Hypersensitivity of an Enzyme Reaction to Solvent Water†,‡

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Received March 16, 1993; Revised Manuscript Received June 14, 1993®

ABSTRACT: The hydrolytic activity of calf intestinal adenosine deaminase is reduced sharply, but reversibly, in the presence of added methanol, ethanol, acetonitrile, or dioxane. This decrease in k_{cat}/K_m appears to be related to diminished water content in the presence of each of these cosolvents. No agreement between cosolvents is observed if enzyme activity is plotted as a function of viscosity or dielectric constant; nor do these cosolvents act as conventional reversible inhibitors. The K_m value of adenosine and the K_i values of a substrate analogue (6-dimethylaminopurine ribonucleoside) and a powerful competitive inhibitor (6-hydroxy-1,6-dihydropurine ribonucleoside) increase with decreasing solvent water content, but k_{cat} is unaffected. Values of $1/K_m$ and $1/K_i$ increase with roughly the 9th power of the concentration of water and show no sign of approaching a maximum value as the concentration of water approaches 55 M. These results are consistent with an equilibrium between an abundant, inactive, relatively dehydrated form of the enzyme and a rare, relatively hydrated form of the enzyme. Only the hydrated form of the enzyme, containing at least nine more water molecules than the dehydrated form, appears to be capable of binding substrates or competitive inhibitors. Possible physiological consequences of this behavior, in a tissue in which water is transported in large quantities, are considered.

Ordinarily, it is a simple matter to detect the formation of enzyme–substrate complexes, by examining the influence of substrate concentration on the rate of product formation. Information concerning the affinities of hydrolases and lyases for substrate water has been more difficult to obtain, because the concentration of substrate water cannot be treated as an isolated variable. Most proteins depend on solvent water for maintenance of their native structures, and the interpretation of results obtained in solvent mixtures is clouded by the fact that the composition of a mixture of water with another solvent cannot usually be changed without altering its viscosity and polarity. Nevertheless, the behavior of simple model compounds leaves little doubt that solvent interactions vary greatly in their strengths and directional preferences over the irregular surface of a protein. It would be of interest to know whether the active site of an enzyme constitutes an environment that is attractive, or unattractive, to the most common of all substrates in enzyme reactions, water itself.

In view of the experimental difficulties mentioned above, it is somewhat surprising to find that early experiments on glycosidases, in solvent mixtures containing ethanol or sucrose, showed rates of reaction that seemed to vary in simple porportion to the first power of the concentration of water over a considerable range (Nelson & Schubert, 1928). Haldane (1930) suggested that these results might imply the existence of a high K_m value for substrate water. Much later, enolase (Westhead & Malmström, 1955) and carbonic anhydrase (Pocker & Janjic, 1989) were found to show reversible losses of activity in the presence of organic cosolutes that could be understood in terms of changing viscosity or reductions in solvent water content.

This paper describes the effects of some cosolvents on the action of calf intestinal adenosine deaminase. Earlier, Kurz et al. (1987) showed that k_{cat}/K_m for adenosine varied in inverse proportion to the relative viscosity of solutions containing sucrose. Examining the enzyme's activity in mixtures containing methanol, ethanol, acetonitrile, and dioxane, we observed very different behavior, as described below.

MATERIALS AND METHODS

Calf intestinal adenosine deaminase was purchased from Boehringer Mannheim Corp. as a suspension in ammonium sulfate and was dialyzed against sodium phosphate buffer (10^{-4} M, pH 7.0, containing 1 mM 2-mercaptoethanol). Adenosine, nebularine, and 2-amino-6-chloropurine ribonucleoside were purchased from Sigma Chemical Co., and solvents of the highest purity available (HPLC grade) were purchased from Fisher Scientific Co.

