Enzyme−Substrate Complexes of Adenosine and Cytidine Deaminases: Absence of Accumulation of Water Adducts†

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ABSTRACT: Adenosine deaminase has been reported to bind the product inosine (the substrate for the reverse reaction) as inosine 1,6-hydrate, considered similar in structure to the transition state for adenosine deamination (Wilson & Quiocho, 1994). Accumulation on the enzyme of inosine 1,6-hydrate would be surprising, because this compound is an actual intermediate, probably approaching the transition state, in oxygen exchange between water and the C═O group of inosine, a reaction previously shown to be catalyzed by adenosine deaminase (Wolfenden & Kirsch, 1968). The equilibrium constant for conversion of ES to ES‡, in the oxygen exchange reaction, is less than 10^{-12}. To investigate the structure of enzyme-bound inosine in a different way, we labeled deoxyinosine with ^{13}C, expecting an upfield shift of 70−110 ppm if significant rehybridization to sp^3 had occurred at the carbonyl group. Instead, the results show a very small shift (∼1.3 ppm), indicating that C-6 of 2′-deoxyinosine retains its sp^2 hybridization after binding by calf intestinal adenosine deaminase. In a separate series of experiments, [4,5-^{13}C]-2′-deoxyuridine was synthesized and found to retain its sp^2 hybridization at C-4, after binding by *Escherichia coli* cytidine deaminase, an enzyme that catalyzes ^{18}O exchange from water into uridine. These findings are consistent with the general expectation, based on the unfavorable equilibrium of activation of enzyme-bound substrates, that enzymes should not accumulate appreciable concentrations of intermediates whose free energies approach that of the transition state in substrate transformation.

When enzymes bind substrates, their mutual affinity increases as they approach the transition state, lowering the activation barrier that limits the rate of reaction. Stages along the path toward the transition state are difficult to define, because the many available pathways across the free energy surface tend to be operationally indistinguishable (Hammett, 1970). However, in those numerous cases where k_{cat}/K_m is large enough to approach the diffusion limit, the frequency of successful enzyme−substrate encounter indicates that the enzyme acts by binding "ground state" forms of the substrate that are abundant in solution. Mechanisms that would require that an enzyme bind forms of a substrate that are not reasonably populous can be ruled out, because enzyme−substrate encounter would then be too infrequent to explain the second-order rate constants that are observed (Wolfenden, 1974).

In view of the forces at work in an enzyme's active site, the possibility remains that, after binding, an enzyme could accumulate observable amounts of a bound form of the substrate that differs significantly in structure from the form that is most abundant in solution. Some substrates, for example, have been shown to undergo appreciable changes in carbonyl stretching frequency or ^{13}C chemical shift upon binding by enzymes (for examples, see Belasco & Knowles, 1980; Kurz et al., 1992).

The present paper describes an investigation of the structures of substrates bound by adenosine and cytidine deaminases. These proficient catalysts of hydrolytic deamination are believed to act by stabilizing high-energy intermediates formed by covalent addition of water at the carbon atom at which displacement is to occur. The resulting intermediates decompose by elimination of ammonia (or a variety of other leaving groups), with the release of inosine or uridine, respectively (Scheme 1A).

When a hydrogen atom replaces the amino leaving group of the substrates adenosine or cytidine, as in purine ribonucleoside or pyrimidin-2-one ribonucleoside, elimination cannot occur. Instead, a water adduct accumulates as an inhibitor at the active site (Scheme 1B), as revealed by ^{13}C NMR and X-ray diffraction from single crystals of ADA[1] (Kurz & Frieden, 1987; Jones et al., 1989; Wilson et al., 1991) and CDA (Frick et al., 1989; Xiang et al., 1995). Correction for the very high energetic cost of hydration (8−10 kcal/mol in free energy) reveals that the rare covalent hydrates of purine ribonucleoside and pyrimidin-2-one ribonucleoside are extremely tightly bound by adenosine and cytidine deaminases, respectively, with affinities approaching the values that distinguish the altered substrate in the transition state from the substrate in the ground state (for a review, see Wolfenden & Kati, 1991).

Adenosine deaminase was recently reported to bind the reaction product inosine as its 1,6-hydrate (Scheme 1C), a *gem*-diol that appears to be even more closely analogous than those considered above, to highly activated intermediates in substrate deamination (Wilson & Quiocho, 1994). Indeed, the structure of the proposed *gem*-diol would bear an even closer resemblance to a tetrahedral intermediate in oxygen

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[1] Abbreviations: ADA, adenosine deaminase; CDA, cytidine deaminase.

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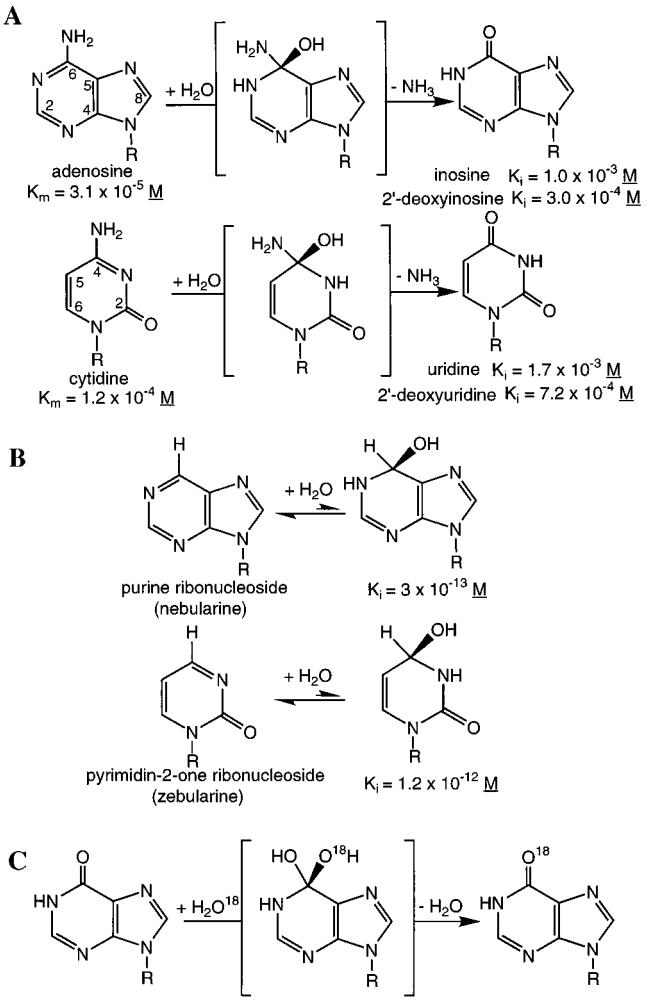
Scheme 1[a]



