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Adenosine Deaminase Converts Purine Riboside into an Analogue of a Reactive Intermediate: A ^{13}C NMR and Kinetic Study†

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ABSTRACT: The ^{13}C NMR spectra of [2-^{13}C]- and [6-^{13}C]purine ribosides have been obtained free in solution and bound to the active site of adenosine deaminase. The positions of the resonances of the bound ligand are shifted relative to those of the free ligand as follows: C-2, −3.7 ppm; C-6, −73.1 ppm. The binary complexes are in slow exchange with free purine riboside on the NMR time scale, and the dissociation rate constant is estimated to be 13.5 s^{-1} from the slow exchange broadening of the free signal. In aqueous solution, protonation of purine riboside at N-1 results in changes in ^{13}C chemical shift relative to those of the free base as follows: C-2, −4.9 ppm; C-6, −7.9 ppm. The changes in chemical shift that occur when purine riboside binds to the enzyme indicate that the hybridization of C-6 changes from sp^2 to sp^3 in the binary complex with formation of a new bond to oxygen or sulfur. A change in C-2 hybridization can be eliminated as can protonation at N-1 as the sole cause of the chemical shift changes. The kinetic constants for the adenosine deaminase catalyzed hydrolysis of 6-chloro- and 6-fluoropurine riboside have been compared, and the reactivity order implies that carbon–halogen bond breaking does not occur in the rate-determining step. These observations support a mechanism for the enzyme in which formation of a tetrahedral intermediate is the most difficult chemical step. Enzymic stabilization of this intermediate may be an important catalytic strategy used by the enzyme to lower the standard free energy of the preceding transition state.

Adenosine deaminase (EC 3.5.4.4) catalyzes the hydrolysis of (deoxy)adenosine to (deoxy)inosine. Purine riboside, which lacks a leaving group at C-6, is an unreactive adenosine analogue and a potent reversible inhibitor of the enzyme (Wolfenden et al., 1969).

The structures of both the ligand and the enzyme are perturbed when purine riboside binds to adenosine deaminase (Kurz & Frieden, 1983; Kurz et al., 1985). The binding is accompanied by a large UV difference spectrum, and the solvent isotope effect on the binding constant, $K_i(H_2O)/K_i(D_2O) = 1.5$, is quite substantial. Studies of acrylamide quenching of protein fluorescence indicate decreased solvent accessibility of protein tryptophan residues in enzyme–inhibitor complexes (Kurz et al., 1985).

In order to account for the UV difference spectra and solvent isotope effect data, we previously proposed three possible structures for the complexed purine riboside (Kurz & Frieden, 1983): protonation at N-1, covalent adduct formation (possibly with a sulfhydryl residue) at C-2, or covalent adduct formation at C-6 (Figure 1). We now present evidence from ^{13}C NMR which supports covalent adduct formation or hydration at C-6.

This observation together with new data on the relative reactivity of 6-F- and 6-Cl-substituted purine ribosides supports a mechanism for the enzyme in which formation of a tetra-

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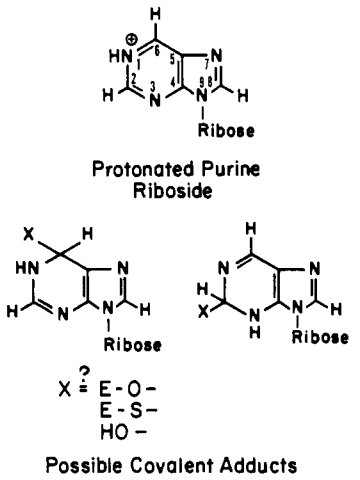

FIGURE 1: Possible structures of purine riboside bound to adenosine deaminase.

hedral intermediate is the most difficult chemical step.

Materials and Methods

Materials. Adenosine deaminase (calf intestine) was obtained from Boehringer Mannheim Co. Deoxycoformycin was obtained from Dr. John Douros of the Developmental Therapeutic Program, Chemotherapy, National Cancer Institute. [^{13}C]Paraformaldehyde and [1-^{13}C]ethyl cyanoacetate were products of MSD Isotopes. 6-Chloropurine riboside was obtained from Sigma Chemical Co.

Enzyme concentrations were determined by active site titration with deoxycoformycin as described elsewhere (Kurz et al., 1985).

6-Fluoropurine riboside was prepared from 6-chloropurine riboside in two steps. Trimethyl(9-β-D-ribofuranosylpurin-6-yl)ammonium chloride, prepared from 6-chloropurine riboside according to Kiburis and Lister (1971), was converted to 6-fluoropurine riboside according to the procedure of Lister and Kiburis (1978).

[2-^{13}C]Purine riboside was prepared from [^{13}C]paraformaldehyde in four steps.

(1) [2-^{13}C]Inosine was prepared from N^1-(β-D-ribofuranosyl)-5-aminoimidazole-4-carboxamide (AICA riboside, Sigma Chemical Co.) according to a procedure similar to that described by Meyer and Wong (1981) in their preparation of [2-^{13}C]adenosine 5′-phosphate. AICA riboside (1 g, 3.9 mmol) was dissolved in 6 mL of 2 M NaOH. [^{13}C]Paraformaldehyde (120 mg, 3.9 mmol) and 0.4 g of 10% Pd/C (Aldrich Chemical Co.) were added, and the solution was refluxed for 30 min until H_2 evolution had ceased. Up to three more additions of paraformaldehyde (60 mg, 1.9 mmol) were made at 30-min intervals until the 248.5 nm/267 nm absorbance ratio of a filtered and neutralized sample of the reaction mixture reached a value >2.3 (2.49 theoretical). Multiple additions of paraformaldehyde are required because formaldehyde disproportionation competes with the desired reaction. After filtration and dilution to 100 mL with water, the reaction mixture was applied to a 75-mL column of AG 1-X8, formate form (Bio-Rad). The column was eluted with a linear gradient of 0–0.2 formic acid in a total volume of 1 L. AICA riboside and a yellow impurity eluted before inosine. Fractions containing inosine with an absorbance ratio >2.4 were combined and lyophilized. Anhydrous inosine (64% yield) was obtained by recrystallization of the dry material from 8:2 (v/v) ethanol:water.