Initial rates of deamination of adenosine were routinely determined in sodium phosphate buffer (0.05 M, pH 7.0), with ionic strength adjusted to 0.15 with NaCl, by monitoring the decrease in UV absorbance at 260 nm ($\Delta\epsilon_M = -7.8 \times 10^3$ (Beaven et al., 1955)) in cuvettes of 1-cm light path, thermostatted at 25 °C, using a Hewlett-Packard 8452A diode array spectrophotometer. If the initial absorbance at 260 nm exceeded 1.4, then a cuvette of shorter light path was used or the reaction was monitored at a higher wavelength where the total absorbance was lower. For measurements of the elevated K_i values of nebularine in solvent mixtures in which it absorbed strongly at 260 nm, it was more convenient to monitor the enzyme's activity using the alternate substrate 2-amino-6-chloropurine ribonucleoside, whose deamination to guanosine results in a decrease in absorbance at 308 nm ($\Delta\epsilon_m = -7.1 \times 10^3$). Rate constants were calculated using extinction coefficients for reactant and product measured independently in each of the solvent mixtures at the wavelength in question.

Changes in the UV absorption spectrum of nebularine, upon binding of this inhibitor by adenosine deaminase, were determined by first recording the spectrum of identical volumes of purine ribonucleoside and enzyme in otherwise identical solutions in different compartments of a tandem cuvette thermostatted at 25°. The solutions were then mixed, the spectrum was again recorded, and the earlier spectrum was subtracted to obtain a difference spectrum. Changes were

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† This work was supported by Grant No. GM-18325 from the National Institutes of Health.

‡ This paper is dedicated to Dr. John C. Parker, whose scientific work provided an inspiration for this investigation.

® Abstract published in *Advance ACS Abstracts,* August 15, 1993.

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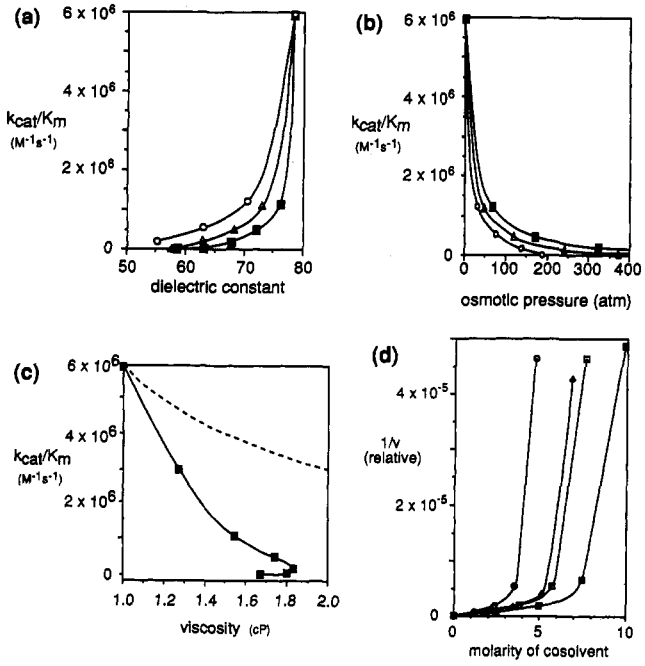

FIGURE 1: (a) Dependence of k_{cat}/K_m on the dielectric constant of assay solutions containing added methanol (■), dioxane (○), and ethanol (△). (b) Dependence of k_{cat}/K_m on osmotic pressure, adjusted by addition of methanol (■), dioxane (○), and ethanol (△). (c) Dependence of k_{cat}/K_m on the viscosity of assay solutions containing methanol. The dotted line shows the effect expected if the rate of reaction varied as a linear function of viscosity. (d) Inhibition of the deamination of adenosine (3×10^{-6} M) by methanol (■), dioxane (○), ethanol (△), and acetonitrile (□). Simple reversible inhibition would yield straight lines.

instantaneous and did not progress further during the period (typically 30 min) required to complete the experiment. Spectra of unmixed purine ribonucleoside or adenosine deaminase were found to change by less than 5% in passing from 0% to 20% methanol. High absorbances precluded using purine ribonucleoside concentrations greater than 2.4×10^{-4} M, and due to the large increase in K_i as the water content of the solvent was reduced, measurements of difference spectra were limited to solutions containing at least 80% water.