[a] (A) Proposed mechanism of action of adenosine and cytidine deaminases, involving direct water attack on adenosine or cytidine to generate a tetrahedral intermediate. K_i values of the nucleoside and deoxynucleoside reaction products, determined as competitive inhibitors in this study, are also listed [a considerably higher value of 1.6×10^{-4} M for inosine, reported earlier by Wolfenden (1969), appears to have arisen from a spectrophotometric error]. (B) Competitive inhibitors formed by covalent hydration of nebularine (for ADA) and zebularine (for CDA). (C) Proposed mechanism of exchange of water into inosine.

exchange from water into inosine, a reaction earlier found to be catalyzed by adenosine deaminase (Wolfenden & Kirsch, 1968). Similarly, cytidine deaminase has recently been shown to catalyze oxygen exchange from water into uridine (D. Carlow, personal communication).

Equilibria of substrate activation at enzyme active sites, although many orders of magnitude more favorable than equilibria of activation in free solution, remain extremely unfavorable in even the fastest enzyme reactions. In general, accumulation of an enzyme complex with any species closely resembling the transition state in free energy and in structure, in preference to forms resembling the substrate in the ground state, is therefore unexpected. Accordingly, inosine is not expected to accumulate as its 1,6-hydrate at the active site of adenosine deaminase.

For that reason, it seemed worthwhile to reexamine the structure of bound inosine using a different method. ^{13}C NMR spectroscopy has been useful in clarifying the behavior of carbonyl groups in other systems, such as the controversial interaction between trypsin and bovine pancreatic trypsin inhibitor. Early diffraction studies seemed to suggest that the bovine pancreatic trypsin inhibitor was bound by trypsin as a tetrahedral addition compound, analogous in structure to a tetrahedral intermediate in hydrolysis of the scissile bond in bovine pancreatic trypsin inhibitor (Rühlmann et al., 1973; Blow et al., 1974; Sweet et al., 1974). However, addition was shown to be highly unfavorable energetically (Guthrie, 1974), and NMR studies of ^{13}C-labeled peptides later established that no significant rehybridization had occurred at the supposed site of addition (Hunkapiller et al., 1979; Baillargeon et al., 1980; Richarz et al., 1980).[2]

A change at the scissile carbon atom from sp^2 to sp^3 hybridization is expected to result in a major upfield shift in the resonance of that carbon atom, as in earlier studies of the interaction of [6-^{13}C]purine ribonucleoside with adenosine deaminase (Kurz & Frieden, 1987; Jones et al., 1989). In the present work, we labeled 2′-deoxyinosine uniformly with ^{13}C in the heterocyclic ring, in order to observe the chemical shift of the critical carbon atom, before and after binding by calf intestinal adenosine deaminase. We also prepared [4,5-^{13}C]-2′-deoxyuridine, in order to determine whether C-4 of deoxyuridine retains its sp^2 hybridization after binding by *Escherichia coli* cytidine deaminase. 2′-Deoxynucleosides were chosen for this purpose, because they are somewhat more tightly bound than the corresponding ribonucleosides (See Results and the K_i values in Scheme 1A), favoring complex formation under the conditions of the NMR experiments.

EXPERIMENTAL PROCEDURES

Calf intestinal adenosine deaminase (EC 3.5.4.4) was purchased from Boehringer Mannheim Corp., in ammonium sulfate suspension: its specific activity after dialysis in potassium phosphate buffer (0.05 M, pH 7.0) was approximately 200 units/mg at 25 °C. *E. coli* cytidine deaminase was produced from the expression of *E. coli* SS6130 containing the wild-type *cdd* gene encoded plasmid whose plasmid-borne *cdd* gene is completely derepressed (Smith et al., 1994). The subcloning and expression of the *cdd* gene and the purification of cytidine deaminase have been described previously (Carlow et al., 1995). Nucleoside 2-deoxyribosyltransferase II (EC 2.4.2.6) was a gift from Dr. Steven Short (Glaxo Wellcome Co.) (Smar et al., 1991). Formamide-^{13}C (99%) was purchased from Isotec Inc., and [4,5-^{13}C]uracil (99%) was purchased from Cambridge Isotope Laboratories. 2′-Deoxyadenosine, inosine, and uridine were purchased from Sigma Chemical Co.

Uniformly labeled [^{13}C]adenine was prepared as described by Morita et al. (1968), using ^{13}C-labeled formamide as the starting material. The resulting crude ^{13}C-labeled adenine was purified by reverse-phase HPLC, using a Whatman Partisil 10 ODS-2 column. The product exhibited UV and ^1H and ^{13}C NMR spectra consistent with those observed for adenine at natural abundance (Breitmaier & Voelter, 1974; Chenon et al., 1975b). 2′-Deoxyadenosine, labeled uniformly with ^{13}C labeled on the heterocyclic ring, was synthesized

[2] Serine and cysteine proteases, as well as carboxypeptidases and aminopeptidases, are believed to stabilize tetrahedral intermediates in substrate hydrolysis, but not to such an extent that these intermediates actually accumulate. In contrast, inhibitory aldehydes, ketones, and boronic acids form relatively stable adducts with enzyme nucleophiles or with water at the active site. These compounds were designed to reduce or eliminate the thermodynamic hill that must be climbed when nucleophiles add to a peptide bond (for references, see Radzicka & Wolfenden, 1995).