(2) [2-^{13}C]Triacetylinosine. Inosine (0.75 g, 2.8 mmol) was dried at 80 °C overnight in vacuo along with required glassware. Anhydrous pyridine (9 mL, Pierce Chemical Co., silylation grade) and acetic anhydride (7 mL) were added, and the suspension was stirred at 37 °C protected by a drying tube until all the solid was dissolved (~3–4 h). The solution was evaporated to an oil in vacuo, which upon repeated evaporations from ethanol yielded a white solid that was subsequently recrystallized from absolute ethanol (90% yield).

(3) [2-^{13}C]-6-Chloropurine riboside was prepared on the basis of the method described by Robins and Basom (1973). Inert atmosphere techniques were practiced throughout. Triacetylinosine (0.76 g, 1.9 mmol) was dissolved in 30 mL of dry CH_2Cl_2 (Aldrich anhydrous, gold label) in a three-neck flask equipped with an efficient condenser. A mixture of 1.5 mL (21 mmol) of $SOCl_2$ (freshly opened bottle), 0.77 mL of anhydrous dimethylformamide (Aldrich anhydrous, gold label), and 18 mL of CH_2Cl_2 was prepared in a dropping funnel. The thionyl chloride solution was added to the reaction over a 2-h period while the solution was heated with stirring in an oil bath at 55 °C. The reaction was monitored by thin-layer chromatography (TLC) on Kieselgel 60 F_{254} plastic sheets (EM Reagents) developed with 5:1 (v/v) $CHCl_3$:MeOH. A total of 5–6 h was required to go to completion. The cool reaction mixture was added dropwise to 50 mL of a vigorously stirred, ice-cold solution containing 60 g of $NaHCO_3$/L. The phases were separated, retaining the organic layer. The aqueous layer was washed with two 10-mL portions of CH_2Cl_2, and the combined organic layers were dried over $MgSO_4$. The filtered solution was evaporated to dryness in vacuo.

The dry solid was dissolved in 2 mL of CH_2Cl_2 and transferred to a reaction flask containing 15 mL of anhydrous methanol saturated with anhydrous NH_3 at 0 °C. The flask was protected by a serum cap and drying tube. The transfer syringe and original flask were rinsed with an additional 2 mL of CH_2Cl_2, and the rinse was added to the ammonia solution. Additional NH_3 was then allowed to bubble through the stirred solution for 5 min after which the drying tube and gas inlet tubes were removed. The solution was stored on ice overnight. The cloudy solution was evaporated to dryness with several reevaporations from methanol. The solid was dissolved in methanol. Sil G 60 (3 g of 70–230 mesh, EM) was added, and the suspension was evaporated to dryness. The solid was transferred to the top of an 80-mL Sil G 60 column and eluted with 80 mL of CH_2Cl_2 and then with 5:1 (v/v) $CHCl_3$:MeOH. Fractions were monitored by TLC as previously. Fractions containing 6-chloropurine riboside (trace adenosine) were combined and evaporated to dryness. Recrystallization from absolute ethanol yielded pure 6-chloropurine riboside (yield 53%, major loss is in the deprotection step).

(4) [2-^{13}C]Purine riboside was prepared by catalytic hydrogenation at atmospheric pressure of the 6-Cl compound according to Brown and Weliky (1953). Care was taken not to exceed the theoretical hydrogen uptake. The product was recrystallized from absolute ethanol (yield 56%).

[6-^{13}C]Purine riboside was synthesized on the basis of a modified pyrimidine synthesis of hypoxanthine (Roblin et al., 1945; Taylor & Cheng, 1960), the oxynucleoside synthesis of Dudycz and Wright (1984) with the final conversion of triacetylinosine to purine riboside following the [2-^{13}C]purine riboside protocol described above.

(1) [4-^{13}C]-2-Mercapto-4-hydroxy-6-aminopyrimidine. A solution of 13.5 mL of NaOMe in MeOH (24 wt %, Aldrich) and 30 mL of absolute ethanol was prepared in a dry three-neck flask under argon. [1-^{13}C]Ethyl cyanoacetate (4.7 mL; 44 mmol) was added by syringe and stirred 15 min at room temperature. Thiourea (3.45 g, 45 mmol) was added, and the

solution stirred at room temperature for 1 h. The mixture was then heated for 2.5 h under reflux. During this time the solution, which was originally very pale yellow, turned green, then yellow, and finally forming a white precipitate. After evaporation to dryness, the solid was dissolved in 40 mL of boiling 0.02 M KOH and filtered to remove a small amount of black material. Glacial acetic acid (9 mL) was added to yield the pyrimidine as a white solid (quantitative yield).

(*2*) [*4-*^{13}*C*]*-2-Mercapto-4-hydroxy-5-nitroso-6-aminopyrimidine.* The product from the previous step (5.0 g, 35 mmol) was dissolved in 110 mL of 0.31 M NaOH. A solution of 2.5 g (36 mmol) of $NaNO_2$ in 15 mL of water was added. Glacial acetic acid (3.6 mL) was added dropwise with swirling. The solution was stoppered and stirred magnetically for 20 h at room temperature. The red-orange product was collected on a sintered funnel and washed 3 times with 30 mL of boiling acetone and twice with 30 mL of boiling ethanol (yield 78%).

