but topomerization of the D or L homomers still takes place quite rapidly through the low-energy transition state (Figure 1).

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Fractionation Factor for Hydrogen Isotopes at the Aqueous Ligand of Cobalt in Co(II)-Substituted Bovine Carbonic Anhydrase

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The accurate interpretation of the solvent deuterium isotope effect in the hydration of CO_2 catalyzed by carbonic anhydrase requires a knowledge of the fractionation factor of the reactant state of the enzyme's active site,[1] which is a species of water bound to the metal of carbonic anhydrase.[2] The NMR method of Gold[3] and Kresge and Allred[4] for the determination of reactant state fractionation factors is based on the chemical shift which results from the rapid exchange of protons between solute and solvent. When this exchange is sufficiently rapid, a single resonance is observed with chemical shift that is the weighted average of the chemical shift at the solute site and the solvent site in the absence of exchange, information from which a fractionation factor can be obtained. This method has not been applied to proteins because of the complexity of the proton NMR envelope and also because the concentration of protein required to give detectable changes in chemical shifts is so large as to exceed protein solubility or at least to introduce errors from solute–solute interactions. This report demonstrates that these difficulties can be overcome by modifying the method of Gold[3] and Kresge and Allred[4] to use the relaxation rate, $1/T_1$, of the exchanging ligands of a paramagnetic metal in a metalloenzyme, such as cobalt in Co(II)-substituted carbonic anhydrase. Previous studies of Fabry et al.[5] have determined that the proton relaxation of water in the presence of Co(II)-carbonic anhydrase occurs in the rapid exchange limit for which $1/T_1$ observed for water protons is a weighted average between solvent water and the species of water at the inner coordination sphere of cobalt in the enzyme's active site. Furthermore, the relaxation rate of protons in bulk solvent is near 0.3 s^{-1} and that of the species of water bound to the cobalt is near 6×10^4 s^{-1}.[5] This means that the effect of the cobalt-substituted enzyme on the observed $1/T_1$ occurs at concentrations of enzyme (10^{-4}–10^{-3} M) which are practicable. I report here that the fractionation factor is unity, within experimental error, for the species of water bound to the metal at the active site of Co(II)-substituted bovine, red-cell carbonic anhydrase.

Bovine, red-cell carbonic anhydrase, purchased from Sigma Chemical Co., was purified by the affinity chromatography procedure of Khalifah et al.[6] The apoenzyme was prepared by dialysis using dipicolinic acid[7] followed by thorough dialysis. The amount of apoenzyme present was estimated from the absorbance at 280 nm, and the cobalt-substituted enzyme was obtained by adding an equimolar amount of $CoSO_4$. The enzyme was dialyzed further for 3 days against five changes of a large volume of distilled water. Enzyme samples were prepared by using glassware rinsed in EDTA and adding lyophilized Co(II)-carbonic anhydrase to solutions containing 0.033 M of Na_2SO_4 and 50 mM of [tris(hydroxymethyl)amino]methane adjusted to pH(D) 8.2 with H_2SO_4 (D_2SO_4) and NaOH (NaOD). This value of pH(D) is the uncorrected pH meter reading. Experiments were performed at 23 ± 1 °C on samples in equilibrium with air using a Jeol Co. FX100 spectrometer at a resonance frequency of 100 MHz. Each relaxation time of water protons was determined by a three-parameter, nonlinear least-squares fit of 11 measurements of relaxation recovery after a 180–90° pulse sequence.

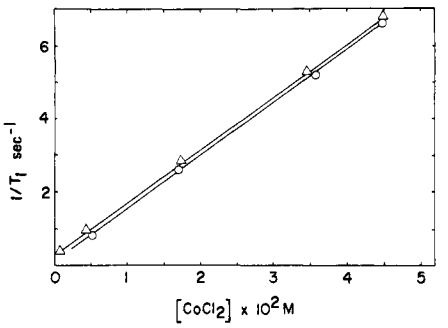

Figure 1. Proton magnetic relaxation rate $1/T_1$ at 100 MHz of solvent water as a function of concentration of $CoCl_2$ in aqueous solutions with atom fraction of deuterium in solvent $n = 0.10$ (△) and $n = 0.75$ (○). Samples were unbuffered solutions in equilibrium with air and measured at 23 ± 1 °C.