Viscosities of sucrose–water and methanol–water mixtures were measured at the temperature of each experiment, using calibrated Cannon-type viscometers purchased from Baxter Inc. Volumes of mixing were determined by comparing the measured density of each mixture with the density that would have been expected if there had been no volume of mixing, and in no case did they exceed 1% of the final volume. Osmotic pressures of methanol–water mixtures were calculated from values recorded for the freezing point depression (*CRC Handbook*, 1972). Dielectric constants of methanol–water mixtures were interpolated from the values collected by Akerlöf (1932). Dielectric constants of dioxane–water mixtures were interpolated from those recorded by Akerlöf and Short (1936). Osmotic pressures of dioxane–water mixtures were obtained from the tables of Scatchard et al. (1936) and Havorka et al. (1936).

RESULTS

The catalytic activity of adenosine deaminase was sharply reduced in the presence of added methanol, ethanol, dioxane, and acetonitrile. Losses in activity in these aqueous mixtures, amounting to more than 99.9% in some cases, were fully reversed when the concentration of organic solvent was reduced to less than 1% by dilution into water. Inactivation was reversible, so that, even in the most extreme cases, the activity

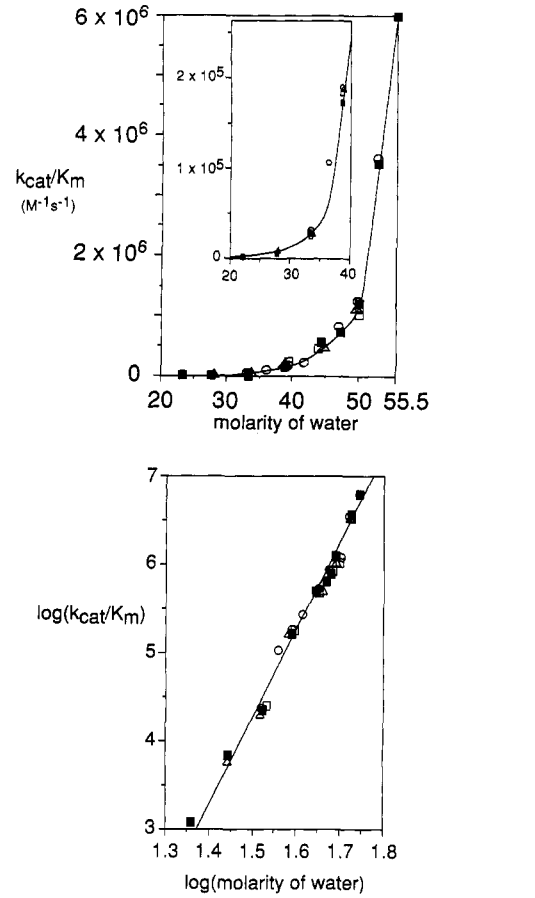

FIGURE 2: (a, top) Dependence of k_{cat}/K_m on the water content of solutions containing added methanol (■), dioxane (○), ethanol (△), and acetonitrile (□). The inset shows values on an expanded vertical scale. (b, bottom) Dependence of the logarithm of k_{cat}/K_M on the logarithm of solvent water content, in solutions containing methanol (■), dioxane (○), ethanol (△), and acetonitrile (□).

remaining could be measured easily by increasing the enzyme concentration.

When k_{cat}/K_m was plotted as a function of varying dielectric constant, osmotic pressure, or viscosity, differing results were obtained for each of the various cosolvents, as shown in panels a, b, and c of Figure 1. In a test for competitive or noncompetitive inhibition, $1/v$ was plotted as a function of the concentration of added cosolvent. Figure 1d shows that strong curvature was observed in each case, inconsistent with the behavior expected if these compounds acted as simple reversible inhibitors.

When k_{cat}/K_m was plotted as a function of water content, however, virtually identical curves were obtained in mixtures containing added methanol, ethanol, dioxane, or acetonitrile, as shown in Figure 2a. The inset of Figure 2a shows that this coinciding behavior was observed even in mixtures that produced extremely low reaction rates. In other experiments (not shown), when any two of these cosolvents were present in pairs, the observed activity was the same as the activity observed with only a single cosolvent was present, provided that water content was the same. A plot of the logarithm of k_{cat}/K_m, as a function of the logarithm of the water content, yielded a straight line with a slope of 9 (Figure 2b). Values of k_{cat}, obtained from double-reciprocal plots, and rate measurements at adenosine concentrations 5–10-fold in excess of K_m showed that this variation arose entirely from changes in K_m, whereas k_{cat} did not vary significantly as a function of changing water content (Figure 3).