Table 1: ^{13}C Chemical Shift Values for C-2, C-4, C-5, C-6, and C-8 of Deoxyinosine Alone in Solution and the Bound and Unbound Pairs by Adenosine Deaminase

	alone in solution		unbound		enzyme bound		
carbon	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	$\Delta\delta$[a] (ppm)
C-2	142.8	35.2 $J_{^{13}C-^{1}H}$	142.9	34.3 $J_{^{13}C-^{1}H}$	142.3	38.3 $J_{^{13}C-^{1}H}$	−0.6
C-4	145.1	62.2 $J_{^{13}C-^{13}C}$	145.2	61.2 $J_{^{13}C-^{13}C}$	149.5	62.8 $J_{^{13}C-^{13}C}$	+4.3
C-5	120.7	62.9 $J_{^{13}C-^{13}C}$	120.7	61.5 $J_{^{13}C-^{13}C}$	116.0	62.5 $J_{^{13}C-^{13}C}$	−4.7
		85.4 $J_{^{13}C-^{13}C}$		83.9 $J_{^{13}C-^{13}C}$		84.1 $J_{^{13}C-^{13}C}$	
C-6	155.5	85.5 $J_{^{13}C-^{13}C}$	155.5	83.6 $J_{^{13}C-^{13}C}$	154.2	84.3 $J_{^{13}C-^{13}C}$	−1.3
C-8	136.7	33.5 $J_{^{13}C-^{1}H}$	136.7	29.8 $JJ_{^{13}C-^{1}H}$	138.7	38.0 $J_{^{13}C-^{1}H}$	+2.0

[a] $\Delta\delta$ is δ of enzyme bound minus δ of unbound.

enzymatically from this material using nucleoside deoxyribosyltransferase II. 2′-Deoxycytidine (1 mM), as the deoxyribosyl donor, and ^{13}C-labeled adenine (3 mM), as the acceptor, were incubated overnight in 50 mM phosphate buffer, pH 6.0 at 35 °C, with 1 unit/mL transferase. After the reaction mixture had been deproteinized by ultrafiltration (Microcon 10, Amicon Corp.) at 14000*g* for 10 min, cytosine, 2′-deoxycytidine, adenine, and 2′-deoxyadenosine were separated by reverse-phase HPLC. The labeled 2′-deoxyadenosine was then converted enzymatically to 2′-deoxyinosine using adenosine deaminase. Cytosine and deoxycytidine were eluted with 100% water, followed by a linear gradient of methanol in which adenine was eluted at approximately 8% methanol, and 2′-deoxyadenosine was eluted at approximately 15% methanol.

[4,5-^{13}C]Deoxyuridine was synthesized by a similar procedure, using 2′-deoxyadenosine as the deoxyribosyl donor and [4,5-^{13}C]uracil as the acceptor. The mixture was separated by reverse-phase HPLC, and uracil and deoxyuridine were eluted with 100% water. The purified, labeled deoxyribonucleosides showed UV and ^{13}C NMR spectral characteristics identical with those of the authentic deoxyribonucleosides at natural abundance, and consistent with chemical shift assignments in the literature (Chenon et al., 1975a; Ellis et al., 1973).

Carbon-13 NMR spectra were observed at 125.77 MHz, using a Bruker AMX500 spectrometer equipped with 10 mm Bruker VSP broadband probe, at 22 °C. Chemical shifts (δ) are reported in ppm relative to [2-^{13}C]acetate (2 mM), used as an internal standard with its δ set at 20.0 ppm. The CDA/[4,5-^{13}C]-2′-deoxyuridine system was observed without ^{1}H decoupling, and the ADA/[2,4,5,6,8-^{13}C]-2′-deoxyinosine system was observed with the GARP sequence ^{1}H decoupling. For the ADA/[2,4,5,6,8-^{13}C]-2′-deoxyinosine system, ^{1}H decoupling was incomplete, and residual $^{13}C-^{1}H$ coupling remained. The buffer employed for both systems was potassium phosphate (50 mM, pH 7.0) in 20% D_2O (for internal lock). Data acquisition times varied from 90 min to 14 h, depending on the concentration of ^{13}C-enriched materials.

The specific activity of both ADA and CDA, measured after NMR experiments, was identical with that measured before NMR experiments. The activity of ADA was monitored by following the decrease in UV absorbance as adenosine was converted to inosine, at 260 nm ($\Delta\epsilon = -7800$ M^{-1} cm^{-1}), in potassium phosphate buffer (0.1 M, pH 7.0) at 25 °C. The specific activity of ADA used in the NMR experiment was 190 ± 15 units/mg. The activity of CDA was monitored by following the decrease in UV absorbance as cytidine was converted to uridine, at 280 nm ($\Delta\epsilon = -39\,000$ M^{-1} cm^{-1}), in potassium phosphate buffer (0.1 M, pH 7.0) at 25 °C. The specific activity of CDA used in the NMR experiment was 680 ± 25 units/mg. K_i values of inosine and deoxyinosine for ADA, and of uridine and deoxyuridine for CDA, were determined under similar conditions, at substrate concentrations equal to 0.1 and 0.2 times K_m, at several inhibitor concentrations.

Changes in the UV absorption spectrum of inosine and uridine, upon binding by ADA and CDA, respectively, were determined by first recording the spectrum of enzymes and inhibitors individually (1 mM each), using cuvettes of 0.1 mm light path (Wilmad Glass Inc.). Equal volumes of enzyme and inhibitor of identical concentration (2 mM each) were then mixed, the spectrum was again recorded, and spectra obtained earlier for the unmixed components were subtracted to obtain a difference spectrum. Spectra of inosine and uridine were also recorded with incremental addition of several organic solvents.

RESULTS

^{13}C NMR Spectra of [2,4,5,6,8-^{13}C]-2′-Deoxyinosine. The partially ^{1}H decoupled ^{13}C spectrum of [2,4,5,6,8-^{13}C]-2′-deoxyinosine (panel A of Figure 2 and Table 1) shows resonances at 120.7, 136.7, 142.8, 145.1, and 155.5 ppm, corresponding to C-5, C-8, C-2, C-4, and C-6, respectively. These assignments were based on the published ^{13}C chemical shifts for hypoxanthine (Breitmaier & Voelter, 1974; Chenon et al., 1975b) and inosine (Chenon et al., 1975a). Due to the uniform ^{13}C-labeling of the heterocyclic ring of deoxyinosine, $^{13}C-^{13}C$ spin−spin couplings are observed for C-4, C-5, and C-6 (panel A of Figure 2 and Table 1). Due to the incomplete ^{1}H decoupling, partial $^{13}C-^{1}H$ couplings are observed for C-2 and C-8.

