | \(f(r_s)\) (maximal frequency dependence) |
|---------|------------|------------|--------------------------------|--------------------------------|
| Xylose isomerase (146 \(\mu\text{M}\)) | 815 | 1.290 | 12.3 ± 2.4 | 13.6 ± 7.8 |
| Xylose isomerase (32 mm) | 370 | 1.400 | 14.7 | 5.8 |
| Mn\(^{2+}\) (276 \(\mu\text{M}\)) | 815 | 1.174 | 9.9 | 21.4 |

*Conditions and sample preparation as described in Table I.*

*The normalization factor \(1/f\) equaled \([\text{xylitol}]^[\text{Mn}^{2+}]\).*

*With the high affinity of the enzyme for Mn\(^{2+}\) and xylitol (5) and a low affinity of xylitol for Mn\(^{2+}\).*

**Determination of Correlation Time \(\tau_s\) which Modulates Dipolar Interaction between Enzyme-bound Mn\(^{2+}\) and \(\alpha\)-D-Xylose and Xylitol**—The paramagnetic contribution to the longitudinal (20) and transverse (21) relaxation rates for Mn\(^{2+}\)-ligand interactions are given by:

\[
1/T_{1p} = q(T_{1w} + \tau_w) + 1/T_{1x}.
\]

\[
1/T_{2p} = q(T_{2w} + \tau_w) + 1/T_{2x}.
\]

where \(q\) is the stoichiometry of bound ligand to the bound Mn\(^{2+}\), \(\tau_w\) is the residence time of the coordinated ligand, \(T_{1x}\) and \(T_{2x}\) are the relaxation times of the coordinated ligands, \(f = [\text{paramagnetic species}] / [\text{ligand}]\), and \(1/T_{1x}\) is the small outer sphere contribution to the relaxation rates. In the present case \(1/T_{1x}\) is negligible as determined by the effect of the enzyme-manganese-xylitol complex on the relaxation rates of D-xylose (Fig. 4). The observed frequency dependence of \(1/T_{1p}\) (Tables I and IV) and the finding that \(1/T_{2p}\) is significantly less than \(1/T_{2p}\) (Tables I and III) indicate that \(\tau_s < T_{1x}\), i.e., that values of the relaxation rates are not limited by ligand exchange (8,
22). Hence $1/T_{1p}$ is well approximated by $q/T_{1w}$. Since neither positive nor negative cooperativity was seen upon titration of the xylose isomerase-Mn$^{2+}$ complex with $\alpha$-xylose (5), we make the simplest assumption that the stoichiometry $q$ for bound $\alpha$-d-xylose is one per bound Mn$^{2+}$. Although alternative more complicated coordination schemes cannot be excluded, this assumption is supported by the water proton relaxation rates of the binary and ternary complexes (5).

The experimental values of $1/T_{1p}$ at 100 and 220 MHz (Tables I and IV) can be used to evaluate the correlation time, $\tau_c$, for the Mn$^{2+}$-proton interaction. In the limit of fast exchange the dipolar term of the Solomon-Bloembergen equation (10, 23, 24) is given by

$$\frac{1}{f(T_{1p})} = \frac{g}{T_{1w}} = g \left( \frac{C}{r} \right)^2 \cdot f(\tau_c) \quad (4)$$

where $r$ is the ion-proton internuclear distance and $C$ is a product of constants proportional to the spin state and average $g$ value of the metal ion. The correlation function, $f(\tau_c)$, is defined in Equation 5.

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_0^2 \tau_c^2} + \frac{7\tau_c}{1 + 3\omega_0^2 \tau_c^2} \quad (5)$$

The correlation function includes terms for the Larmor precession frequencies for nuclear ($\omega_n$) and electron ($\omega_e$) spins and the correlation time for dipolar interaction ($\tau_c$). For Mn$^{2+}$ and its complexes, $\omega_0^2 \tau_c^2 \gg 1 \gg 3\tau_c$ while $\omega_e^2 \tau_c^2 \leq 1$. Hence the second term or the right side of Equation 5 is negligible (7). From Equations 4 and 5 it can be seen that the experimental values of $1/T_{1p}$ at the two frequencies provide the ratio $f(\tau_c)$ at 100 MHz/T$_{1p}$ at 220 MHz and the correlation time $\tau_c$ can be calculated.

