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Evidence from Nitrogen-15 and Solvent Deuterium Isotope Effects on the Chemical Mechanism of Adenosine Deaminase†

Paul M. Weiss, P. F. Cook,‡ J. D. Hermes,§ and W. W. Cleland*
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706
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ABSTRACT: We have determined ^{15}N isotope effects and solvent deuterium isotope effects for adenosine deaminase using both adenosine and the slow alternate substrate 7,8-dihydro-8-oxoadenosine. With adenosine, ^{15}N isotope effects were 1.0040 in H_2O and 1.0023 in D_2O, and the solvent deuterium isotope effect was 0.77. With 7,8-dihydro-8-oxoadenosine, ^{15}N isotope effects were 1.015 in H_2O and 1.0131 in D_2O, and the solvent deuterium isotope effect was 0.45. The inverse solvent deuterium isotope effect shows that the fractionation factor of a proton, which is originally <0.6, increases to near unity during formation of the tetrahedral intermediate from which ammonia is released. Proton inventories for $1/V$ and $1/(V/K)$ vs percent D_2O are linear, indicating that a single proton has its fractionation factor altered during the reaction. We conclude that a sulfhydryl group on the enzyme donates its proton to oxygen or nitrogen during this step. pH profiles with 7,8-dihydro-8-oxoadenosine suggest that the pK of this sulfhydryl group is 8.45. The inhibition of adenosine deaminase by cadmium also shows a pK of ~9 from the pK_i profile. Quantitative analysis of the isotope effects suggests an intrinsic ^{15}N isotope effect for the release of ammonia from the tetrahedral intermediate of ~1.03 for both substrates; however, the partition ratio of this intermediate for release of ammonia as opposed to back-reaction is 14 times greater for adenosine (1.4) than for 7,8-dihydro-8-oxoadenosine (0.1). The most likely chemical mechanism for adenosine deaminase involves protonation of N-1 by the sulfhydryl group while water adds to C-6 with general base assistance by a histidine with pK = 5.24. The pK values for 7,8-dihydro-8-oxoadenosine are 3.0 and 8.8 and for 7,8-dihydro-8-oxoinosine 7.8 and 11.1. The shape of a plot of $(V/K)K_i$ vs pH is shown to be a very sensitive criterion for the stickiness of a substrate, and by this test adenosine is not sticky.

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) catalyzes the hydrolytic cleavage of adenosine to inosine and ammonia, as well as the elimination of a number of other groups from the 6-position of purine ribonucleosides. Two chemical mechanisms have been proposed for adenosine deaminase. Orsi et al. (1972) suggested that direct addition of an enzymic sulfhydryl group to C-6 of adenosine was the first step, with ammonia release from this tetrahedral intermediate leaving an enzyme-bound intermediate that was in turn displaced by water to yield inosine. Kurz and Frieden (1983) suggest protonation of N-1 by the enzyme sulfhydyl group during attack at C-6 by water, with ammonia release from the tetrahedral intermediate giving inosine directly.

To help gain evidence on the chemical mechanism, and in particular the participation of a sulfhydryl group, we have measured the ^{15}N kinetic isotope effects in H_2O and D_2O and the solvent deuterium isotope effect for both the normal substrate adenosine and the slow alternate substrate 7,8-dihydro-8-oxoadenosine. The larger isotope effects observed with the latter facilitate quantitative analysis and have allowed us to establish without doubt that a sulfhydryl group is involved in the mechanism and transfers its proton to oxygen or nitrogen prior to ammonia release.

Materials and Methods

Materials. Adenosine deaminase from calf intestinal mucosa in glycerol, glutamate dehydrogenase from bovine liver in glycerol, α-ketoglutarate, reduced β-nicotinamide adenine dinucleotide (DPNH), and adenosine were from Sigma. NaOD and *N*-bromoacetamide were from Aldrich. D_2O (99.9 atom % D) was from Cambridge Isotope Laboratories. Assay of ammonia was with the Nessler's-based Sigma Ammonia Color Reagent. AG 50W-X8 cation-exchange resin was from Bio-Rad.

7,8-Dihydro-8-oxoadenosine (6-Amino-9-β-D-ribofuranosyl-9H-purin-8(7H)-one). Tri-*O*-acetyladenosine (II) was prepared according to Bredereck and Martini (1947). A total of 50 g (187 mmol) of adenosine (I) was dissolved in 740 mL of dry pyridine. To this was added 530 mL of acetic anhydride, and this solution was allowed to stir at 25 °C for 2.5 h. Solvent was removed in vacuo and the residue washed twice with 300 mL of ethanol. The yellowish syrup was recrystallized from 150 mL of cold ethanol at 4 °C. The white crystals were collected and dried under vacuum to yield 47 g of II (64% yield from I). Thin-layer chromatography of this

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‡Present address: Department of Biochemistry, North Texas State University, Denton, TX 76203.
§Present address: Department of Chemistry, Harvard University, Cambridge, MA 02138.