^{13}C NMR Spectra of ADA−[2,4,5,6,8-^{13}C]-2′-Deoxyinosine Mixtures. NMR spectra of free ADA (1.05 mM), and of enzyme plus 1.2, 3.4, and 10.1 molar equiv of [2,4,5,6,8-^{13}C]-2′-deoxyinosine, are shown in Figure 1. 2′-Deoxyinosine is bound by the enzyme, as a competitive inhibitor with $K_i = 3 \times 10^{-4}$ M at pH 7.0, as shown in separate inhibition experiments. The ADA−deoxyinosine complex was found to be in slow exchange with free deoxyinosine, on the NMR time scale. Despite moderate line broadening, pairs of peaks corresponding to the bound and unbound forms of deoxyinosine are evident in each of the spectra shown in Figure 1, and in expanded form in Figure 2. At the highest concentration of deoxyinosine (1.02 mM ADA/10.3 mM deoxyinosine, panel D of Figure 1), all signals are reasonably distinct and sharp, allowing unambiguous assignments of the bound and unbound pairs of each of the five labeled carbons in [2,4,5,6,8-^{13}C]deoxyinosine. New peaks arising from the addition of deoxyinosine to ADA are confined to the region between ∼115 and ∼155 ppm, where signals from the free form of [2,4,5,6,8-^{13}C]deoxyinosine reside.

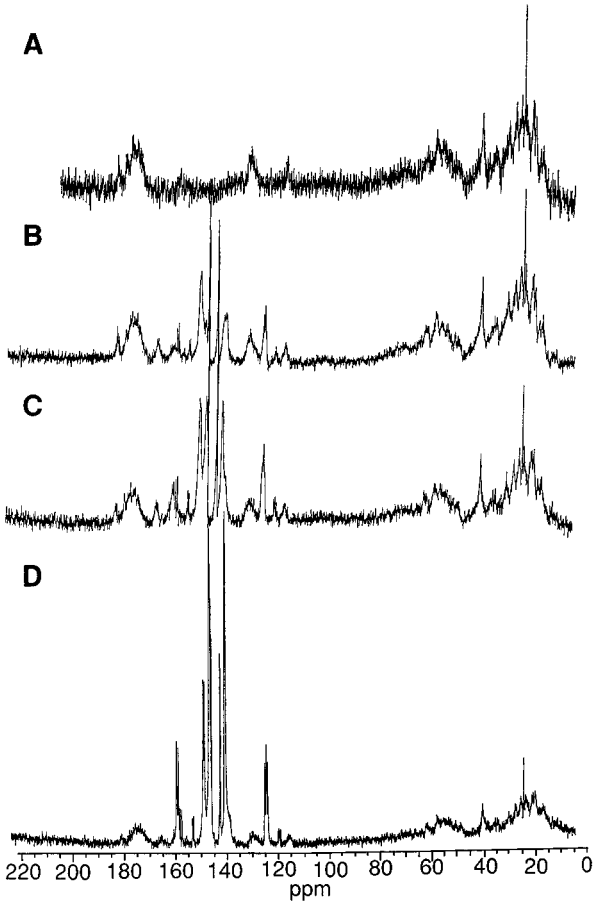


FIGURE 1: ^{13}C NMR spectra: (panel A) 1.05 mM ADA; (panel B) 1.05 mM ADA + 1.3 mM [2,4,5,6,8-^{13}C]deoxyinosine; (panel C) 1.04 mM ADA + 3.5 mM [2,4,5,6,8-^{13}C]deoxyinosine; (panel D) 1.02 mM ADA + 10.3 mM [2,4,5,6,8-^{13}C]deoxyinosine. All spectra were obtained with partial ^1H decoupling at 22 °C in the 10-mm probe of Bruker AMX500 spectrometer resonating at 125.77 MHz. Samples were prepared in 50 mM potassium phosphate buffer, pH 7.0 in 20% D_2O, with approximately 2 mM [2-^{13}C]sodium acetate as the chemical shift reference (δ = 20.0 ppm). Data acquisition times were 1, 5.5, 3.25, and 1.25 h for the spectra shown in panels A, B, C, and D, respectively.

Figure 2 compares the signals of [2,4,5,6,8-^{13}C]deoxyinosine in free form (panel A) and in solution with ADA with pairs of bound and unbound peaks (panel B). The chemical shifts and the $^{13}C-^{13}C$ coupling constants (Table 1) of the unbound form of deoxyinosine were in good agreement with those observed for free inosine. Peaks were assigned to the bound form of deoxyinosine based on their relative chemical shifts and, more convincingly, on the $^{13}C-^{13}C$ coupling constants. The $^{13}C-^1H$ coupling constants of C-2 and C-8 are subject to greater uncertainty, because partial ^1H decoupling was used, and because signals arising from the bound form overlapped signals from unbound inosine. However, the conclusions of this study are unaffected if these two assignments are reversed. Binding of [2,4,5,6,8-^{13}C]-deoxyinosine by ADA does not alter the chemical shifts of deoxyinosine appreciably. In particular, formation of the binary complex results in an upfield shift of only 1.3 ppm at the reaction center, C-6 (Table 1). More significantly, no new signal appears in the region 45−85 ppm expected for a change from sp^2 to sp^3 hybridization.[3]

^{13}C NMR Spectrum of Free [4,5-^{13}C]-2′-Deoxyuridine. The non-proton-decoupled ^{13}C spectrum of [4,5-^{13}C]-2′-deoxyuridine (panel A of Figure 3 and Table 2) shows signals at 97.8 and 164.3 ppm, corresponding to C-5 and C-4,

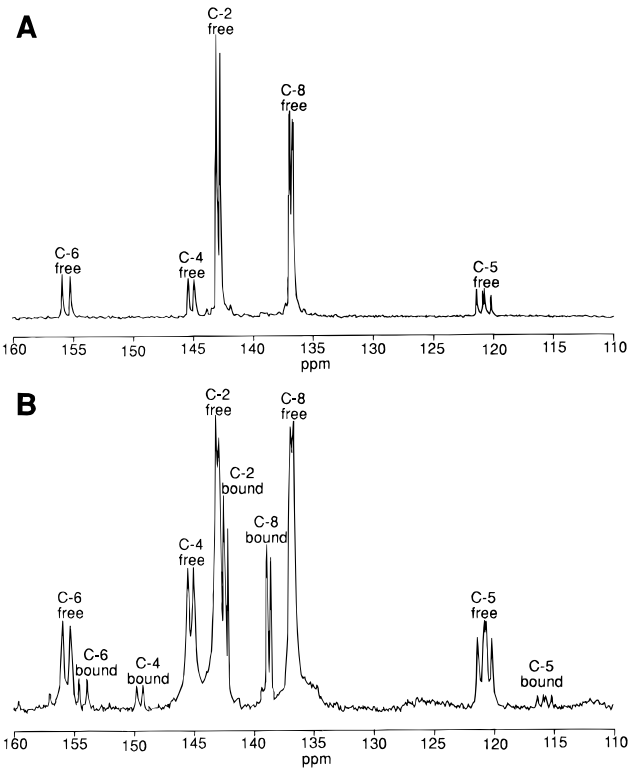


FIGURE 2: ^{13}C NMR spectra of [2,4,5,6,8-^{13}C]deoxyinosine alone (panel A); and in the presence of ADA (1.12 mM ADA/12.5 mM deoxyinosine), in the regions where the signals were observed (panel B, expanded version of the spectrum shown in panel D of Figure 1). Spectra were obtained under similar conditions as described in Figure 1 legend. Pairs of bound and unbound peaks for the binary system (panel B) were assigned based on the signals of deoxyinosine alone (panel A) and the coupling constants in Table 1.