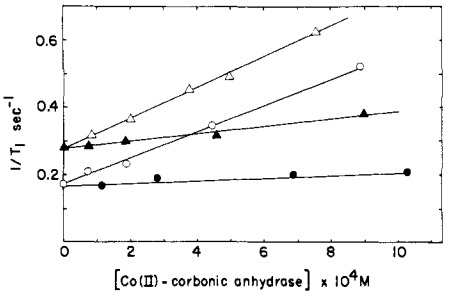

Figure 2. Proton magnetic relaxation rate $1/T_1$ at 100 MHz and 23 ± 1 °C of solvent water as a function of concentration of Co(II)-substituted, bovine, red-cell carbonic anhydrase. All solutions contained 0.033 M of Na_2SO_4 and 50 mM [tris(hydroxymethyl)amino]methane sulfate at pH(D) 8.2 and were in equilibrium with air. pH(D) is the uncorrected pH meter reading. Samples with $n = 0.10$ contained no methazolamide (△) and 4.9×10^{-3} M methazolamide (▲). Samples with $n = 0.75$ contained no methazolamide (○) and 4.9×10^{-3} M methazolamide (●).

Figure 1 demonstrates the linear dependence of the relaxation rate $1/T_1$ of solvent water protons on the concentration of hexaaquo, paramagnetic Co^{2+} ions, an effect which is interpreted in terms of an electron-nuclear dipole–dipole interaction.[8] For concentrations of $CoCl_2$ up to 0.045 M, this effect was the same in solutions with atom fraction of deuterium in solvent, n, of 0.10 and 0.75, based on the identical slopes in Figure 1. There was also a linear dependence of $1/T_1$ for water protons on the concentration up to 1 mM of cobalt(II)-carbonic anhydrase at pH(D) of 8.2 (Figure 2). These data show that this concentration dependence is nearly the same for two solvent compositions, $n = 0.10$ (least-squares slope in Figure 2 is 4.6×10^2 M^{-1} s^{-1}) and $n = 0.75$ (slope is 3.9×10^2 M^{-1} s^{-1}). That this effect is in large part due to the paramagnetic cobalt at the active site is demonstrated by the relaxation rates measured in the presence of 1.0 mg/mL (4.9×10^{-3} M) of methazolamide, a specific inhibitor of carbonic anhydrase ($K_I \sim 2 \times 10^{-8}$ M), the sulfonamide group of which binds to the metal at the active site displacing the species of water bound there.[2] This inhibition does not completely abolish the paramagnetic contribution of cobalt to the relaxation rate; the paramagnetic component of the relaxivity is reduced by about

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85% under conditions of complete inhibition by the sulfonamides acetazolamide and ethoxzolamide.[5,9] However, the component of the relaxation rate not inhibited by methazolamide also showed a linear dependence on enzyme concentration in the experiments reported here (Figure 2): for $n = 0.10$ the least-squares slope from Figure 2 is 1.1×10^2 M^{-1} s^{-1}; for $n = 0.75$, 46 M^{-1} s^{-1}. From the data in Figure 2, the difference in slopes $1/T_1$ between the uninhibited and inhibited Co(II)-carbonic anhydrase at $n = 0.10$ was $(3.5 \pm 0.3) \times 10^2$ M^{-1} s^{-1} and at $n = 0.75$ was $(3.4 \pm 0.3) \times 10^2$ M^{-1} s^{-1} (least-squares slope and 95% confidence limit). No correction has been made for the contribution of dissolved O_2 to relaxation rates. For these samples in equilibrium with air, the effect on $1/T_1$ of O_2 is about 0.1 s^{-1}, a sizable fraction of the observed rates. This and the small paramagnetic contribution to $1/T_1$ at proton resonance frequencies above 10 MHz compared to lower resonance frequencies[5,9] are disadvantages in these data.