(*3*) [*4-*^{13}*C*]*-2-Mercapto-4-hydroxy-5,6-diaminopyrimidine.* The product from the previous step (4.0 g, 23 mmol) was dissolved in 100 mL of 1 N NaOH. The solution was cooled to 5–15 °C, and 16.2 g of sodium dithionite (Fisher, specially purified) was added a little at a time with stirring such that the temperature did not rise over 30 °C. After about 2 h sufficient additional 1 N NaOH (50–75 mL) was added slowly until the solution was only slightly turbid. After 20 h of stirring at room temperature, activated charcoal was added and the solution filtered. The pH of the solution was adjusted to pH 5 with glacial acetic acid. The precipitate was collected on a sintered funnel and washed with H_2O, EtOH, and EtOEt (yield 84%).

(*4*) [*4-*^{13}*C*]*-4-Hydroxy-5,6-diaminopyrimidine Hydrochloride.* The product from the previous step (3.5 g, 22 mmol) was suspended in 85 mL of a 0.5% solution of Na_2CO_3. Raney nickel (12 g under 3 mL of H_2O, Aldrich) was rinsed into the solution with an additional 25 mL of 0.5% Na_2CO_3. The solution was refluxed for 2 h. The hot solution was filtered, and the catalyst was rinsed with 25 mL of 0.5% Na_2CO_3. The solution was acidified with 1 N HCl to pH 2–3 and treated twice with charcoal until the solution was almost colorless, rinsing the charcoal each time with warm water. After evaporation to dryness in vacuo, the solid was recrystallized from 50% EtOH/H_2O with testing as to the solubility of the remaining solid (51% yield).

(*5*) [*6-*^{13}*C*]*Hypoxanthine.* The diaminopyrimidine (2.0 g, 12 mmol) and required glassware were dried over P_2O_5 at 60 °C in a heated vacuum desiccator. Triethylorthoformate (19 mL, Aldrich) and acetic anhydride (10 mL) were added, and the suspension was refluxed for 4 h protected by a drying tube. The solid was collected and rinsed with EtOH. Recrystallization was from boiling water with charcoal treatment (yield 89%).

(*6*) [*6-*^{13}*C*]*Triacetylinosine.* Inert atmosphere techniques were practiced throughout this procedure. Hypoxanthine (0.5 g, 3.7 mmol in a 100-mL three-neck flask), tetra-*O*-acetylribose (1.3 g, 4.1 mmol in a 30-mL Mininert vial, Aldrich), and required apparatus were dried over P_2O_5 overnight at 60 °C in a heated vacuum desiccator and allowed to come to room temperature under vacuum. Acetonitrile (15 mL, Aldrich anhydrous, gold label) was added by syringe to the sugar in the Mininert flask, and the solution was stirred magnetically until the sugar dissolved. Bis(trimethylsilyl)acetamide (2 mL, 8.1 mmol, Aldrich) was added to a suspension of the hypoxanthine in 15 mL of acetonitrile under argon. After 15-min reflux, the sugar solution was added to the clear silylated purine solution followed by *S*-(trimethylsilyl) trifluoromethanesulfonate (0.9 mL, 4.7 mmol, Aldrich). The mixture was refluxed for 5 h under argon. The cool solution was evaporated to a thick syrup, which partially solidified on reevaporation from EtOH. The residue was treated with 100 mL of $CHCl_3$ and 50 mL of H_2O. The aqueous phase was neutralized by the addition of a saturated solution of Na_2HPO_4 in water while being stirred vigorously. After separation of the layers, extraction was repeated until no more product was obtained in the $CHCl_3$ layer. Extracts were monitored by HPLC on a 3.9 × 300 mm μPorasil column (Waters) eluted at 1 mL/min with $CHCl_3$–MeOH–AcOH (98:2:0.2). After the combined extracts were dried over $MgSO_4$ and evaporated to dryness, the solid was dissolved in a small amount of $CHCl_3$ and applied to a 100-mL Sil G column. Elution with $CHCl_3$ removed several yellow impurities. Triacetylinosine was eluted with 98:2 $CHCl_3$:MeOH. The fractions were monitored by HPLC. Pure fractions were combined and reduced to dryness, and the solid was recrystallized from absolute EtOH (yield 39%). Impure fractions and the mother liquor can be rechromatographed to give additional product with up to a 60% yield eventually obtained. Recrystallization of impure fractions is not satisfactory.

Nuclear Magnetic Resonance Spectroscopy. Carbon-13 spectra were obtained on a Bruker WH-360 spectrometer operated at 90.56 MHz with broad-band decoupling or a Varian XL-300 spectrometer operated at 75.93 MHz with Waltz decoupling. A 10-mm multinuclear probe was used. The sample temperature was maintained at 10 °C. A 45° pulse with a 1-s recycle time was used. Coupled spectra (with NOE) were obtained by gating the decoupler off during the acquisition time (~0.8 s) and on during the delay time (~0.2 s). The final sample composition included 20% D_2O (for internal lock), 0.1–0.2 M acetonitrile (as internal chemical shift reference), and a final enzyme concentration of 100–200 mg/mL (1–2 mM active sites) in 50 mM potassium phosphate buffer containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 6.95. Spectra acquired on the WH-360 required 16 h of data collection while those of the XL-300 required 8 h. Chemical shifts were internally referenced to the acetonitrile cyano resonance, which was given a value of 118.90 ppm relative to tetramethylsilane (Me_4Si). The IUPAC-recommended convention was followed (IUPAC, 1976). In all cases, magnetic field inhomogeneity limited the resolution to ~1 Hz as monitored by the line width of the acetonitrile (internal reference) methyl resonance. All the line widths of protein-bound resonances exceeded this by a factor of ~50. Therefore for protein-bound resonances, the line widths at half-height reflect the true transverse T_2 relaxation time.