respectively. These assignments were based on the ^{13}C chemical shifts reported for uracil by Ellis et al. (1973). $^{13}C-^{13}C$ coupling is observed between C-4 and the adjacent labeled carbons, and the ^1H attached to C-5 gives rise to $^{13}C-^1H$ splittings not only on C-5 but on C-4 as well (panel A of Figure 3 and Table 2). As a result, both peaks appear as a doublet of doublets, with differing coupling constants.

^{13}C NMR Spectrum of CDA−[4,5-^{13}C]-2′-Deoxyuridine Mixtures. The ^{13}C NMR spectrum of CDA alone (1.08 mM) and with 13.8 molar equiv of [4,5-^{13}C]deoxyuridine is shown in Figure 3, panels A and C, respectively. Separate inhibition experiments showed that 2′-deoxyuridine is bound as a competitive inhibitor, with $K_i = 7.3\ (\pm 1) \times 10^{-4}$ M at pH 7.0. As in the ADA−2′-deoxyinosine system, the CDA−2′-deoxyuridine complexes were found to be in slow exchange with free 2′-deoxyuridine on the NMR time scale. Insets a and b of panel C show the expanded spectral regions of [4,5-^{13}C]-2′-deoxyuridine in free and CDA-bound form,

[3] The ^{13}C resonance of the hydrated form of inosine is expected to move to higher field upon rehybridization. For example, the ^{13}C chemical shift of the hydrated form of nebularine yielded a 75 ppm upfield shift from that of nebularine (Kurz & Frieden, 1987). The ^{13}C resonance of the tetrahedral center of dimethoxymethane is 94.2 ppm (Stothers, 1972), whereas those of the corresponding sp^2 hybridized compounds, acetone and acetaldehyde, are 205.2 and 200.5 ppm, respectively (Levy et al., 1980). Thus, an upfield shift in the range of 70−110 ppm at the C-6 atom of inosine would be expected if rehybridization from sp^2 to sp^3 had occurred. The presence of Zn at the active site is not expected to modify the ^{13}C resonance of inosine very much. Observations on carboxypeptidase suggest that any perturbation by the Zn atom of the C-6 chemical shift is likely to be small compared with that involved in the rehybridization from sp^2 to sp^3 (Palmer et al., 1982).

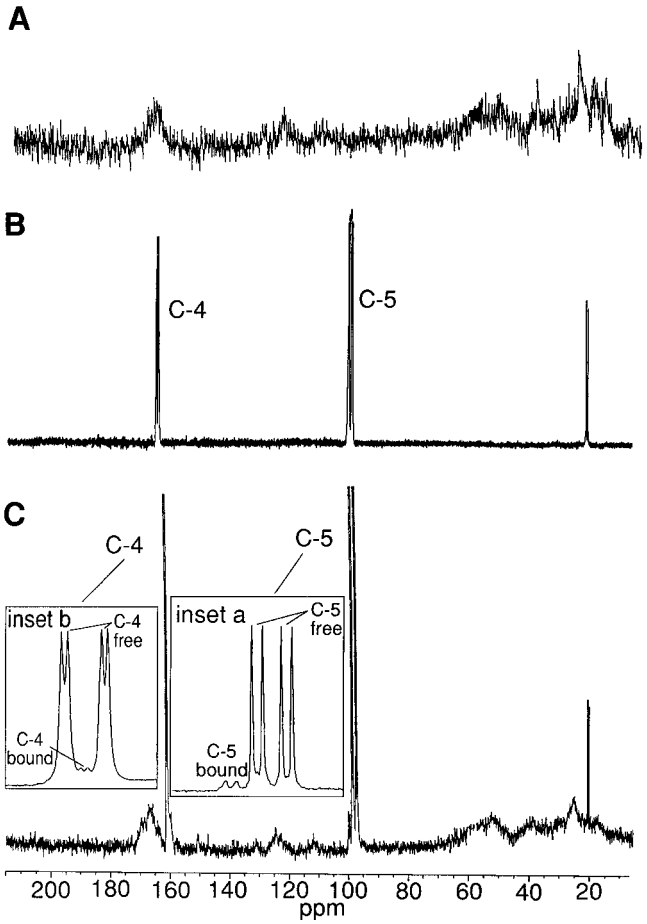


FIGURE 3: ^{13}C NMR spectra: (panel A) 1.08 mM CDA; (panel B) 5 mM [4,5-^{13}C]deoxyuridine; and (panel C) 1.02 mM CDA + 14.9 mM [4,5-^{13}C]deoxyuridine; data acquisition times were 1 h, 20 min, and 14 h, respectively. All spectra were obtained at 22 °C in the 10-mm probe of Bruker AMX500 spectrometer resonating at 125.77 MHz. Samples are prepared in 50 mM potassium phosphate, pH 7.0 in 20% D_2O, with approximately 2 mM [2-^{13}C]sodium acetate as the chemical shift reference (δ = 20.0 ppm). Insets a and b of panel C show expanded signals for C-5 and C-4, respectively, of [4,5-^{13}C]deoxyuridine.