The value of $\tau_c$, $5.12 \times 10^{-10}$ s, for the enzyme-Mn$^{2+}$-a-D-xylose complex, was calculated assuming that $\tau_c$ is independent of frequency between 100 and 220 MHz and in close agreement with values reported for the paramagnetic effects of Mn$^{2+}$ on proton relaxation rates in enolase (9) and pyruvate kinase (22) complexes. Furthermore, this value of $\tau_c$ is characteristic of values reported for $\tau_c$ (the longitudinal electron spin relaxation time) of Mn$^{2+}$ (25). This is consistent with $\tau_c$ being the dominant term in $\tau_c$, as reported for several enzyme-Mn$^{2+}$ complexes (9, 26, 27). Alternatively, if a maximal dependence of $\tau_c$ on frequency between 100 and 220 MHz is assumed (28), a value of $\tau_c$ equal to $2.11 \times 10^{-10}$ s at 100 MHz is obtained. Analysis of Equations 4 and 5 shows that this range of values of $\tau_c$ results in only a ±7% uncertainty in calculated distances (see below).

For the xylose isomerase-Mn$^{2+}$-xylitol complex the value of $\tau_c$, $4.47 \times 10^{-10}$ s, was calculated assuming that $\tau_c$ is independent of frequency between 100 and 220 MHz. If maximal frequency dependence is assumed, the value calculated is $5.9 \pm 4.0 \times 10^{-10}$ s. The range of values of $f(\tau_c)$ for these extreme values of $\tau_c$ is significantly smaller (Table IV) and leads to an overall uncertainty in the distance of less than ±8% because of the sixth root relationship in Equation 4.

**Average Distance between Xylose Isomerase-bound Mn$^{2+}$ and Magnetic Nuclei of a-D-Xylose and Xylitol**—The average manganese to $\alpha$-C$_1$ proton distance in the xylose isomerase-Mn$^{2+}$-a-D-xylose complex may be calculated from Equations 4 and 5 (7, 8, 23, 24) with $C = 812$ Å s$^{-1/2}$ for Mn$^{2+}$-proton interactions (8). Assuming $q = 1$ and complete occupancy of enzymatic sites by $\alpha$-d-xylose, and employing values of $f(\tau_c)$ and $f(T_{1p})$ determined from the experimental data (Fig. 2, Tables I and V), the calculated manganese-$\alpha$-C$_1$ proton distance is 10.4 ± 0.7 Å. The assumption of complete occupancy by $\alpha$-d-xylose must be qualified, however. The isomerase-catalyzed reaction is at equilibrium in the NMR experiments, and the product d-xylulose will compete for binding to the enzyme. The close agreement observed for the values for the $K_a$ of d-xylose (10 mM) and the dissociation constant for an equilibrium mixture of ternary complexes (8.1 mM) (5) argues that d-xylulose and d-xylose bind with similar affinities. Furthermore, the $K_a$ values for d-glucose and d-fructose determined under other conditions are comparable. The anomeric specificity of the enzyme must also be considered. One conclusion from kinetic studies (4) was that any interaction of $\beta$-d-xylose with xylose isomerase must be very weak compared to that of the $\alpha$ anomer. On the other hand, $\alpha$-methyl-d-xyloside and $\beta$-methyl-d-xyloside were found by equilibrium measurements to bind to the enzyme-Mn$^{2+}$ complex with equally low affinities (6), which suggests that the $\beta$-d-xylose may bind in a nonproductive manner. Kinetic investigations (4) showed that the enzymatically formed anomer of the product d-xylulose predominates (81%) at equilibrium, although this anomer has not been identified.