Chemical shift changes upon protonation of [2-^{13}C]- and [6-^{13}C]purine riboside were obtained by measuring the chemical shift referenced to acetonitrile in a series of phosphoric acid buffers, I = 0.1, containing 20% D_2O. The apparent pH was not corrected for the D_2O content. The data were fitted by nonlinear regression to eq 1, where δ_{obsd} is the

$$\delta_{obsd} = \frac{a_{H^+}\delta_{PrH^+} + K_a\delta_{Pr}}{a_{H^+} + K_a} \quad (1)$$

observed chemical shift, δ_{PrH^+} is the chemical shift of the protonated base, a_{H^+} is the hydrogen ion activity calculated from the pH, δ_{Pr} is the chemical shift of the free base, and K_a is the apparent ionization constant.

Stopped-Flow Experiments. All stopped-flow experiments were performed on a Durrum stopped-flow instrument with a 2-cm cell thermostated at 20 °C. The experiments were performed at a wavelength of 267 nm and a slit width of 0.3

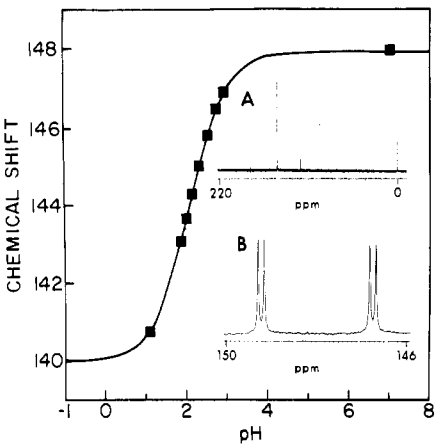

FIGURE 2: Effect of pH on the chemical shift of [6-^{13}C]purine riboside. Chemical shift (■) of [6-^{13}C]purine riboside at 10 °C in a series of phosphoric acid buffers containing 20% v/v D_2O at the indicated apparent pH. The solid line is the least-squares fit of the data to eq 1, yielding a pK_a value of 2.07 and a chemical shift change upon protonation of −7.9 ppm. (Inset A) Decoupled spectrum of [6-^{13}C]purine riboside containing 0.2 M acetonitrile (peaks at 118.9 and 0.9 ppm). (Inset B) Coupled spectrum of [6-^{13}C]purine riboside.

mm. The concentrations of adenosine and purine riboside stock solutions were determined after appropriate dilution from their absorbance (at 259 nm for adenosine and 263 nm for purine riboside) with a Cary 118 spectrophotometer. Full time course data were collected continuously and stored in digital mode for later recall and analysis.

Kinetic constants from full time course data were determined as previously described (Kurz & Frieden, 1983) by fitting progress curves (using a nonlinear least-squares regression analysis; Zimmerle and Frieden, unpublished data) to the appropriate kinetic scheme with the programs KINSIM and FITSIM on a Digital Electronics Corp. microVAX II. KINSIM allows simulation of kinetic mechanisms by numerical integration (Barshop et al., 1983). FITSIM (Zimmerle and Frieden, unpublished data) was used with the Marquardt algorithm (Marquardt, 1963) for the nonlinear least-squares analysis. KINSIM was also used to explore what mechanisms are kinetically equivalent (i.e., yield the same progress curves under a given set of experimental conditions).

Initial velocity experiments for the hydrolysis of 6-chloropurine riboside and 6-fluoropurine riboside were performed at 20 °C on a recording Gilford 240 spectrophotometer. A $\Delta\epsilon$ of 5.91 mM^{-1} cm^{-1} at 250 nm was used for 6-chloropurine riboside hydrolysis and of 5.33 mM^{-1} cm^{-1} at 248 nm was used for 6-fluoropurine riboside hydrolysis. Kinetic constants were obtained by fitting data to eq 2 with nonlinear regression analysis.

$$V_i = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

RESULTS

^{13}C NMR Spectra of the Free Ligand. The decoupled ^{13}C spectra of [2-^{13}C]purine riboside and [6-^{13}C]purine riboside (inset A of Figure 2) each show a single resonance at 151.8 and 147.9 ppm, respectively. The coupled (with NOE) spectrum of the [6-^{13}C]purine riboside (inset B of Figure 2) shows a primary coupling from the 6-proton of 187.1 Hz and a secondary coupling from the 2-proton of 9.7 Hz.

In sufficiently acidic solutions, the positions of the resonances are affected by the protonation that is known to occur at N-1 (Izatt et al., 1971). The effects of pH on the C-6

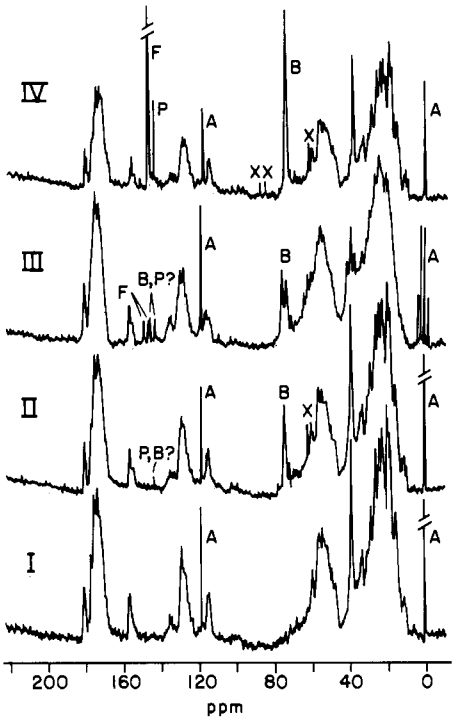

FIGURE 3: ^{13}C NMR spectra: (panel I) 0.9 mM adenosine deaminase, Waltz-decoupled spectrum; (panel II) 2.55 mM adenosine deaminase, 1.91 mM [6-^{13}C]purine riboside, Waltz-decoupled spectrum; (panel III) 3.02 mM adenosine deaminase, 3.2 mM [6-^{13}C]purine riboside, coupled spectrum; (panel IV) 2.26 mM adenosine deaminase, 4.54 mM [6-^{13}C]purine riboside, Waltz-decoupled spectrum. All spectra were obtained at 10 °C in the 10-mm probe of a Varian XL-300 spectrometer. The solutions contained 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 6.95, 20% (v/v) D_2O as lock solvent, and 0.1 M acetonitrile as internal chemical shift reference (except III, which contained 0.2 M acetonitrile). (A) Resonances of acetonitrile internal reference; (B) resonances of protein-bound purine riboside; (F) resonance of free purine riboside; (P) resonance position of purine; (X) resonances of degradation products of purine riboside.