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compound in $CHCl_3$ on 0.1-mm precoated cellulose-F plates in 1% acetic acid revealed a single UV-absorbing spot.

8-Bromo-2′,3′,5′-tri-*O*-acetyladenosine (III) was prepared by method A of Holmes and Robins (1964). The 47 g (120 mmol) of II and 50 g (362 mmol) of *N*-bromoacetamide were dissolved in 500 mL of dry $CHCl_3$. This solution was refluxed for 5 h at which time the solvent was removed in vacuo and the dark red syrup was dissolved in 1 L of ethyl acetate. Two 500-mL portions of this solution were each extracted with 250 mL of 690 mM sodium dithionate followed by 250 mL of a saturated sodium bicarbonate solution. The ethyl acetate extract was dried over sodium sulfate for 6 h and filtered, and the solvent was removed in vacuo. Recrystallization from tetrahydrofuran yielded 10.3 g (18% yield from II) of III.

8-Bromo-6-(*N*-acetylamino)-2′,3′,5′-tri-*O*-acetyladenosine (IV), 6-(*N*-acetylamino)-9-β-D-ribofuranosyl-9*H*-purin-8-(7*H*)-one (V), and 6-amino-9-β-D-ribofuranosyl-9*H*-purin-8-(7*H*)-one (7,8-dihydro-8-oxoadenosine) (VI) were synthesized according to Holmes and Robins (1965). The 10.3 g (22 mmol) of III was added to a solution of 21 mL of pyridine in 42 mL of acetic anhydride. After being stirred for 8 h at 50 °C, the solution was cooled to room temperature, 105 mL of ethanol added, and the solvent removed in vacuo. The residue was washed 3 more times with ethanol, and IV was isolated as a glass. The crude IV was dissolved in 62 mL of methanol, and to this was added 206 mL of methanolic ammonia (prepared by bubbling dry ammonia gas through methanol until saturated) and 10 mL of water. The resulting solution was stirred at 25 °C for 11 h. The solvent was removed in vacuo and the residue dissolved in boiling water. Recrystallization at 4 °C, filtering, and air-drying yielded 1.7 g (24% yield from III) of a light brown powder. Thin-layer chromatography as described above indicated a single UV-absorbing spot. The UV absorption data for this compound were identical with those of Holmes and Robins (1965) for compound V. The 1.7 g (5.3 mmol) of V was dissolved in 50 mL of 1 M NaOH and this solution refluxed for 6 h, cooled to room temperature, and neutralized with 6 M HCl. Diluted aliquots again exhibited identical UV absorption with that seen by Holmes and Robins (1965) for compound VI. This solution was frozen in liquid nitrogen and lyophilized to yield a light brown powder.

The 125-MHz, proton-coupled, ^{13}C NMR spectra of this compound and of adenosine were obtained in D_2O/NaOD (dioxane as external standard). Comparison of the two spectra indicated the expected resonances for 7,8-dihydro-8-oxoadenosine (the resonance for C-8 became a singlet at δ 163 vs a doublet centered at δ 141 in adenosine). The p*K*'s of 7,8-dihydro-8-oxoadenosine and 7,8-dihydro-8-oxoinosine (prepared by completely deaminating a sample of 7,8-dihydro-8-oxoadenosine with adenosine deaminase) were obtained by following the changes with pH in the absorbance at 290 nm (7,8-dihydro-8-oxoadenosine) or 285 nm (7,8-dihydro-8-oxoinosine). These data were fitted to eq 4, giving p*K*'s of 3.0 ± 0.1 and 8.8 ± 0.2 for 7,8-dihydro-8-oxoadenosine and 7.8 ± 0.6 and 11.1 ± 0.1 for 7,8-dihydro-8-oxoinosine. For the isotope effect and initial velocity studies described below 7,8-dihydro-8-oxoadenosine was used without further purification; however, a sample was purified by ion-exchange chromatography to obtain a proton NMR spectrum. A solution of 7,8-dihydro-8-oxoadenosine was titrated to pH 2.1 with 88% formic acid and applied to a column of AG 50W-X8 (ammonium form). After being washed with water, 7,8-dihydro-8-oxoadenosine was eluted with 20 mM ammonia. Fractions indicating UV absorbance by thin-layer chromatography were pooled, and the ammonia/H_2O was removed in vacuo. The 270-MHz ^1H NMR spectrum of 7,8-dihydro-8-oxoadenosine in DMSO-d_6[1] (TMS as internal standard) showed a broad singlet at δ 6.7 (2 H) from the amino group and a broad singlet at δ 10.5 (1 H) from the proton on N-7. Both of these resonances agree well with those observed by Holmes and Robins (1965). The kinetic parameters with adenosine deaminase for both the purified and unpurified 7,8-dihydro-8-oxoadenosine were identical.