Table 2: ^{13}C Chemical Shift Values for C-4 and C-5 of Deoxyuridine Free and Cytidine Deaminase Bound

	free in solution[a]		enzyme bound		$\Delta\delta$[b]
carbon	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	(ppm)
C-4	164.3	10.4 $J_{^{13}C\text{-}^{1}H}$	164.3 (centered at	11.0	0
		65.4 $J_{^{13}C\text{-}^{13}C}$	one set of doublets)	$J_{^{13}C\text{-}^{1}H}$	
C-5	97.8	65.4 $J_{^{13}C\text{-}^{13}C}$	99.8 (centered at	63.2	+2.0
		177.6 $J_{^{13}C\text{-}^{1}H}$	one set of doublets)	$J_{^{13}C\text{-}^{13}C}$	

[a] The chemical shifts and coupling constants for the unbound form of deoxyuridine in the deoxyuridine/CDA spectrum are identical to those of deoxyuridine free in solution. [b] $\Delta\delta$ is δ of enzyme bound minus δ of unbound.

respectively. Chemical shifts and coupling constants of the two labeled carbon atoms in the unbound form are virtually the same as those of the free form of [4,5-^{13}C]deoxyuridine. An additional doublet, associated with each of the two labeled carbons in the CDA−[4,5-^{13}C]-2′-deoxyuridine spectrum, appears with a coupling constant characteristic of the carbon atom with which it is associated (insets of panel C, Figure 3, and Table 2). These signals were therefore assigned to the CDA-bound form of [4,5-^{13}C]deoxyuridine. The other set of doublets for each carbon is obscured by overlapping signals from the unbound nucleoside. No other peaks were evident in the rest of the spectrum.[3,4]

DISCUSSION

Comparison with Crystallographic Results. There is a clear discrepancy between the results of the present NMR experiments with calf intestinal adenosine deaminase, indicating that deoxyinosine is bound intact, and the crystallographic results reported earlier for the human enzyme, interpreted as indicating that inosine is bound in covalently hydrated form (Wilson & Quiocho, 1994). The present experiments were conducted at pH 7, at which the enzyme is maximally active, and spectra at various stages of titration were gathered over a total interval of 11 h during which the enzyme retained full catalytic activity. In contrast, the crystallographic observations were performed at pH 4.2, where the enzyme exhibits roughly 25% of its maximal activity (Sharff et al., 1992). Our attempts to repeat the NMR experiments at lower pH, under conditions more closely comparable with those used in crystallization, were frustrated by loss of activity and precipitation of the calf intestinal enzyme at the high concentrations required for NMR experiments.

Unlike adenosine deaminase, bacterial cytidine deaminase yields useful crystals under the same conditions as those used in our titration with deoxyuridine, in which the enzyme is also maximally active. In agreement with the present NMR results, the crystal structure of the uridine−CDA complex indicates that uridine retains its planar character, with O-4 of uridine remaining in the plane of the ring (Xiang and Carter, manuscript in preparation).

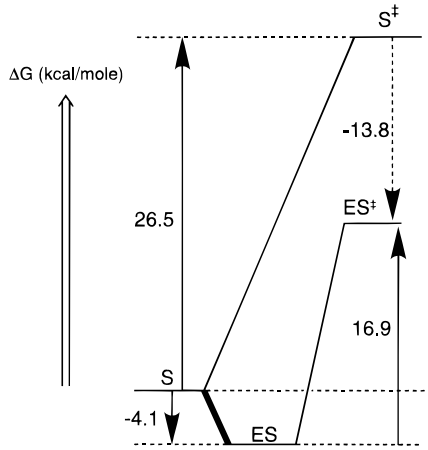
Is a Hydrated Intermediate Expected To Accumulate? The reasons for the discrepancy between the present NMR findings, indicating that 2′-deoxyinosine and 2′-deoxyuridine are bound unaltered, and those of earlier crystallographic results, suggesting that inosine is bound as the 1,6-hydrated species, are unclear. However, it is of general interest to consider whether enzymes are likely to accumulate substrates in highly activated forms, and of specific interest to consider the expected stability of inosine 1,6-hydrate.

It might seem natural to suppose that, if adenosine deaminase binds the competitive inhibitor purine ribonucleoside as its 1,6-hydrate, then the enzyme might bind the reaction product inosine in the same way. There appear to be more fundamental reasons, however, for expecting that such an intermediate should not accumulate in detectable amounts on adenosine deaminase, if its catalytic properties are to resemble those of a real enzyme. Let us first estimate the value of the equilibrium constant for substrate activation at the enzyme's active site, and then consider how closely inosine 1,6-hydrate approaches in free energy the $S^{\ddagger}$ for substrate transformation.

Equilibrium Constant for Substrate Activation at the Enzyme's Active Site. When any enzyme's turnover number is divided by kT/h, it becomes evident that the equilibrium constant for conversion of the ground state complex (ES) to the transition state complex ($ES^{\ddagger}$) remains extremely unfavorable at the enzyme's active site, even though it is much more favorable than the equilibrium constant for substrate activation in free solution. Scheme 2 illustrates the situation

[4] We observed enzyme-induced UV spectral shifts to higher wavelength in complexes of ADA with inosine, and of CDA with uridine, but similar perturbations in the UV spectrum were also observed in the presence of 1,4 dioxane. Thus, it appears likely that these perturbations in UV spectrum arise from changes in physical environment, rather than from chemical changes.

Scheme 2[a]



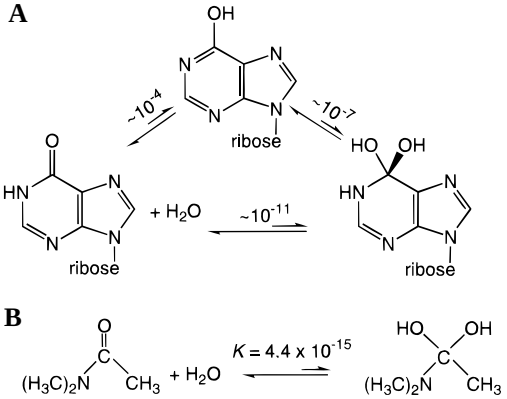
[a] The likelihood of accumulation on adenosine deaminase of a water adduct of inosine can be considered using a free energy diagram depicting the energetics of nonenzymatic conversion from S to S‡ compared with that from ES to ES‡ at 25 °C, assuming that inosine 1,6-hydrate is analogous in structure to the transition state ES‡, as assumed by Wilson and Quiocho (1994). In this diagram, S is inosine, S‡ is the transition state for nonenzymatic ^{18}O exchange into inosine, ES is the enzyme−inosine complex in the ground state, and ES‡ is the enzyme−inosine complex in the transition state (see text). The experimental values (solid arrows) suggest that the altered substrate in the transition state is bound (broken arrow) with a K_d value of 7.6×10^{-11} M ($\Delta G_{binding} = -13.8$ kcal/mol).

for oxygen exchange into inosine (Wolfenden & Kirsch, 1968).[5] The equilibrium constant for conversion of the enzyme−substrate complex in the ground state, to the enzyme complex with the altered substrate in the transition state, can be estimated by dividing the turnover number (~2.6 s^{-1}) by kT/h, yielding $K^{\ddagger} = 4.2 \times 10^{-13}$, equivalent to +16.9 kcal/mol in free energy. Thus, on the enzyme, the equilibrium of conversion of a ground state-like species to any transition state-like species is expected to be extremely unfavorable.