chemical shift are shown in Figure 2. The solid line is the nonlinear least-squares fit of the data to eq 1. The chemical shift change upon protonation for C-2 is −4.9 ± 0.1 ppm while that for C-6 is −7.9 ± 0.1 ppm. The apparent pK_a, 2.07 ± 0.05 (uncorrected for temperature and deuterium content), is consistent with previously determined values (Brown & Weliky, 1953; Kurz & Frieden, 1983).

^{13}C NMR Spectra of the Bound Ligand. Purine riboside binds tightly to the enzyme, $K_{diss} \sim 3$ μM (Kurz & Frieden, 1983). In the presence of a small excess of enzyme at a concentration of ~2 mM, all the ligand is bound to the enzyme, and the stable binary complex can be studied without possible ambiguities arising from chemical exchange with free ligand.

The ^{13}C NMR spectra of free enzyme, enzyme plus 0.75 equiv of [6-^{13}C]purine riboside, and enzyme plus 2 equiv of [6-^{13}C]purine riboside are shown in panels I, II, and IV of Figure 3, respectively. In panel III is shown the coupled spectrum with NOE of the binary complex in the presence of a small excess of ligand. The primary enzyme-bound resonance of [6-^{13}C]purine riboside is found at 74.8 ppm, a shift of −73.1 ppm (upfield) from the position of the resonance of the free ligand. The position of this bound resonance is not affected by the presence of excess ligand. The line width is 65 Hz. The coupling constant for the primary bound resonance is 171 Hz (panel III).

In spectra containing substoichiometric concentrations of ligand (panel II), a small resonance with a line width of ~20 Hz is found at 144.5 ppm.[1] After ~15 000 transients, the

ratio of the intensities of the resonances at 74.8 and 144.5 ppm has a value of 9:1 while after 30 000 transients that ratio has decreased to 7:1. The coupling constant for this small resonance (panel III) is ~183 Hz with no evidence for secondary couplings. However, in spectra containing excess ligand, the resonance at 144.5 ppm grows more intense with time; its apparent line width narrows in decoupled spectra, and secondary couplings of ~9 Hz appear in coupled spectra. For comparison, the 6-carbon of purine is found to resonate at 144.5 ppm with a primary coupling constant of 183 Hz and a secondary coupling constant of 9 Hz.

Except for the much smaller chemical shift change for the bound ligand, similar decoupled spectra are obtained for the [2-^{13}C]purine riboside (data not shown). The primary enzyme-bound resonance of [2-^{13}C]purine riboside is found at 148.1 ppm, a shift of −3.7 ppm (upfield), and its position is unaffected by the presence of excess ligand. The line width of the bound resonance is 60 Hz. There is no indication of more than one bound species in spectra of this complex. The small peak resonating at the chemical shift of purine has a narrow line width.

In samples containing excess ligand, several narrow lines gradually appear in the spectra during the long accumulation times for these experiments. With the possible exception of the line at 144.5 ppm whose apparent line width depends upon experimental conditions,[1] the sharpness of these lines, which grow in intensity with time, suggests that they are not enzyme bound. It is likely that the presence of trace amounts of contaminating nucleosidases and other degradative activities in these adenosine deaminase preparations account for these new lines.

The line width of the free ligand is found to be increased by 4.3 Hz in the presence of an equal amount of the bound ligand. Under these conditions of slow exchange broadening, eq 3 applies, where $\Delta\nu_{1/2,obsd}$ is the line width of the free ligand

$$\pi\Delta\nu_{1/2,obsd} = \pi\Delta\nu_{1/2,free} + k_{-1} \quad (3)$$

observed in the presence of exchange, $\Delta\nu_{1/2,free}$ is the line width for the free ligand in the absence of exchange (in the absence of enzyme), and k_{-1} is the rate constant for dissociation of the ligand from the complex. Our data imply a value of 13.5 s^{-1} for k_{-1}. The dissociation constant for purine riboside, as determined kinetically (Kurz & Frieden, 1983), is 3 μM and gives an apparent association rate constant, k_1, of 4.5 μM^{-1} s^{-1}. Because of the continuous degradation of the free ligand by contaminating activities, it is difficult to be certain of the exact concentration of free purine riboside in these experiments, and thus it is difficult to estimate the error associated with these rate constants. The value of k_{-1} calculated above may be regarded as an upper limit and the value of k_1 as a lower limit.

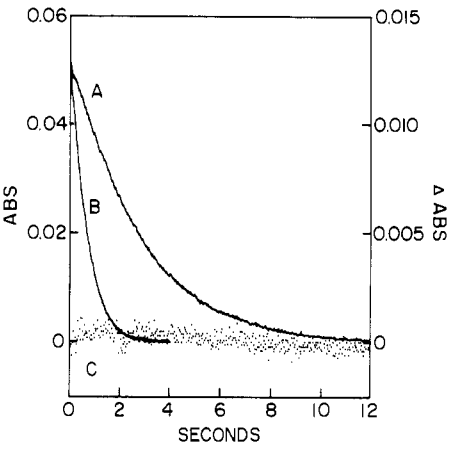

FIGURE 4: Progress curves for adenosine hydrolysis. (B) Progress curve in the absence of purine riboside; 0.21 μM enzyme and 11 μM adenosine monitored at 267 nm. (A) Progress curve in the presence of purine riboside; concentrations are identical with those in (B) except that the substrate syringe also contained 17 μM purine riboside. (C) Difference between the time course depicted in (A) and one in which 17 μM purine riboside was preincubated with the enzyme for 1 h prior to initiation of the reaction. Experiments were performed at 20 °C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA, pH 6.95.