Nomenclature. The nomenclature used is that of Northrop (1977), in which isotope effects on kinetic or thermodynamic parameters are defined by leading superscripts. Since all deuterium isotope effects determined in this study were solvent deuterium ones, the superscript D refers to such effects. For example, $^{15}(V/K)_D$ is the ^{15}N isotope effect in D_2O, and $^D(V/K)$ is the solvent deuterium isotope effect. For a further discussion of nomenclature, see Cook and Cleland (1981).

Initial Velocity Studies. Initial velocity studies were performed at 25 °C with a Cary 118 spectrophotometer. Reaction rates were monitored by following the rate of change in absorbance at either 265 nm with adenosine or 272 nm with 7,8-dihydro-8-oxoadenosine. At pH 7.1, the maximal change in absorbance between adenosine and inosine is $\Delta\epsilon_{265} = -8330$ M^{-1}, while that between 7,8-dihydro-8-oxoadenosine and 7,8-dihydro-8-oxoinosine is $\Delta\epsilon_{272} = -4550$ M^{-1} (Simon et al., 1970). For pH(D) profiles, Δε was determined as a function of pH(D) and solvent composition. No change in Δε was observed between H_2O and D_2O; however, Δε did vary significantly with pH(D), and the corrected values were used when initial velocities were calculated. A coupled assay for NH_3 with DPNH, α-ketoglutarate, and glutamate dehydrogenase was used for all end-point determinations. In this case, measurement was at 340 nm and $\epsilon = -6220$ M^{-1} for DPNH disappearance. These end points were used to establish adenosine and 7,8-dihydro-8-oxoadenosine concentrations. pH(D) profiles were determined with the following buffers for the indicated pH(D) ranges: acetate (4.5–6.0), MES (5.8–6.6), PIPES (6.4–7.0), HEPES (7.0–7.8), TAPS (7.8–8.8), CHES (8.8–9.5), and CAPS (9.5–10.5). The pH and pD (pH meter reading + 0.4) values were measured before and after the assays were carried out and found not to be different. Assays in D_2O were made up of components from stock solutions also in D_2O. Adenosine deaminase by glycerol was diluted into D_2O buffer. Comparison with similar dilutions of adenosine deaminase into H_2O buffers indicated no effect of D_2O on enzyme activity. The pD of solutions in D_2O was adjusted with NaOD.

^{15}N Kinetic Isotope Effects. The $^{15}(V/K)$ isotope effects were determined by isotope ratio mass spectrometric analysis of the ammonia (as N_2) from complete or low-conversion (10–20% reaction) samples by use of the natural abundance of ^{15}N as the label. The complete-conversion samples give the mass ratio in the original substrate, while the low-conversion samples reflect the isotopic discrimination.

All solutions were checked for ammonia content by either the glutamate dehydrogenase or Nessler's assay. Typically, for low-conversion samples, 30-mL solutions of 25 mM adenosine or 7,8-dihydro-8-oxoadenosine were prepared in 50 mM buffer in H_2O or D_2O at the desired pH(D) in well-sealed

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; DMSO, dimethyl sulfoxide; TMS, trimethylsilane.

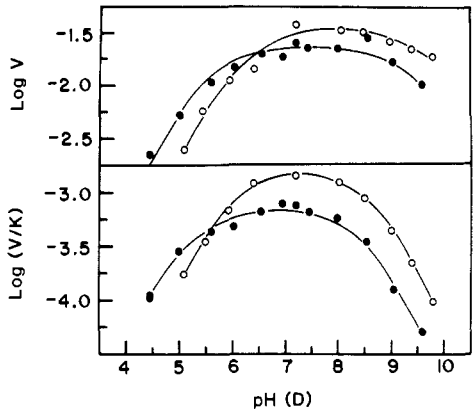


FIGURE 1: pH(D) profiles for 7,8-dihydro-8-oxoadenosine. Closed circles represent data in H_2O and open circles, D_2O. Velocities at each pH(D) value were fitted to eq 1, and the curves in the figure are drawn from fits of V or V/K to eq 2. The points are experimental values.

vials. To initiate both the H_2O and D_2O reactions containing adenosine, 5 units of adenosine deaminase was added. The 7,8-dihydro-8-oxoadenosine reactions were initiated with 29 units (H_2O) and 18 units (D_2O) of adenosine deaminase. The progress of all low-conversion reactions was followed by withdrawing aliquots and assaying for ammonia with the Nessler's assay. At appropriate times, reactions were quenched with concentrated H_2SO_4 [pH(D) to <1]. High-conversion samples were run with 3 mM substrate, initiated with 40 units of adenosine deaminase, and incubated 4–6 h (greater than 10 times the time found required to reach 100% reaction) prior to quenching. The ammonia was isolated, oxidized to N_2, and analyzed as described by Hermes et al. (1985).