Based on these considerations we can make corrections of the calculated distances. A minimal correction is required if only $\alpha$-d-xylose and the enzymatically active anomer of d-xylulose compete with equal affinity for binding. Of the total concentration of sugars present at equilibrium, 84% will be $\alpha$-xylose (29), and the enzymatically active form constitutes 33% of this (18). The enzymatically active anomer of d-xylose will be only 13% of the total concentration of sugar at equilibrium. Thus $\alpha$-d-xylose represents 68% of the total concentration of enzymatically active sugars and would occupy this fraction of the binding sites. The experimentally determined $f/T_{1p}$ would then be lower by a factor of 1/0.68 or 1.47 than would be found with complete occupancy by $\alpha$-d-xylose. However, because of the sixth root relationship of Equation 4 the 1.47-fold uncertainty in $f/T_{1p}$ leads to only a ±7% uncertainty in the distances, and the corrected Mn$^{2+}$-$\alpha$-C$_1$ proton distance is 9.8 Å. A maximal correction is obtained by proposing that all forms of d-xylose and d-xylose bind with equal affinities, but only $\alpha$-d-xylose protons reflect the paramagnetic relaxation effects of enzyme-bound Mn$^{2+}$. In this case $\alpha$-d-xylose constitutes 28% of the total sugar concentration at equilibrium and the corrected distance is 8.4 Å. From these considerations the overall range of corrected distances from enzyme-bound Mn$^{2+}$ to the C$_1$ proton of $\alpha$-d-xylose (9.1 ± 0.7 Å, Table V) is too great for direct coordination to the C$_1$-hydroxyl group of the $\alpha$-d-xylose sub-

| Table V | Correlation functions and distances calculated for ternary xylose isomerase-Mn$^{2+}$-ligand complexes |
|---------|---------------------------------------------------------------|
| Ligand  | $1/T_{1p}$ | $f(T_{1p})$ | $\tau_c$ |
|---------|-------------|-------------|----------|
| $\alpha$-d-xylose | 225 ± 44 | 6.2±13.9 | 9.1 ± 0.7$^*$ |
| Xylitol | 551 ± 116 | 5.9±21.4 | 9.4 ± 0.7 |

$^*$ Value at 100 MHz.
$^*$ Calculated from $T_{1p}$ at 100 MHz/$T_{1p}$ at 220 MHz assuming maximal frequency dependence of $\tau_c$.
$^*$ Calculated from $T_{1p}$ at 100 MHz/$T_{1p}$ at 220 MHz assuming no frequency dependence of $\tau_c$.
$^*$ Corrected for occupancy as discussed in text.

* R. A. Johnson, personal communication.
strate. Preliminary experiments using partially deuterated α-D-xylose to simplify the NMR spectrum suggest that the line-broadening and radio frequency powers required to saturate the C₆, C₅, and C₄ protons agree within experimental error indicating identical values of 1/ₜₑₓ for these 3 protons. Since ₛ and ṭₑₓ are the same for these protons the enzyme-bound Mn²⁺ ion must be equidistant from these 3 protons. Hence no portion of the α-D-xylose substrate is coordinated directly to the enzyme-bound Mn²⁺.

In the case of xylitol the average distance from the enzyme-bound manganese to terminal methylène protons in the xylose isomerase·Mn²⁺·xylitol complex may be calculated in a similar manner (Table V). Assuming ᵣₚ = 1 and complete occupancy of enzymatic sites by the inhibitor (ₚ = 0.51 mm (5)), and employing values of ₁/ₜₑₓ and ₁/ₜₑₓ summarized in Table IV, the calculated manganese-terminal protons distance is 9.4 ± 0.7 Å (Table V). Since only a single isomer of xylitol, an analog of the open chain intermediate, is present in solution no correction for occupancy is necessary. The calculated distance (9.4 ± 0.7 Å) overlaps with the corrected distance to the protons of α-D-xylose. The role of the Mn²⁺ ion in activating xylose isomerase thus appears to be structural rather than catalytic. Danno had reached similar conclusions from kinetic studies of the enzyme from Bacillus coagulans (30).

The range of calculated distances suggests that an enzyme·Mn²⁺·X·Y·α-D-xylose complex is formed in which two small ligands such as water molecules, or one portion of the protein intervenes between the bound metal ion and the bound substrate. The previously reported decrease in the relaxation rate of water protons when α-D-xylose or its analogs bind to the enzyme·Mn²⁺ complex (5) may reflect a decrease in ṭₑₓ due to a conformational change in the protein, or a decrease in ₛ due to the number of rapidly exchanging water ligands on Mn²⁺ due to occlusion of the active site. From the value of ṭₑₓ determined here, the previously determined value of ₁/ₜₑₓ of water protons (₁/ₜₑₓ = 1.12 × 10⁸ s⁻¹) (5) and from the Mn²⁺ to water proton distance of 2.87 ± 0.05 Å from x-ray data (26), it is estimated that 1.4 ± 0.3 fast exchanging water ligands remain coordinated to the enzyme-bound Mn²⁺ in the ternary α-D-xylose complex. Hence the results of the present and previous study (6) together suggest that the intervening ligands X and Y may be water molecules.