The validity of arguments such as those above as well as for most of the other conclusions reached from these data requires that the bound ligand is in reversible equilibrium with the free ligand and that both it and the enzyme remains substantially intact during the course of our experiments. The time and concentration scales of kinetic and NMR experiments are very different. Within the signal/noise limitations of early scans, the size of the bound resonance remains constant from the time it is first detected (~15 min from mixing the sample). Furthermore, after ~8 h in the spectrometer, addition of excess *unlabeled* purine riboside results in an immediate decrease in the size of the bound resonance and an increase in the size of the resonance at the position of the unbound ligand whose intensity very slowly diminishes with time as that of the purine resonance grows (data not shown).

However, in order to further check that the enzyme–purine riboside species present (at near steady state) in kinetic experiments are the same as those present at equilibrium in NMR experiments, the effect of preincubation of purine ribosine with the enzyme on the full time course hydrolysis of adenosine was examined. In Figure 4, the solid curve A is the progress curve for the hydrolysis of adenosine in the presence of purine riboside. The solid curve B is the progress curve for the hydrolysis of adenosine in the absence of purine riboside. The dots (C) are the differences (at each time point) between the progress curve shown in A and one obtained under identical conditions except that purine riboside was incubated with the enzyme for 1 h before being mixed with adenosine. Except for a small decrease in specific activity, kinetic runs made on samples after ~3 days of exposure to the ligand in the spectrometer were normal in all respects.

Kinetic Constants for 6-Chloro- and 6-Fluoropurine Riboside. For mechanisms involving a tetrahedral intermediate, the order of reactivity of substrates with a halogen leaving group can provide some insight into whether the formation or decay of the intermediate is more rate determining (Miller, 1968). The kinetic constants for 6-chloro- and 6-fluoropurine ribosides were obtained by fitting initial velocity data to eq 2 with a nonlinear regression. For 6-chloropurine riboside,

[1] We are not certain of the identity of this small peak. Its line width, ~20 Hz, suggests that it is protein bound. Its line width is considerably less than that of the primary bound resonance, 61 Hz, suggesting that it either is experiencing a different magnetic environment or is less tightly constrained in the active site. In experiments containing excess ligand, the narrower line which grows with time at this position is undoubtedly attributable to free purine produced by contaminating activities. There are two possible origins for the broadened resonance. It could reflect the presence of bound, structurally unmodified purine riboside in equilibrium with the primary form at 74.8 ppm. The small shift of 3–4 ppm could be attributed to effects of the active site environment. If this is the case, the equilibrium constant between the two forms exceeds 10:1 in favor of the form at 74.8 ppm. Alternatively, the resonance at 144.5 ppm could reflect the presence of bound purine produced by the contaminating activities which degrade the free ligand. The binding constant of purine to the enzyme is undoubtedly very much less than that of the nucleoside (Wolfenden et al., 1977). The resolution of this question will have to await the availability of enzyme preparations free from these degradative activities.

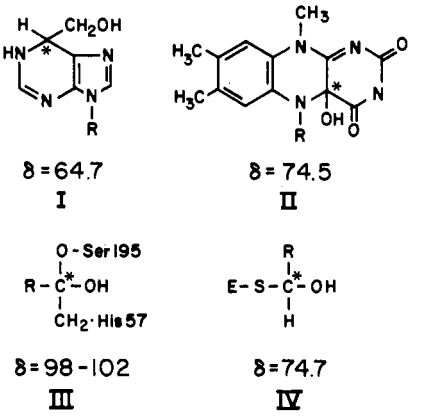

FIGURE 5: Models for the 6-^{13}C chemical shift of purine riboside bound to adenosine deaminase. (*) represents the carbon analogous to the 6-C of purine riboside, and the δ value below the structure is its ^{13}C chemical shift. (I) Methanol photoadduct of purine riboside. (II) 4a-Hydroxy-5-alkyl-4a,5-dihydroflavin. (III) Chloromethyl ketone adduct of the serine proteases. (IV) Thiohemiacetal adduct of aldehydes with papain.

K_m is found to be 330 ± 10 μM and V_{max} is 96 ± 1 s^{-1}.[2] For 6-fluoropurine riboside, K_m is found to be 667 ± 32 μM and V_{max} is 486 ± 39 s^{-1}.

DISCUSSION

Structure of Bound Purine Riboside. The changes in ^{13}C chemical shifts and the reduction of size of the primary coupling constant to the 6-proton (Levy et al., 1980) observed when purine riboside binds to adenosine deaminase indicate a change in hybridization at C-6 from sp^2 to sp^3 with a new bond formed to a heteroatom, most likely oxygen or sulfur (X in Figure 1). Formation of the C-6 methanol photoadduct of purine riboside (I in Figure 5), which approximately models the proposed structural changes, results in chemical shift changes of −93.4 and −2.7 ppm at C-6 and C-2, respectively. The change at C-6 is greater in this model than that observed when purine riboside binds to adenosine deaminase. A smaller change, such as we observe, is expected if the new bond is formed with a heteroatom such as oxygen or sulfur rather than with carbon as in the methanol photoadduct. Typically, sp^3 carbons attached to hydroxyl groups resonate in the ~70 ppm region. An example of a tetrahedral oxygen containing species in a nitrogen heterocycle is provided by the 4a-carbon of 4a-hydroxy-5-alkyl-4a,5-dihydroflavins (II in Figure 5), which resonates at 74.5 ppm (Ghisla et al., 1978).