For the nonenzymatic reaction ($k = 2 \times 10^{-7}$ s^{-1}), the equilibrium constant for activation is 3.2×10^{-20}, corresponding to a free energy of activation of +26.5 kcal/mol. From these values, and the measured K_i value of inosine (1.0×10^{-3} M, $\Delta G_{binding} = -4.1$ kcal/mol), it can be estimated that the altered substrate in the transition state is bound with a K_d value of no greater than 7.6×10^{-11} M ($\Delta G_{binding} = -13.8$ kcal/mol).

Equilibrium Constant for 1,6-Hydration of Inosine. How unstable is the covalent hydrate of inosine in solution, and how closely does it approach the transition state for oxygen exchange from water into inosine? Although inosine 1,6-hydrate is much too unstable to accumulate in detectable amounts in aqueous solution, an indication of its thermodynamic stability in neutral solution can be obtained from known values of equilibrium constants for related reactions. Scheme 3 outlines an approach to this question, in which the reaction is divided into two stages: (1) tautomerization of inosine to its enolic form, followed by (2) addition of water to the enol tautomer to yield inosine 1,6-hydrate. The product of the equilibrium constants for these two processes should be thermodynamically equivalent to the equilibrium constant for direct addition of water to the common, keto, tautomer of inosine.

Scheme 3[a]



[a] (A) The equilibrium constant for 1,6-hydration of inosine can be estimated from the product of equilibrium constants for keto−enol isomerization of inosine (~10^{-4}; Medeiros & Thomas, 1971; Psoda & Shugar, 1971; Chenon et al., 1975b; Elguero et al., 1976) and for 1,6-hydration of nebularine [1.1×10^{-7}, taking the activity of pure water as unity (Jones et al., 1989)]. (B) Equilibrium of hydration of *N*,*N*-dimethylacetamide (values of Guthrie, 1974).

(1) In neutral aqueous solution, inosine exists almost entirely as its 6-keto tautomer, and the enolic tautomer is so rare that it has escaped detection by the most sensitive spectroscopic methods (Medeiros & Thomas, 1971; Psoda & Shugar, 1971; Chenon et al., 1975b). Thus, the equilibrium constant for conversion of the 6-keto to the 6-hydroxy tautomer of inosine is extremely unfavorable, as in the case of other rare tautomers of the nucleic acid bases, and of pyridin-2-one (for discussion, see Elguero et al., 1976), and seems likely to be in the neighborhood of 10^{-4} or less.[6]

(2) The equilibrium constant for 1,6-addition of water to unsubstituted purine ribonucleoside, obtained by the pseudobase method, is 1.1×10^{-7} (Jones et al., 1989). A hydroxyl

[5] Even in much more rapid enzyme reactions, equilibria of activation at the active site remain extremely unfavorable. For example, k_{cat} attains a value of 4×10^7 s^{-1} when catalase acts on H_2O_2 (Ogura, 1955), but $K^{\ddagger}$ (the equilibrium constant for conversion of the enzyme−substrate complex in the ground state, to the enzyme complex with the altered substrate in the transition state) remains less than 10^{-5}. If equilibria of activation were not so unfavorable, then highly activated intermediates approaching the transition state might accumulate at significant concentrations in equilibrium with the enzyme−substrate complex in the ground state. However, these complexes would decompose at rates approaching kT/h, rendering their structural determination problematic.

[6] The equilibrium constant for converting the keto form of inosine to the enol form has not been established, but the concentration of the enol is too low to be observable in water. 2-Pyridone has an equilibrium constant for enolization of ca. 10^{-4} in water, but in the vapor phase, its value approaches unity (Beak et al., 1976). 9-Alkylhypoxanthines, in contrast, remain entirely in the keto form, even in the vapor phase (Nowak et al., 1978). If the same solvent effect is operative, then the extent of enolization of inosine in water is probably lower than that of 2-pyridone.

[7] In pteridine and other heterocyclic systems, a $-CH_3$ group reduces the equilibrium constant for covalent hydration by a factor of roughly 100 (for a review, see Albert & Armarego, 1965). Preliminary analysis of the behavior of other aromatic and aliphatic systems suggests that substitution of −H by −OH, at the −CH═N− bond, is likely to leave unchanged, or decrease, its tendency toward covalent hydration (Prof. Peter Guthrie, personal communication).

[8] The C═O bond lengths in uridine (1.233 Å) and inosine (1.22 Å) (Voet & Rich, 1970) are similar to those in acetamide (1.22 Å, Kuchitsu, 1995), and the lengths of the adjacent C−N bonds are also similar, consistent with the possibility that electronic properties of these systems may also be similar.

[9] In contrast with these actual reaction intermediates, hydrated forms of purine ribonucleoside and pyrimidin-2-one ribonucleoside, in which hydrogen replaces the leaving amino group (see the introduction), are able to accumulate at the active site because their equilibria of hydration are much less unfavorable (~10^{-7} and 10^{-6} M, respectively) and can be overcome by forces of attraction at the active site.

substituent, replacing the hydrogen atom at C-6 of purine ribonucleoside, would be expected to enhance the equilibrium constant for addition slightly by electron withdrawal, but to hinder addition by steric effects.[7] In combination, these steric and inductive effects of a 6-OH substituent are expected to cancel each other, or slightly disfavor water addition to the enol tautomer of inosine, compared with water addition to purine ribonucleoside. Thus, the equilibrium constant for water addition to the 6-OH tautomer of inosine is unlikely to be greater than 10^{-7} and could be considerably less.

These two equilibrium constants, corresponding to processes (1) and (2), can be multiplied to yield an overall equilibrium constant of 10^{-11} or less, for addition of water to the carbonyl group of the common keto tautomer of inosine, as shown in Scheme 3A [for comparison, Scheme 3B shows the equilibrium constant for the structurally similar hydration of *N*,*N*-dimethylacetamide, reported by Guthrie (1974)].[8] Thus, accumulation of 1,6-hydrated inosine as an enzyme-bound species, with an apparent K_i value of 1.7×10^{-3} M for the keto tautomer (Scheme 1A), would imply that the enzyme's true dissociation constant from 1,6-hydrated inosine is 10^{-14} M or less. Such an extraordinary affinity for the putative *gem*-diol seems unlikely and unnecessary, because it would greatly surpass the enzyme's apparent binding affinity, estimated above, for the altered substrate in the transition state for oxygen exchange from water into inosine (8×10^{-11} M).