Data Analysis. Reciprocal initial velocities were plotted versus the reciprocal of the substrate concentration, and the data were fitted by the least-squares method assuming equal variances for the values of v or log y by a digital computer and the Fortran programs of Cleland (1979). Individual saturation curves were fitted to eq 1. pH profiles in which

$$v = VA/(K + A) \quad (1)$$

the log of the parameter decreased both above pK_2 with a slope of −1 and below pK_1 with a slope of 1 were fitted to eq 2, where

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \quad (2)$$

c is the pH(D)-independent plateau value. $^D(V/K)$ and ^DV values were thus calculated from the ratio of C values in H_2O and D_2O. pH profiles in which the log of the parameter decreased above pK_1 with a slope of −1 were fitted to eq 3.

$$\log y = \log [C/(1 + K_1/H)] \quad (3)$$

pH profiles that showed a plateau at low and high pH were fitted to eq 4, where Y_L and Y_H are the low-pH and high-pH

$$\log y = \log [(Y_L + Y_H K_1/H)/(1 + K_1/H)] \quad (4)$$

plateau values, respectively. To determine ^{15}N kinetic isotope effects, eq 5 was used, where R_p is the $^{15}N/^{14}N$ ratio in the

$$^{15}(V/K) = \log (1 - f)/\log (1 - fR_p/R_0) \quad (5)$$

product at the fraction of reaction f and R_0 is the mass ratio in the initial substrate (determined by total conversion to ammonia).

Results and Discussion

pH Profiles. 7,8-Dihydro-8-oxoadenosine is a slow alternate substrate for adenosine deaminase [V reported as 10% that of adenosine, with K_m twice as high (Simon et al., 1970); V was 7% that of adenosine in our hands at pH 7.2, with a K_m of 40 μM vs. 20 μM for adenosine]. The V/K pH(D) profiles with 7,8-dihydro-8-oxoadenosine indicate pK values of 5.24 ± 0.08 and 8.45 ± 0.07 in H_2O and 5.99 ± 0.04 and 8.60 ± 0.03 in D_2O (Figure 1).

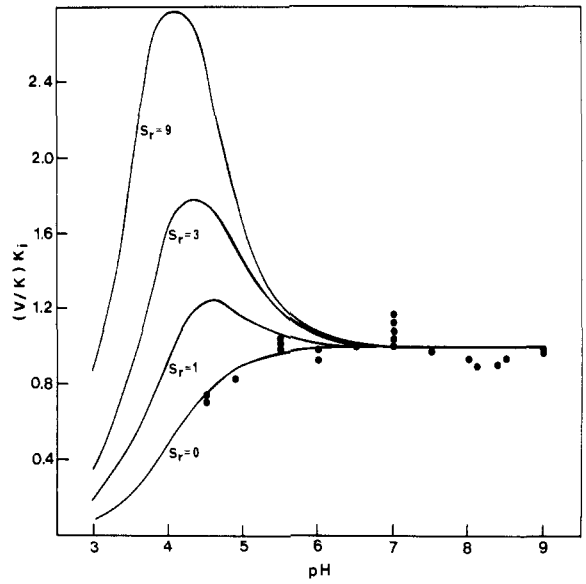


FIGURE 2: Calculated pH profiles for $(V/K)K_i$ for various stickiness ratios (S_r) with adenosine as substrate. The curves were calculated from eq 6, while the data points are normalized from Kurz and Frieden (1983) for the inhibition by purine riboside vs adenosine.

Stickiness of Adenosine. A sticky substrate is one that reacts to give products as fast or faster than it dissociates, and the stickiness ratio S_r is the ratio of these rates (Cleland, 1986). When the substrate is sticky, the pK's in the V/K profile are usually displaced outward from their true values. With adenosine as the substrate, Orsi et al. (1972) reported pK's of 5.7 and 8.7 for V/K at 37 °C, while Kurz and Frieden (1983) reported the low-pH pK to be 5.0 at 20 °C. The latter workers also determined a pK of 4.1 from the $(V/K)K_i$ profile for the inhibitor purine riboside and pK's of 3.8 and 4.7 from pH profiles for k_{off} and k_{on} for the inhibitor 1,6-dihydro-6-(hydroxymethyl)purine riboside. These data show that protonation of the enzymic group with pK ~5 leads to decreased binding of the inhibitors, but does not totally prevent it, while the pK of the enzymic groups is displaced by binding of an inhibitor to ~4. This situation predicts that the pH profile of $(V/K)K_i$ should be given by

$$(V/K)K_i = \frac{a(1 + H/10^{-5})}{(1 + H/10^{-4})(1 + H/10^{-x})} \quad (6)$$

where H is hydrogen ion concentration, a is a constant, and x is the pK observed in the V/K profile. If $a = 1$, the curves in Figure 2 are obtained by assuming various stickiness ratios for adenosine and that the pK in the V/K profile will be displaced to the low-pH side by log $(1 + S_r)$ (Cleland, 1986). The data of Kurz and Frieden (1983) for the inhibitor purine riboside have been normalized and included in Figure 2. Clearly these data match the predicted curve for $S_r = 0$, and thus adenosine is not sticky.[2] The shape of the pH profile of $(V/K)K_i$ is very sensitive to stickiness, and thus such profiles

[2] The same conclusion follows from the close correspondence of the pK in the V/K profile and that for the profile of k_{on} for the inhibitor 1,6-dihydro-6-(hydroxymethyl)purine riboside, but the shape of the $(V/K)K_i$ profile is a more sensitive test for stickiness because all of the information comes from the same set of kinetic data.