To monitor an equilibrium, rather than a kinetic, property of the enzyme, the affinity of adenosine deaminase for the

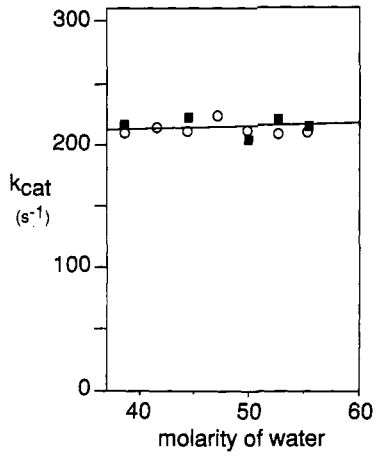

FIGURE 3: Effect of solvent water content on k_{cat} in mixtures containing methanol (■) and dioxane (○).

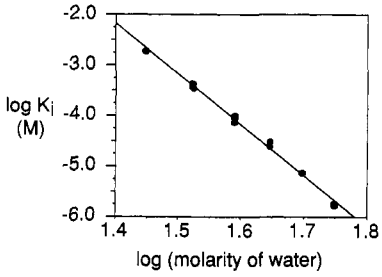

FIGURE 4: Dependence of the K_i value of nebularine on the concentration of water, in solutions containing methanol, using 2-amino-6-chloropurine ribonucleoside as the substrate (see text).

competitive inhibitor nebularine was examined as a function of solvent water content. Adenosine and nebularine absorb at similar wavelengths, and this coincidence, together with the sharp increase in K_m that occurred as water concentrations were decreased, limited the range over which K_i values of nebularine could be determined using adenosine. This limitation was circumvented by using the alternate substrate 2-amino-6-chloropurine ribonucleoside, whose deamination to guanosine results in a decrease in absorbance at 308 nm ($\Delta\epsilon_m = -7.1 \times 10^3$) comparable in magnitude with the decrease observed at 260 nm for deamination of adenosine ($\Delta\epsilon_m = -7.8 \times 10^3$). Separate experiments (not shown) indicated that the rate of dechlorination of this substrate, like the rate of deamination of adenosine, varied with roughly the 9th power of the concentration of water in methanol–water mixtures, at a substrate concentration (4×10^{-5} M) substantially below K_m (3×10^{-4} M in water, determined in separate experiments). Single assays at this relatively low substrate concentration were used to find the inhibitor concentration that reduced the rate of reaction by 50%, and this was taken as roughly equivalent to the K_i value. In this way, inhibition could be estimated in solvent mixtures with a water content as low as 50%.

Figure 4 shows that the logarithm of $1/K_i$ for nebularine, plotted as a function of the logarithm of water content, yielded a straight line with a slope of 10. Double-reciprocal plots yielded similar results, although the range of water concentrations that could be studied was limited by the increase in K_m and K_i values that occurred under these conditions.

In separate experiments, enzyme-bound nebularine, found earlier to be found entirely as the 1,6-hydrated species in water (Kurz & Frieden, 1987), showed the same changes in UV absorption spectrum in solutions of reduced water content, when sufficient nebularine was present to saturate the enzyme (Figure 5). Thus, although reduced water content resulted

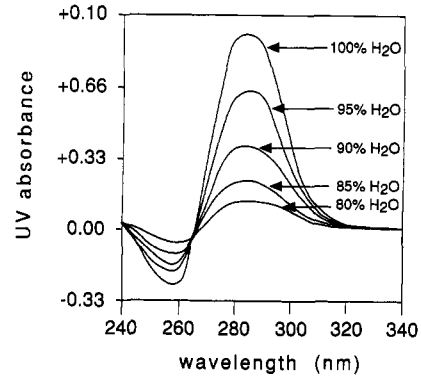

FIGURE 5: Change in the UV difference spectrum between bound and unbound nebularine (2.4×10^{-5} M) in the presence of adenosine deaminase (4.5×10^{-5} M), in solutions containing added methanol. The difference spectrum changed in proportion to the fraction of the enzyme that was inhibitor-bound, as estimated from the K_i values in Figure 4. The change in pure water was reproduced in 80% water, when the nebularine concentration was increased to 2.4×10^{-4} M, well in excess of K_i (data not shown).