The fractionation factor ϕ for the species of water bound to cobalt expresses the ratio of the preference of this site for deuterium over protium compared with the preference of a solvent site for deuterium over protium:[10]

$$\phi = \frac{[Co(OHD)]/[Co(OH_2)]}{[HOD]/[H_2O]}$$

Here it is assumed that the rule of the geometric mean holds.[11] Gold[3] and Kresge and Allred[4] have described an NMR method for determining fractionation factors based on the fact that when the chemical shift of a rapidly exchanging proton is collapsed into a single peak, its chemical shift is the weighted average, based on mole fraction, of the chemical shifts in the absence of exchange. This and the definition of the fractionation factor is sufficient to determine ϕ from NMR data (for a review see ref 10). The relaxation rate $1/T_1$ of a nucleus exchanging between two sites, in the fast exchange limit, is also a weighted average of the values of $1/T_1$ for each site in the absence of exchange.[12] Hence, the method of Gold[3] and Kresge and Allred[4] is directly applicable to the data of Figure 2. For two experiments using solvents of different deuterium content, the slopes of plots of $1/T_1$ vs. mole fraction of solute are related:

$$\frac{\text{slope 1}}{\text{slope 2}} = \frac{(1 - n + \phi n)_2}{(1 - n + \phi n)_1}$$

Application of this equation to the data in Figures 1 and 2 demonstrates that ϕ is unity for protons exchanging from the inner coordination sphere of cobalt in the aquometal complex, $Co(H_2O)_6^{2+}$, and in bovine Co(II)-carbonic anhydrase $\phi = 1.05 \pm 0.17$ for those protons which are displaced by methazolamide from the inner coordination sphere of cobalt.

A fractionation factor of unity for both the hexaaquo cobalt complex and the cobalt-substituted enzyme is consistent with an exchange of water between the inner coordination sphere of the metal and solvent at pH 8.2, although this experiment does not determine whether protons alone or the entire water molecule is exchanging (see, however, ref 12). The conclusion that the proton exchange observed by NMR occurs from water in the inner coordination site of cobalt in cobalt(II)-carbonic anhydrase was also reached by Fabry et al.[5] on the basis of kinetic arguments and the observed pH dependence of the relaxation rate of the exchanging protons. However, the determination in this report of a fractionation factor of unity is not in itself unambiguous evidence for the existence of a rapidly exchanging water bound to metal. Although the fractionation factor observed for hydroxide ion in aqueous solution is 0.47–0.56,[10] it is believed to arise from the hydrogens of solvating water molecules strongly hydrogen bonded to the hydroxide ion. The fractionation factor of hydrogen in the hydroxide ion itself is estimated to be close to unity.[13,14] Hence, the fractionation factor of unity determined in this study is also consistent with a hydroxide bound to cobalt at the active site which is not as extensively or as strongly solvated as hydroxide ion in solution. Although these fractionation factors cannot be used to distinguish between cobalt-bound water and hydroxide in cobalt(II)-carbonic anhydrase, it can be concluded that fractionation factors for nuclei exchanging rapidly from paramagnetic sites can be determined from nuclear magnetic relaxation rates.

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Total Synthesis and Stereochemical Identity of the $C_{18}H_{32}O_5$ Degradation Product from Boromycin

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Boromycin **1** is a unique boron-containing antibiotic[1] whose constitutional structure was determined by X-ray studies[2] and shown to be that of a novel macrodiolide containing a D-valine ester and a Böeseken complex of boric acid. Limited chemical degradation studies[2] on des-valine-des-boron-boromycin involving hydroxylation of the double bond followed by periodate oxidation gave acetaldehyde, an acid, $C_{13}H_{24}O_4$, and a neutral compound, $C_{18}H_{32}O_5$, which was assigned structure **2**, on the basis of its mode of formation. As part of our studies directed toward the total synthesis of boromycin[3] and the structurally related aplasmomycin,[4] we report herein a highly stereoselective, total synthesis of **2**, thereby unambiguously confirming its structure. Together with the glycolic acid unit, this constitutes the entire upper half of boromycin. We also describe methodology that allows the attachment of such a unit and hence the generation of a valuable intermediate toward our intended final goal.

Examination of the structure in question (Scheme I) reveals elements of carbohydrate-type symmetry[5] by virtue of the presence of the tetrahydrofuran and tetrahydropyran rings. Clearly, the major synthetic challenges in attaining this structure consist in devising practical routes to appropriately functionalized chiral precursors to the two ring subunits **3** and **4**, in elaborating suitable appendages, and in exploring methods for their union. Among several bond-forming possibilities, it was decided that the formation of the 9′–10′ bond (boromycin numbering), hence the creation of the neopentyl-type alcohol function, would constitute a viable protocol. Following extensive model studies it was also convened that the critical union of the two subunits would utilize a sulfoxide intermediate (**3**) (X = SOPh) and the aldehyde **4**.

Subunit 3. The chiral tetrahydrofuran unit **3** can be envisaged to arise by one of several possible routes formally leading to a

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