Table I: ^{15}N Kinetic Isotope Effects for Adenosine Deaminase

substrate	solvent	pH(D)	$^{15}(V/K)$	no.[a]
adenosine	H_2O	5.2	1.0043 ± 0.0001	2
adenosine	H_2O	6.0	1.0032 ± 0.0002	3
adenosine	H_2O	7.0	1.0039 ± 0.0002	3
adenosine	H_2O	7.2	1.0048[b]	2
adenosine	H_2O	9.8	1.0039 ± 0.0001	2
adenosine	D_2O	7.2	1.0023 ± 0.0001	2
7,8-dihydro-8-oxoadenosine	H_2O	8.0	1.0150 ± 0.0001	3
7,8-dihydro-8-oxoadenosine	D_2O	8.0	1.0131 ± 0.0002	3

[a] Number of determinations. A single determination involved one low-conversion sample and at least two complete conversion samples. The average of all $^{15}(V/K)$ values for adenosine in H_2O is 1.0040 ± 0.0005. [b] The two values were identical.

should be used when one wishes to determine the stickiness of a substrate. When protonation of the enzymic group does totally prevent the binding of the inhibitor, the curves in Figure 2 will not drop at low pH but will rise to new plateau values at low pH.

Evidence for Participation of a Sulfhydryl Group. On the basis of inactivation with *p*-(chloromercuri)benzoate above a pK of 8.6, Orsi et al. (1972) postulated that the pK of 8.7 seen in the adenosine V/K profile was due to a catalytic sulfhydryl group. The pH profile 7,8-dihydro-8-oxoadenosine was repeated from pH 7 to pH 10.5 in the presence of 25% dimethylformamide, and the pK of 8.45 increased by 0.5 pH unit (the pH-independent plateau values of V and V/K were 71% and 19% of those without dimethylformamide). This solvent perturbation shows that the catalytic group is a neutral acid, which is consistent with a thiol residue.

Cadmium ion was found to inhibit adenosine deaminase with an inhibition constant of ~56 nM at pH 9.0. The pK_i profile showed maximum inhibition above a pK of ~9.5, but the profile had a slope of ~2 below this pK, suggesting two thiol groups on the enzyme with pK's near 9.5. Ronca et al. (1967) found that, in sodium dodecyl sulfate denatured adenosine deaminase, 2.1 sulfhydryl groups were titratable with *p*-(chloromercuri)benzoate; however, only one was titratable in active enzyme, with a corresponding loss of activity. Possibly Cd^{2+} is small enough to interact with the second sulfhydryl group as well as with the catalytic one.

The shift in the high pK of the V/K profile in D_2O vs H_2O was 0.15 pH unit. A sulfhydryl would be expected to show a shift of ~0.18, and thus the observed shift is also consistent with an –SH residue. The shift in the low pK was 0.75 pH unit, suggesting a carboxylate or histidine residue. A histidine has been proposed on the basis of solvent perturbation data that showed the group to be a cationic acid (Orsi et al., 1972). This group and the sulfhydryl group appear necessary for catalysis rather than for binding of 7,8-dihydro-8-oxoadenosine, as similar pK's were obtained in the pH(D) profile of V (Figure 1). The shifts of the pK values in D_2O are similar to those in the V/K profile.

The proton inventories (i.e., variation with fraction of D_2O) for V and V/K with 7,8-dihydro-8-oxoadenosine were determined at pH(D) 7.2 (Figure 3). Both $1/V$ and $1/(V/K)$ vs percent D_2O were linear. The reciprocal of the parameter is plotted whenever a net inverse solvent isotope effect is observed, as is the case here, and linearity indicates involvement of a single proton whose fractionation factor changes appreciably during the reaction (Schowen, 1978).

^{15}N Isotope Effects. The ^{15}N isotope effect on the adenosine deaminase reaction was essentially pH independent over the pH range 5.2–9.8 (Table I), although V/K decreased at both low and high pH with pK's of 5–5.7 and 8.7 (Kurz & Frieden, 1983; Orsi et al., 1972). Since V also decreased at low pH,