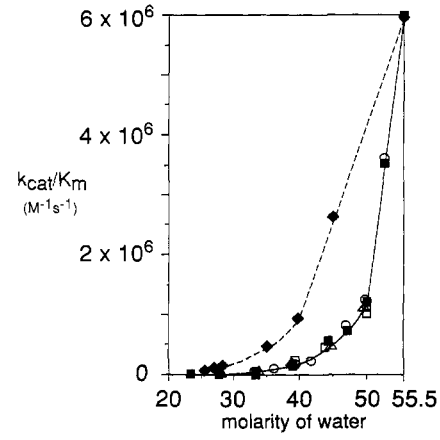

FIGURE 6: Comparison of the effect of water content on k_{cat}/K_m in the presence of sucrose (◆) with the greater effect observed in the presence of methanol (■), dioxane (○), ethanol (△), or acetonitrile (□).

in weaker binding of nebularine, all the nebularine that was bound appeared to be covalently hydrated in the enzyme–inhibitor complex.

In contrast with the results obtained in aqueous mixtures of methanol, ethanol, dioxane, and acetonitrile, sucrose was found to produce much smaller reductions in enzyme activity than the smaller cosolutes at the same water concentrations, as shown by the broken line in Figure 6. As demonstrated earlier by Kurz et al. (1987), K_m/k_{cat} varies linearly in sucrose–water mixtures as a function of relative viscosity, in the range from 1 to 3 cP. Extending their measurements, we found that this relationship was maintained to viscosities as high as 75 cP at 25 and 45 °C (data not shown).

DISCUSSION

In the present work, several cosolutes were found to interfere reversibly with the action of calf intestinal adenosine deaminase. Earlier, Kurz et al. (1987) had observed that in the presence of sucrose or ficoll, k_{cat}/K_m for adenosine varied in inverse proportion to the relative viscosity. We were able to duplicate their findings, extending the measurements to considerably higher viscosities. When we examined the enzyme's behavior in mixtures containing cosolutes of lower molecular weight, however, reductions in enzyme activity were much more pronounced. In the presence of methanol, ethanol, acetonitrile, or dioxane, enzyme activity was sharply, but

reversibly, reduced, and each of them reduced the rate of reaction to roughly the same extent if k_{cat}/K_m was plotted as a function of the water content of the solvent (Figure 2).

Is Water Content the Critical Variable? We were initially reluctant to suppose that water content might be the critical variable in determining the influence of these cosolvents on k_{cat}/K_m and considered changing viscosity, solvent polarity, or conventional inhibition as possible origins of the effects observed. However, it was noted that the various cosolvents produced similar effects at very different viscosities (Figure 1c). Moreover, the K_i value of nebularine, an equilibrium property, was affected in the same way as was the K_m value of adenosine, whereas viscosity changes might have been expected to have affected rates rather than equilibria. Futhermore, viscosity first increased and then decreased with increasing concentrations of methanol, whereas enzyme activity decreased continuously with increasing concentrations of methanol (Figure 1c). Thus, it seemed unlikely that viscosity alone was responsible for the observed effects. Next, we noted that methanol, dioxane, and acetonitrile produced the same reduction in reaction rate in mixtures whose dielectric constants differed markedly (Figure 1a), suggesting that solvent polarity was not the sole determinant of reaction rates. Third, the widely differing structures of these compounds did not suggest the existence of a common inhibitory binding site. Further, such a site might be expected to discriminate between methanol and ethanol on the basis of their differing polarities, but no such distinction was obvious. Finally, each of these cosolvents showed marked curvature in plots of $1/v$ as a function of cosolute concentration, in a manner not predicted for a conventional competitive or noncompetitive inhibitor (Figure 1d), so that reversible inhibition in the conventional sense did not seem to be occurring.

In contrast, panels a and b of Figure 2 show, first, that the reduction in water content required to reduce k_{cat}/K_m by 50% was the same for each cosolvent and, second, that the dependence of the reaction rate on the 9th order of the water content of the solvent mixtures was the same in each case. In other experiments, the same behavior was observed in binary and ternary solvent mixtures. These results do not, of course, rule out the possibility that some combination of the other variables, or some other variable that remains to be identified, could be at work. However, no one of these variables appears to fit all of these observations as well as does the water content.