With the enzyme from L. brevis the comparable paramagnetic effect of the enzyme-bound Mn²⁺ on ₁/ₜₑₓ of the C-1 proton of α-D-xylose (Table II) suggests a similar structure of its ternary complex.

**Kinetic and Thermodynamic Properties of Ternary Complexes of Xylose Isomerase, Mn²⁺ and α-D-Xylose**—The kinetic and thermodynamic properties of the substrate complexes detected by NMR are summarized in Table VI. As discussed below, these observations are consistent with the participation of these complexes in catalysis. First, from Equations 2 and 3 it can be seen that the fastest relaxation rate sets a lower limit on the ligand exchange rate ₁/ₜₑₓ:

\[ ₁/ₜₑₓ \leq ₁/ₜₑₓ \leq ₁/ₜₑₓ \]  

(6)

The value of ₁/ₜₑₓ sets a lower limit on ₁/ₜₑₓ or ṭₑₓ, the rate constant for dissociation of the ternary complex in the following kinetic scheme:

α-D-Xylose + E-Mn²⁺ → E-Mn²⁺ + α-D-xylulose

α-D-Xylose·E-Mn²⁺ → E-Mn²⁺ + α-D-xylose

\[ k_{ₚₑₓ} \]

(7)

By using these rate constants and employing values of ₁/ₜₑₓ and ₁/ₜₑₓ summarized in Table IV, the calculated manganese-terminal protons distance is 9.4 ± 0.7 Å (Table V). Since only a single isomer of xylitol, an analog of the open chain intermediate, is present in solution no correction for occupancy is necessary. The calculated distance (9.4 ± 0.7 Å) overlaps with the corrected distance to the protons of α-D-xylose. The role of the Mn²⁺ ion in activating xylose isomerase thus appears to be structural rather than catalytic. Danno had reached similar conclusions from kinetic studies of the enzyme from Bacillus coagulans (30).

The range of calculated distances suggests that an enzyme·Mn²⁺·X·Y·α-D-xylose complex is formed in which two small ligands such as water molecules, or one portion of the protein intervenes between the bound metal ion and the bound substrate. The previously reported decrease in the relaxation rate of water protons when α-D-xylose or its analogs bind to the enzyme·Mn²⁺ complex (5) may reflect a decrease in ṭₑₓ due to a conformational change in the protein, or a decrease in ₛ due to the number of rapidly exchanging water ligands on Mn²⁺ due to occlusion of the active site. From the value of ṭₑₓ determined here, the previously determined value of ₁/ₜₑₓ of water protons (₁/ₜₑₓ = 1.12 × 10⁸ s⁻¹) (5) and from the Mn²⁺ to water proton distance of 2.87 ± 0.05 Å from x-ray data (26), it is estimated that 1.4 ± 0.3 fast exchanging water ligands remain coordinated to the enzyme-bound Mn²⁺ in the ternary α-D-xylose complex. Hence the results of the present and previous study (6) together suggest that the intervening ligands X and Y may be water molecules.

With the enzyme from L. brevis the comparable paramagnetic effect of the enzyme-bound Mn²⁺ on ₁/ₜₑₓ of the C-1 proton of α-D-xylose (Table II) suggests a similar structure of its ternary complex.