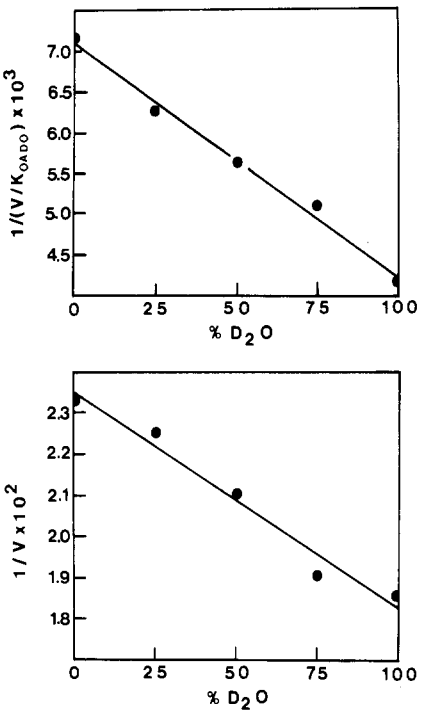


FIGURE 3: Proton inventories for V/K and V with 7,8-dihydro-8-oxoadenosine as substrate at pH(D) 7.2. The reciprocal of each parameter is plotted vs percent D_2O, since the solvent deuterium isotope effect was inverse.

these data are consistent with the conclusion that adenosine is not sticky, and there is no external commitment.

The ^{15}N isotope effect in D_2O was only half that in water [0.23% vs 0.48% at pH(D) 7.2]. Since the solvent deuterium isotope effect on V/K was inverse (0.77 ± 0.06; that is, the value was *higher* in D_2O and $^{D}V = 0.90 \pm 0.06$), we have an unusual combination of isotope effects to use to determine the chemical mechanism of the reaction. The isotope effects for 7,8-dihydro-8-oxoadenosine are even more striking, with large normal ^{15}N isotope effects (Table I) and a highly inverse solvent deuterium isotope effect on V/K (0.45 ± 0.04, with $^{D}V = 0.74 \pm 0.07$). An inverse isotope effect arises because the fractionation factor of one or more hydrogens increases as the reaction proceeds (that is, the equilibrium isotope effect for an early step is inverse, and this step is close enough to equilibrium for the equilibrium isotope effect to be more fully expressed than any primary deuterium isotope effect). Kurz and Frieden (1983) postulated that the inverse isotope effect with adenosine deaminase resulted from participation of an SH group on the enzyme in the reaction. Sulfhydryl groups have fractionation factors of 0.4–0.5, and if the proton is transfered to a nitrogen or oxygen during the reaction (fractionation factor ~1), the equilibrium isotope effect for the transfer will be ~0.5. In addition, the fractionation factor of the hydrogens on the exocyclic amino group in adenosine is 0.95, while the value will be approximately 1.06 in the tetrahedral intermediate containing a protonated amino group, which breaks down to release ammonia (Reuben, 1986). Such an intermediate has been postulated on the basis of strong inhibition (K_i ~760 nM) by 1,6-dihydro-6-(hydroxymethyl)purine riboside, which contains a tetrahedral atom at C-6 (Evans & Wolfenden, 1970), and is really required by the chemistry involved here.

Analysis of Isotope Effects. The decrease in the ^{15}N isotope effects for both adenosine and 7,8-dihydro-8-oxoadenosine in D_2O rules out a concerted mechanism in which the deuterium- and ^{15}N-sensitive steps are the same. We have considered a

number of stepwise mechanisms for the adenosine deaminase reaction and concluded that a sulfhydryl group *must* be involved in the reaction, with its hydrogen being transfered to a nitrogen or oxygen prior to the step in which ammonia is produced. For a two-step mechanism in which there is a tetrahedral intermediate we can write

$$\text{EH–Ado–NH}_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{E–Ado–NH}_3^+ \xrightarrow{k_5} \text{E–Ino} + \text{NH}_3 \qquad (7)$$

While any real chemical mechanism will have more steps than this, the simple model is sufficient for analysis of the isotope effects. For mechanism 7 the ^{15}N isotope effects on V/K will be

$$^{15}(V/K)_H = \frac{^{15}K_{eq3}{}^{15}k_5 + {}^{15}k_3(k_5/k_4)}{1 + k_5/k_4} \qquad (8)$$

$$^{15}(V/K)_D = \frac{^{15}K_{eq3}{}^{15}k_5 + {}^{15}k_3(k_5/k_4)(^Dk_4/^Dk_5)}{1 + (k_5/k_4)(^Dk_4/^Dk_5)} \qquad (9)$$

The solvent deuterium isotope effect will be

$$^D(V/K) = \frac{^DK_{eq3}{}^Dk_5 + {}^Dk_3(k_5/k_4)}{1 + k_5/k_4} \qquad (10)$$

In these equations, we have allowed for isotope effects on all three rate constants in mechanism 7. The value of $^{15}K_{eq3}$ (the equilibrium isotope effect for converting an $-NH_2$ group into an $-NH_3^+$ one) should be 0.9836 (Hermes et al., 1985), while the value of $^DK_{eq3}$ will be at the most $(0.95)^2x/(1.06)^3$, where x is the fractionation factor of the group on the enzyme that donates the extra proton and 0.95 and 1.06 are fractionation factors for hydrogens in $-NH_2$ and $-NH_3^+$ groups (Reuben, 1986). In mechanisms where water has added to give the tetrahedral intermediate in mechanism 7, at least one hydrogen from water will increase its fractionation factor to the value for an alcohol (1.12; Rolston & Gale, 1984), and $^DK_{eq3}$ will be divided by 1.12. Thus if x is near unity, as would be the case if the enzyme group were imidazole or carboxyl, $^DK_{eq3}$ would be 0.76–0.68 (the latter value if water has added), while, if the enzyme group is a sulfhydryl ($x = \sim 0.5$), $^DK_{eq3}$ would be 0.38–0.34.