Both the K_m values of adenosine and the K_i value of the competitive inhibitor nebularine appear to vary as a function of the concentration rather than the activity of water [or the osmotic pressure (Figure 1b), to which water activity is directly related]. Earlier, Westhead and Malmström (1955) observed that the rate of the enolase reaction appeared to vary with water content rather than water activity, following the same curve in the presence of three of the four cosolvents that they tested. More recently, Pocker and Janjic (1989) found that in glycerol–water mixtures k_{cat}/K_m of carbonic anhydrase varied in proportion to the square of water concentration rather than water activity. In chemical studies in mixed solvents, rates of the covalent hydration of dichloroacetone (Bell and Critchlow, 1971) and the hydrolysis of carboxylic acid anhydrides (Davis, 1983) were found to vary with water content, rather than with water activity. To explain the dependence of the rate of ketone hydration on water concentration rather than on water activity, Bell and Critchlow (1971) suggested that the activity *coefficients* of the critical water molecules did not change as the reaction progressed, leaving the concentration of water as the critical variable. Even in these reactions, simpler than those in which proteins participate, considerable uncertainty remains concerning the detailed physical description of the participating water molecules. Some limited speculation may, however, be warranted.

A Physical Model. One might naively expect that a ligand, such as a substrate or an inhibitor, would tend to displace water from an enzyme's active site. However, the sign of the water effect observed in the present experiments is inconsistent with that explanation. Instead, the slope of Figure 2b suggests that numerous water molecules (at least 9) are incorporated in the enzyme–substrate complex, and in the transition state for adenosine deamination, that were absent from the enzyme in the ground state. Since the number of water molecules involved in this process is substantial, and these water molecules must combine with the enzyme if substrates and competitive inhibitors are to be bound, it seems reasonable to infer that some or all of these water molecules do not occupy the position occupied by the substrate water.

From the finding that the enzyme's affinity for inhibitors, and its apparent affinity for substrate ($1/K_m$), increases with water concentrations up to 55 M, with no indication of saturation with water, it seems reasonable to conjecture that much of the native enzyme, well in excess of 50% even in pure water, may exist in the form of a "dehydrated" structure that must combine with numerous water molecules if the reaction is to proceed. According to this scenario, a major fraction of the enzyme begins the reaction in this dehydrated form (Scheme I).

Scheme I

$$E + S + 9W \rightleftharpoons ESW_9 \longrightarrow E + P$$

Somewhat similar inferences have been drawn from the effects of osmotically active agents on equilibrium properties of other proteins, which imply that 10 water molecules are bound during the 1e– reduction of cytochrome oxidase (Kornblatt & Hui Bon Hoa, 1990) and that 60 water molecules accompany the binding of oxygen by hemoglobin (Colombo et al., 1992). Both these proteins are oligomeric, so that subunit contacts may be involved in the effects observed. Subunit contacts can presumably be ruled out in the case of adenosine deaminase, which is monomeric in water and whose specific activity remained concentration-independent under all conditions that we examined.

The enzyme's equilibrium of hydration, to form ESW_9, seems unlikely to be extremely unfavorable, because k_{cat}/K_m (1.4×10^7 s^{-1} M^{-1}) (Hunt & Hoffee, 1982) falls within 2 orders of magnitude of the limits imposed by the expected rate of enzyme–substrate encounter in water. If the "hydrated" form of the enzyme that is required for substrate binding constituted less than 1% of the total enzyme present (assuming a diffusion limit in the neighborhood of 10^9 s^{-1} M^{-1}), then productive encounter would have been expected to constrain k_{cat}/K_m to values lower than 10^7 s^{-1} M^{-1}.