Conclusion. These considerations suggest that although covalent hydrates are formed as high-energy intermediates during catalysis by adenosine and cytidine deaminases, they are unlikely to accumulate at the active sites.[9] The present NMR experiments agree with that conclusion.

We are at a loss to explain the crystallographic findings reported earlier for adenosine deaminase, but suggest that this discrepancy might be resolved if the refinement of the X-ray structure were re-examined.

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NOTE ADDED IN PROOF

Preliminary observations by Raman spectroscopy, comparing free inosine with inosine bound by adenosine deaminase, indicate that the hypoxanthine ring modes of the nucleoside are not altered by binding (Professor Paul Carey and Dr. John Clarkson, personal communication).

REFERENCES

Albert, A., & Armarego, W. L. F. (1965) *Adv. Heterocyclic Chem. 4*, 1−42.
Baillargeon, M. W., Laskowski, M., Jr., Neves, D. E., Porubean, M. A., Santini, R. E., & Markley, J. L. (1980) *Biochemistry 19*, 5703−5710.
Beak, P., Fry, F. S., Lee, J., & Steele, F. (1976) *J. Am. Chem. Soc. 98*, 171−179.
Belasco, J. G., & Knowles, J. R. (1980) *Biochemistry 19*, 472−477.
Blow, D. M., Janin, J., & Sweet, R. M. (1974) *Nature (London) 249*, 54−57.
Breitmaier, E., & Voelter, W. (1974) *Tetrahedron 30*, 3941−3943.
Carlow, D. C., Smith, A. A., Yang, C. C., Short, S. A., & Wolfenden, R. (1995) *Biochemistry 34*, 4220−4224.
Chenon, M.-T., Pugmire, R. J., Grant, D. M., Panzica, R. P., & Townsend, L. B. (1975a) *J. Am. Chem. Soc. 97*, 4627−4635.
Chenon, M.-T., Pugmire, R. J., Grant, D. M., Panzica, R. P., & Townsend, L. B. (1975b) *J. Am. Chem. Soc. 97*, 4636−4642.
Elguero, J., Marzin, C., Katritzky, A. R., & Linda, P. (1976) *The Tautomerism of Heterocycles*, Academic Press, New York.
Ellis, P. D., Dunlap, R. B., Pollard, A. L., Seidman, K., & Cardin, A. D. (1973) *J. Am. Chem. Soc. 95*, 4399−4402.
Frick, L., Yang, C., Marquez, V. E., & Wolfenden, R. (1989) *Biochemistry 28*, 9423−9430.
Guthrie, J. P. (1974) *J. Am. Chem. Soc. 96*, 3608−3615.
Hammett, L. P. (1970) *Physical Organic Chemistry*, pp 116−117, McGraw-Hill, New York.
Hunkapiller, M. W., Forgac, M. D., Yu, E. H., & Richards, J. H. (1979) *Biochem. Biophys. Res. Commun. 87*, 25−31.
Jones, W., Kurz, L. C., & Wolfenden, R. (1989) *Biochemistry 28*, 1242−1247.
Kuchitsu, K. (1995) *CRC Handbook of Chemistry and Physics*, 76th ed., pp 9−24, CRC Press, Boca Raton, FL.
Kurz, L. C., & Frieden, C. (1987) *Biochemistry 26*, 8450−8457.
Kurz, L. C., Drysdale, G. R., Riley, M. C., Evans, C. T., & Srere, P. A. (1992) *Biochemistry 31*, 7908−7914.
Levy, G. C., Lichter, R. L., & Nelson, G. L. (1980) *Carbon-13 Nuclear Magnetic Resonance Spectroscopy*, p 137, Wiley, New York.
Medeiros, G. C., & Thomas, G. J., Jr. (1971) *Biochim. Biophys. Acta 238*, 1−4.
Morita, K., Ochiai, M., & Marumoto, R. (1968) *Chem. Ind. (London)*, 1117.
Nowak, M. J., Szczepaniak, K., Barski, A., & Shugar, A. (1978) *Z. Naturforsch. 33c*, 876−880.
Ogura, Y. (1955) *Arch. Biochem. Biophys. 57*, 288−300.
Palmer, A. R., Ellis, P. D., & Wolfenden, R. (1982) *Biochemistry 21*, 5056−5059.
Psoda, A., & Shugar, D. (1971) *Biochim. Biophys. Acta 243*, 507−513.
Radzicka, A., & Wolfenden, R. (1995) *Science 267*, 90−93.
Richarz, R., Tschesche, H., & Wüthrich, K. (1980) *Biochemistry 19*, 5711−5715.
Rühlmann, A., Kukla, D., Schwager, P., Bartels, K., & Huber, R. (1973) *J. Mol. Biol. 77*, 417−436.
Sharff, A. J., Wilson, D. K., Chang, Z., & Quiocho, F. A. (1992) *J. Mol. Biol. 226*, 917−921.
Smar, M., Short, S. A., & Wolfenden, R. (1991) *Biochemistry 30*, 7908−7912.
Smith, A. A., Carlow, D. C., Short, S. A., & Wolfenden, R. (1994) *Biochemistry 33*, 6468.
Stothers, J. B. (1972) *Carbon-13 NMR Spectroscopy*, pp 138−144, Academic Press, New York.
Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., & Blow, D. M. (1974) *Biochemistry 13*, 4212−4228.
Voet, D., & Rich, A. (1970) *Progress in Nucleic Acid Research and Molecular Biology*, vol. X, pp 183−265, Academic Press, New York.
Wilson, D. K., & Quiocho, F. A. (1994) *Nature (London), Struct. Biol. 1*, 691−694.
Wilson, D. K., Rudolph, F. B., & Quiocho, F. A. (1991) *Science 252*, 1278−1284.
Wolfenden, R. (1969) *Nature 223*, 704−705.
Wolfenden, R. (1974) *Mol. Cell. Biochem. 3*, 207−211.
Wolfenden, R. W., & Kirsch, J. F. (1968) *J. Am. Chem. Soc. 90*, 6849−6850.
Wolfenden, R., & Kati, W. M. (1991) *Acc. Chem. Res. 23*, 209−215.
Xiang, S., Short, S. A., Wolfenden, R. W., & Carter, C. W. (1995) *Biochemistry 34*, 4516−4523.

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