Other models for the observed C-6 chemical shift of 74.8 ppm for adenosine deaminase bound purine riboside are provided by the chemical shifts of two enzyme-bound tetrahedral adducts. The tetrahedral adduct bound to chymotrypsin (III in Figure 5) derived from chloromethyl ketone inactivation was found to resonate at 98–102 ppm depending upon its ionization state (Malthouse et al., 1985). Substitution of a proton for one of the two oxygen substituents in this adduct would be expected to reduce its chemical shift (Levy et al., 1980) to a value more closely resembling that observed for the adenosine deaminase bound purine riboside. The tetrahedral thiohemiacetal bound to papain (IV in Figure 5) derived from the reaction of *N*-acetyl-L-phenylalanylglycinal with the active site cysteine was found to resonate at 74.7 ppm (Malthouse, 1986). While it seems certain that a tetrahedral adduct at C-6 is formed when purine riboside binds to adenosine deaminase, the data are insufficient to elucidate the exact structure of that adduct.

The UV difference spectrum of the purine riboside complex is not incompatible with the proposed 1–6 adducts. The UV difference spectrum of the purine riboside complex (Kurz & Frieden, 1983) shows a maximum at 280 nm with a molar extinction coefficient of ~4300. If the bound ligand is the main contributor to this absorption, then it represents a 17-nm red shift (from 263 nm to 280 nm) with a reduction in molar extinction coefficient (from 7350 to 4300 M^{-1} cm^{-1}) in comparison to the values for the free ligand. Although this is smaller than might be expected, (~30 nm; Smith & Elving, 1962; Jones & Wolfenden, 1986), a difficulty with the interpretation of UV difference spectra is the unknown contribution of possible shifts in the extinction and position of the absorption of enzyme chromophores and the unknown sensitivity of the absorption maxima of the ligand to medium effects such as might prevail in the active site of an enzyme. Changes in UV spectra associated with 1–6 additions also depend upon the charge of the resulting adduct. For example, the maximum absorption of the cation of the sulfite adduct of purine (1,6-dihydro-6-purinesulfonate) shows a ~25-nm *blue* shift from the position of the maximum absorption of either purine cation or the free base (Pendergast, 1975). More appropriate models (i.e., the UV and NMR spectra of hydrates and sulfhydryl adducts of the 1-methylpurinium ion pseudobase under a variety of conditions) are under investigation.

Mechanism of Binding Purine Riboside to Adenosine Deaminase. We have presented evidence that a form of purine riboside that is tetrahedral (sp^3) at C-6 is the predominant species bound to adenosine deaminase at equilibrium and in the kinetic experiments used to determine the overall dissociation constant (Figure 4). A reasonable hypothesis (Jones & Wolfenden, 1986) is that this form is purine riboside hydrate. If that is the case, two kinetic schemes may be proposed for its formation: (1) a mechanism in which purine riboside hydrate is produced after binding of the predominant unhydrated species, and (2) a mechanism (Jones & Wolfenden, 1986) in which the enzyme binds to only the rare hydrated form, which preexists in very small concentrations in free solution. Our data allow us to rule out a mechanism that involves selection by the enzyme of a rare preexisting hydrated form. The second-order rate constant, for association of the rare hydrate with the enzyme, would be required to have a value that greatly exceeds the encounter-controlled limit in aqueous solution, ~10^4 μM^{-1} s^{-1}.

Implications for the Mechanism of Adenosine Deaminase. Three basic mechanisms may be proposed for the hydrolysis reactions catalyzed by adenosine deaminase. (1) Hydrolysis could occur by a single step (S_N2) process in which there was more or less synchronous bond formation to the entering water nucleophile and bond breakage to the leaving group. No intermediate would then be formed. (2) Hydrolysis could occur by a double-displacement mechanism (analogous to that of the serine proteases). Addition of an enzyme nucleophile to form an initial tetrahedral intermediate would then be followed by formation of an enzyme–substrate adduct and release of the first product. Attack of water on the enzyme–substrate adduct in a second step via a second tetrahedral intermediate would then complete the hydrolysis reaction with the release of the second product. (3) Hydrolysis could occur by an addition–elimination process involving direct attack of

[2] Previously reported kinetic constants (Chassy & Suhadolnik, 1967; Baer et al., 1968) for the adenosine deaminase catalyzed hydrolysis of 6-chloropurine riboside differ from those which we have obtained. It is likely that the exact isozyme composition of our sample accounts for the discrepancy between the values of the kinetic constants we have obtained and those reported previously (Murphy et al., 1969).

water and formation of a single tetrahedral intermediate. This mechanism is analogous to that found for many nucleophilic aromatic substitution reactions.

While an S_N2 mechanism is common for aliphatic nucleophilic substitution reactions, it is very rare or nonexistent for aromatic nucleophilic substitution (Miller, 1968). However, in their recent report on the hydration constant for purine riboside in aqueous solution, Jones and Wolfenden (1986) note that "the instability of the water adduct of adenosine may approach that of the transition state for its deamination". If that is the case, no tetrahedral intermediate would be formed; thus an S_N2-like mechanism for adenosine hydrolysis should be considered (Jencks, 1980).