To analyze eq 8–10, we will assume that $^{15}k_3$ and $^Dk_3$ are near unity and thus that $^{15}k_4$ and $^Dk_4$ reflect the equilibrium isotope effect values in the reverse direction.[3] Equations 8–10 with the data for adenosine become

$$1.0040 = \frac{0.9836^{15}k_5 + a}{1 + a} \qquad (11)$$

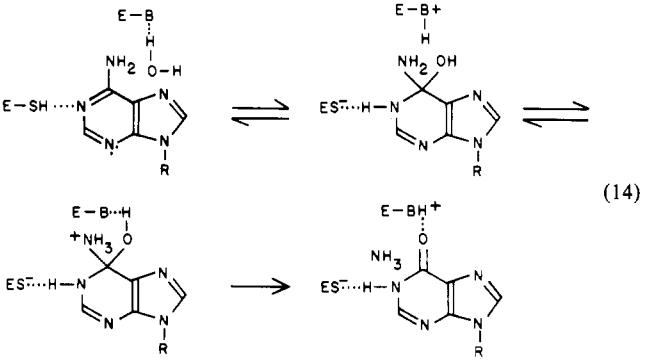
$$1.0023 = \frac{0.9836^{15}k_5 + a/(^DK_{eq3}{}^Dk_5)}{1 + a/(^DK_{eq3}{}^Dk_5)} \qquad (12)$$

$$0.77 = \frac{^DK_{eq3}{}^Dk_5 + a}{1 + a} \qquad (13)$$

where $a = k_5/k_4$. These equations can be solved to give[4] $^DK_{eq3}{}^Dk_5 = 0.45 \pm 0.04$, $a = k_5/k_4 = 1.4 \pm 0.7$, and $^{15}k_5 = 1.026 \pm 0.003$. The value for $^{15}k_5$ is less than expected, since the ^{15}N equilibrium isotope effect for cleavage of the tetrahedral intermediate to give ammonia is 1.0333 (Hermes et al., 1985), and $^{15}k_6$ (the isotope effect for C–N bond formation in the reverse direction) should be slightly normal, because reaction coordinate motion of the nitrogen is involved. In view of the errors involved in this calculation, however, the value of $^{15}k_5$ is probably not significantly different from 1.0333. With the isotope effects of 7,8-dihydro-8-oxadenosine, eq 11–13 give $^DK_{eq3}{}^Dk_5 = 0.39 \pm 0.04$, $a = k_5/k_4 = 0.10 \pm 0.02$, and $^{15}k_5 = 1.0335 \pm 0.0004$[3]. The value of $^{15}k_5$ is better defined by the data in this case, and does come out in the expected range. The major difference between the values for adenosine and 7,8-dihydro-8-oxoadenosine is the ratio of k_5/k_4, which is 14-fold higher for adenosine.

The above solutions to eq 11–13 are quite reasonable, but since $^Dk_5$ can be realistically only be normal (the secondary deuterium isotope effect on the hydrogens of the $-NH_3^+$ group is normal, as would be any primary deuterium isotope effect if other proton motions are involved), this means that $^DK_{eq3}$ must be less than 0.4. Relaxing our requirements that $^Dk_3$ and $^{15}k_3$ are unity does not permit this limit to be raised appreciably, so we are forced to conclude that a sulfhydryl group *is* involved in the reaction, and that its hydrogen undergoes a transfer to nitrogen or oxygen during the reaction but *prior* to ammonia release.

Chemical Mechanism of the Reaction. Several chemical mechanisms for adenosine deaminase are consistent with these results. The first involves protonation of N-1 by the SH group, coupled with addition of water to C-6:



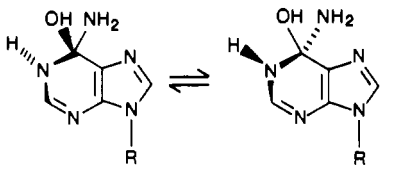
(14)

Alternatively, the last step could involve formation of the enol form of inosine by proton transfer from N-1 to the thiolate group on the enzyme. However, $^DK_{eq3}$ in this mechanism would be $\sim$0.34, so that $^Dk_5$ (the D_2O solvent isotope effect on the final step of ammonia release) would be $\sim$1.2, a reasonable value if the transition state for proton transfer to the enzyme base, shown as E–B, is early, but quite small if the proton on N-1 were also being transfered to the thiolate ($^DK_{eq} \sim 2$).