Possible Chemical Significance of the High K_m Value of Water. In the logarithmic plots of Figures 2b and 4, an optimal fit was observed if approximately 9 water molecules were assumed to be taken up as the substrate was bound. Evidently numerous water molecules participate, directly or indirectly, in the equilibrium process that determines the K_m value of adenosine and the K_i value of nebularine. One of these water molecules may be, but need not be, the actual molecule of water that serves as the substrate. For that reason, these data do not provide firm evidence concerning the active site's affinity (or lack of affinity!) for the actual molecule of water than serves as the substrate. Any binding site for substrate water might become saturated at concentrations lower than the

concentrations that are needed to saturate other water sites that must be saturated in order that nucleoside binding may occur.[1]

Regardless of the physical origin of these phenomena, or the identity of any site at which substrate water may be bound, it is of interest that the lines in Figure 2 pass through 55 M water with no sign of approaching saturation. Evidently the K_m value of water, operationally defined as the concentration that produces half-maximal velocity, is greater than 55 M. It is of interest to inquire why the K_m value of water should be so high. If an enzyme needed to be hydrated in order to function, and if catalytic efficiency had been of overriding importance in the evolution of its catalytic function, then natural selection might have been expected to result in an enzyme in hydrated form. Why should adenosine deaminase have arisen with such an apparent handicap? Zinc is believed to activate water for attack on adenosine at the enzyme's active site (Wilson et al., 1991), and this might seem to imply that the binding of substrate water should be tight.[1] In the absence of other constraints, however, the rate of an enzyme reaction is expected to be affected adversely by strong interactions with a substrate in the ground state, unless they are matched or exceeded in the transition state (Wolfenden, 1972). If the mechanism of action of adenosine deaminase involves ground-state destabilization, then weak binding of substrate water may be an advantage rather than a handicap.

Qualitatively similar relationships have been observed before, although the dependence on solvent water content was less extreme than in the present reaction. Yeast enolase acting on 2-phosphoglycerate, in the presence of dioxane, methanol, or ethanol, appeared not to be saturated with substrate water at 55 M, and more than one water molecule appeared to be involved (Westhead & Malmström, 1955). In the case of carbonic anhydrase, a plot of the logarithm of k_{cat}/K_m as a function of the logarithm of the concentration of water, in glycerol, sucrose, and ficoll solutions, suggested the involvement of two water molecules in the transition state, and there was no indication of saturation with water even at 55 M (Pocker & Janjic, 1989). In carbonic anhydrase, rates of ligand exchange also suggest that substrate water is bound weakly (Taylor et al., 1971; Tu et al., 1981).

Possible Physiological Significance of the High K_m Value of Water. The apparently high K_m value of water, and the remarkable sensitivity of substrate binding by this enzyme to changing water content, may arise from some physiological function of adenosine deaminase that remains to be recognized. An enzyme's reaction rate is maximally responsive to changing concentrations of substrate when the K_m value of that substrate exceeds its physiological concentration. For that reason, natural selection may have tended to favor the evolution of enzymes whose active sites are not saturated with substrate under ordinary conditions [for a discussion, see Cleland (1967)].

Water is so abundant that it might not appear to be among those substrates for which a response of this kind would confer any obvious benefit. However, animal cells regulate water content and volume with precision, at levels substantially lower than 55.5 M, by mechanisms that are not yet fully understood. Colclasure and Parker (1991) have suggested that the rates of certain enzyme reactions, if they were highly sensitive to water concentration, could provide a mechanism by which animal cells sense and regulate their own volumes. Adenosine deaminase is particularly abundant in intestinal mucosa, the source of the enzyme used in this study. Water is transported in large quantities, in both directions, across the intestinal epithelium. In addition, adenosine is a well-known vasodilating agent. These considerations suggest that it may be worthwhile to explore the possibility that adenosine deaminase serves as part of a water-sensing mechanism that may be involved in regulating water transport across the intestinal epithelium.

[1] In preliminary experiments on adenosine deaminase, using the "pulse–chase" procedure of Rose et al. (1974), the product inosine was not significantly enriched in ^{18}O when the enzyme was preincubated with a small volume of $H_2{}^{18}O$ and then mixed rapidly with adenosine in normal water under conditions favoring a single turnover (R. Wolfenden and G. Dzingeleski, unpublished results). This result suggests that substrate water is not "sticky", but tends to dissociate from the enzyme more rapidly than it goes on to form products, as has also been observed for two proteases (Angeles et al., 1992).

ACKNOWLEDGMENT

We are grateful to Drs. Robert A. Argenzio, Larry D. Byers, Kenneth R. Davis, Linda C. Kurz, and John C. Parker for valuable discussions during the preparation of the manuscript.

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