If we make the important assumptions that all adenosine deaminase catalyzed hydrolyses follow the same mechanistic type and that the same step is rate-determining in a multistep mechanism, then all possibilities that involve rate-determining bond breaking to the leaving group can be ruled out. The greater difficulty of breaking a C–F vs a C–Cl bond mandates that if this bond breaking occurs in the rate-determining transition state, then the F-containing substrate should be substantially less reactive than the Cl-containing substrate (Miller, 1968).[3] The very high K_m values for the halogen-containing substrates suggest strongly that the enzyme follows a prior equilibrium mechanism for these substrates and that it is very likely that the chemical transformation is rate-determining. We observe that 6-fluoropurine riboside is hydrolyzed by the enzyme ~7-fold faster than is 6-chloropurine riboside. (It is interesting to note that the V_{max} for the 6-fluoropurine riboside is greater than that for adenosine, 175–200 s^{-1}). Thus the S_N2 mechanism (1) can be eliminated. The only remaining possibilities are a double-displacement mechanism or addition–elimination mechanism in which formation of the (first in the case of double displacement) tetrahedral intermediate is rate-determining.[4]

Originally, Wolfenden (1968) proposed a double-displacement mechanism for adenosine deaminase. Two observations led him to reject this possibility. The first of these was the observation (Evans & Wolfenden, 1973) that adenosine deaminase catalyzes the hydration of pteridine at nearly the same rate as it catalyzes the hydrolysis of 4-aminopteridine. They argued that water attacks the substrate directly (addition–elimination mechanism). However, this observation cannot be used to exclude a double-displacement mechanism with rate-determining formation of the first tetrahedral intermediate leading to a covalent adduct that is rapidly hydrolyzed. The second observation that does strongly argue in favor of an addition–elimination mechanism is the very tight binding of compounds such as (deoxy)coformycin, which have a preformed tetrahedral carbon and sp^3 nitrogen and whose binding does not seem to involve covalent bond formation with an active site residue (Frick et al., 1986). The very tight binding of these compounds led to their designation as transition-state analogues in comparison with the weaker binding of compounds such as purine riboside, which were designated substrate or "ground-state" analogues (Evans & Wolfenden, 1970; Frieden et al., 1980). However, our observations reported here blur the distinction between these two types of inhibitors since the enzyme converts purine riboside into a "transition-state analogue" in the active site. At first glance, purine riboside hydrate would seem to be a closer approximation of the structure of a transition state leading to a tetrahedral intermediate or in fact of the intermediate itself than (deoxy)coformycin. Why then does purine riboside not bind more tightly? Clearly this and other issues would be clarified upon better definition of the structure of bound purine riboside. Hopefully, such structural information will be forthcoming.

Discussion of data relevant to the mechanism of adenosine deaminase would not be complete without consideration of the solvent isotope effect data on purine riboside binding (Kurz & Frieden, 1983). The solvent isotope effect of 1.5 on the dissociation constant of purine riboside (tighter binding in D_2O solution) was attributed to putative protonation of N-1 by an enzyme sulfhydryl residue. In light of our NMR results, this simple explanation must be rejected. Neglecting the very real possibility that the interpretation of solvent isotope effects in enzymatic systems could be extremely complex (Kurz & Frieden, 1983), the observed binding solvent isotope effect suggests a sulfhydryl adduct. As a model, the addition of oxygen nucleophiles such as water or alcohols to the C=O bond of carbonyls is accompanied by inverse isotope effects, ~1.2 (Bone & Wolfenden, 1985), smaller than that which we have observed. Furthermore, solvent isotope effects accompanying hydration of C=N bonds in aromatic heterocyclic systems are close to 1.0 as reported by Davis and Wolfenden (1983) for the hydration constant of pteridine. On the other hand, the inverse solvent isotope effects on addition of sulfhydryls to carbonyls typically have larger values, 2–2.7 (Bone & Wolfenden, 1985), than those for oxygen nucleophiles. Presumably in analogy to the reduced isotope effects in the addition of oxygen nucleophiles to the C=N bond of pteridine, addition of a sulfhydryl to such a C=N bond would likely have a smaller associated solvent isotope effect, perhaps similar to the 1.5 we have previously reported.

Implications for the Catalytic Strategy of Adenosine Deaminase. The high affinity of the enzyme for stable nucleoside analogues already tetrahedral at C-6 (or its equivalent) is well established [Wolfenden (1978) and references cited therein]. We have presented evidence in this work that the enzyme is capable of stabilizing such a tetrahedral species even when it does not preexist. This would require a shift by a factor as great as 10^9 in the position of an unfavorable equilibrium if purine riboside hydrate is the bound form (vide supra). On the basis of a comparison of equilibrium constants for hydrate and thiohemiacetal formation (Kanchuger & Byers, 1979), a stable covalent adduct with an active site sulfhydryl would require a shift of ~10^6. Thus for any of the likely catalytic mechanisms discussed above, the extraordinary ability of this enzyme to stabilize species tetrahedral at C-6 of purine nucleosides translates directly into its ability to stabilize a tetrahedral intermediate for hydrolysis. Stabilization of an intermediate would result in a reduction of the standard free energy of the transition state for its formation through the operation of the Hammond postulate and thus make an important contribution to the catalytic efficiency of the enzyme.

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[3] Including the other halogen leaving groups, Br and I, Chassy and Suhadolnik (1967) found the relative rates (V_{max}) of the enzyme-catalyzed hydrolyses of 6-chloro-, 6-bromo-, and 6-iodopurine ribosides to be in the order 4:2:1. While Miller (1968) found the 6-F to 6-Cl reactivity ratio to be the most diagnostic, most aromatic nucleophilic substitution reactions with rate-determining formation of the tetrahedral intermediate also follow a reactivity order with the other halogens similar to that observed by Chassy and Suhadolnik.

[4] The relative reactivity of 6-F to 6-Cl of ~7 which we have found is smaller than that usually found in most aromatic nucleophilic substitution reactions (Miller, 1968). If we wish to argue strongly that the observed ratio reflects that for a single elementary step, then this reduced ratio probably reflects a very early transition state. An early transition state for formation of a tetrahedral intermediate for this enzyme was first proposed by Wolfenden (1968).

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