### REFERENCES

1. Natake, M. (1966) Agric. Biol. Chem. 30, 887
2. Takasaki, Y., Koshugi, Y., and Kanbayashi, A. (1969) Agric. Biol. Chem. 33, 1527-1534
3. Yamanaka, K. (1969) Biochim. Biophys. Acta 151, 670-680
4. Schrey, K. J., and Rose, J. A. (1971) Biochemistry 10, 1058-1062
5. Schrey, K. J., and Mildvan, A. S. (1972) J. Biol. Chem. 247, 2634-2637
6. Mildvan, A. S. (1973) Adv. Chem. Ser. 100, 390
7. Mildvan, A. S., and Cohn, M. (1970) Adv. Enzymol. Relat. Areas Mol. Biol. 33, 1-70
8. Mildvan, A. S., and Engle, J. L. (1972) Methods Enzymol 26, 654-682
9. Nowak, T., Mildvan, A. S., and Kenyon, G. L. (1973) Biochemistry 12, 1690-1701
10. Fung, C.-H., Mildvan, A. S., and Leigh, J. S., Jr. (1974) Biochemistry 13, 1160-1169
11. Schrey, K. J., and Deshpande, V., and Lybyer, M. D. (1975) Biochemistry 14, 1-70
12. Rose, J. A., O’Connell, E. L., and Mortlock, R. P. (1969) Biochim. Biophys. Acta 178, 376-379
13. Hogue, G., and Cohn, M. (1969) J. Biol. Chem. 250, 7814-7818
14. Berman, H. M., and Cohn, M. (1969) J. Biol. Chem. 249, 3983-3984
15. Shou, D. L., and Mildvan, A. S. (1974) Biochemistry 13, 1171-1178
16. McDonald, G. G., and Leigh, J. S., Jr. (1973) J. Magnetic Res. 9, 358-362
17. Lemieux, R. U., and Stevens, J. D. (1966) Can. J. Chem. 44, 249-262
18. Pigman, W., and Isbell, H. S. (1968) Adv. Carbohydr. Chem. 23, 11-57

---

**Table VI**

| Enzyme source | Kᵣᵃ⁺ | kₑₓ | kₑₓ⁺ | kₑₓ⁻ |
|---------------|-------|-----|------|------|
| Strentomyces  | 8.1   | 3.2 × 10⁵ | 4.0 × 10⁴ | 1.5 × 7.4 |
| Lactobacillus brevis | 2.2   | 1.4 × 10⁵ | 6.4 × 10⁴ | 4.6 |

*From Ref. 5.
*From the ratio kₑₓ/Kᵣᵃ⁺.
*In 16 mm Tris-maleate buffer as described under "Methods.”
*In 50 mm Mes buffer as previously described (5).
*Based on the kinetic data of Yamanaka (3) at 35°C and a molecular weight of 43,000 per active site as found for the Streptomyces enzyme (13, 14).
19. Mildvan, A. S., and Cohn, M. (1966) *J. Biol. Chem.* **241**, 1178-1193
20. Luz, Z., and Meiboom, S. (1964) *J. Chem. Phys.* **40**, 2686-2692
21. Swift, T. J., and Connick, R. E. (1962) *J. Chem. Phys.* **37**, 307-320
22. Nowak, T., and Mildvan, A. S. (1972) *Biochemistry* **11**, 2819-2828
23. Solomon, I. (1955) *Phys. Rev.* **99**, 559-565
24. Solomon, I., and Bloembergen, N. (1956) *J. Chem. Phys.* **25**, 261-266
25. Tinkham, M., Weinstein, R., and Kip, A. F. (1951) *Phys. Rev.* **84**, 848-849
26. Reuben, J., and Cohn, M. (1970) *J. Biol. Chem.* **245**, 6539-6546
27. Grisham, C. M., and Mildvan, A. S. (1974) *J. Biol. Chem.* **249**, 3187-3197
28. Fung, C. H., Mildvan, A. S., Allerhand, A., Komoroski, R., and Scrutton, M. C. (1973) *Biochemistry* **12**, 620-629
29. Stehn, M. W. (1962) *Methods Enzymol.* **5**, 347-350
30. Danno, G. (1971) *Agric. Biol. Chem.* **35**, 997-1006
Proton magnetic relaxation studies of the interaction of D-xylose and xylitol with D-xylose isomerase. Characterization of metal-enzyme-substrate interactions.
JM Young, KJ Schray and AS Mildvan

J. Biol. Chem. 1975, 250:9021-9027.

Access the most updated version of this article at http://www.jbc.org/content/250/23/9021

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/23/9021.full.html#ref-list-1