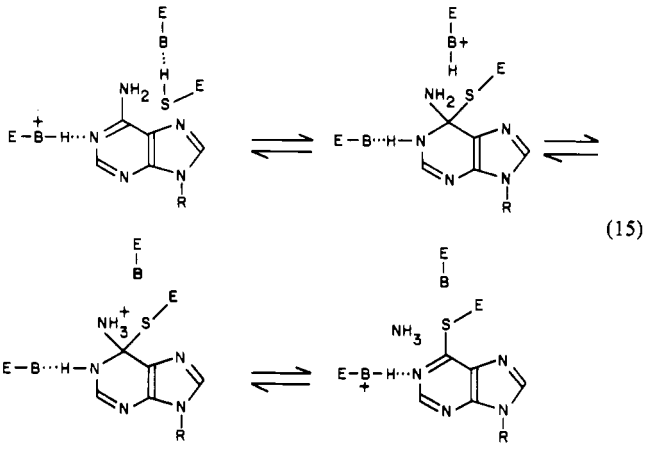
The problem with mechanism 14 is that there is no obvious way in which the enzyme can catalyze the chemistry shown; protonation of N-1 in solution certainly does not render C-6 especially electrophilic. However, if the enzyme were to bend N-1 (and to some extent C-2, since N-1, C-2, N-3, and C-4 must remain coplanar) out of the plane during the conformation change that leads to protonation of N-1 and induces catalysis, the aromaticity of the pyrimidine ring would be destroyed and C-6 would become very electrophilic, thus permitting water attack in the axial position. Bending N-1 out of the plane in the *other* direction would then place the amino group in the axial position from which it could readily leave:

[3] This corresponds to a three-step mechanism in which there are two intermediates, with the partition ratio of the first intermediate strongly favoring forward reaction and the partition ratio of the second intermediate (the one that decomposes to release ammonia) favoring reverse reaction. We have considered all of the possibilities for this more complete model but present only the simpler case, which is consistent with the data. Other choices for partition ratios in the more complex model give either no solutions or unrealistic ones.

[4] The fractional error in each solution parameter was calculated as the square root of the sum of the squares of the fractional errors contributed by each experimental parameter. The fractional error contributed by each experimental parameter was obtained by calculating solutions with each experimental parameter altered by its standard error.

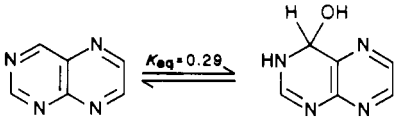


A second mechanism that has been proposed by Orsi et al. (1972) involves direct addition of the sulfhydryl group to C-6 as the result of protonation of N-1:

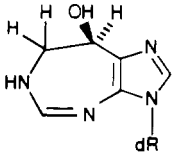


(15)

Water would then replace ammonia in the reverse of this reaction sequence to give inosine and release the sulfhydryl group. $^{D}K_{eq3}$ for such a mechanism would be 0.38, so $^{D}k_5$ would be 1.08. This seems small for a step where a proton is transfered, but we cannot rule out this type of mechanism solely on this basis. This mechanism is made less likely by the fact that the enzyme catalyzes the reversible hydration of pteridine (Evans & Wolfenden, 1973) but does not catalyze



dehydration of, or deuterium or ^{18}O exchange between, water and deoxycoformycin, an intermediate analogue, the 8*R* isomer of which has a K_i value of 2.5×10^{-12} M (Frick et al., 1986):



These data are most easily rationalized on the basis of direct attack of water on C-6 during the adenosine deaminase reaction. The 8*S* isomer binds less tightly than the 8*R* one by a factor of 1.3×10^7 (Schramm & Baker, 1985), but until the binding of amino analogues is evaluated, the stereochemistry of the intermediate cannot be assigned.

At this time we cannot determine which of these chemical mechanisms is correct for adenosine deaminase, although direct water attack on C-6 is most likely (mechanism 14). The fact that 1-deazaadenosine is a tightly bound inhibitor (K_i = 2 μM), while 3-deazaadenosine is neither a substrate nor an inhibitor at 100 μM levels (Ikehara & Fukui, 1974), suggests that N-1 is the position that must be protonated during reaction, but the hydrogen bond to N-3 is important for binding (or perhaps a nitrogen must be present at the 3-position to permit geometric deformation of the ring). In any case, the results reported here show definitely that a sulfhydryl group is involved in the mechanism and its hydrogen doubles its fractionation factor prior to release of ammonia.

Use of Adenosine Deaminase for Remote-Labeled Isotope Effect Studies. The exocyclic amino group of adenosine is a convenient remote label for accurate measurement of isotope effects in other positions of compounds containing adenosine. Thus to determine the primary ^{13}C isotope effect at C-4 of the nicotinamide ring of a pyridine nucleotide in a dehydrogenase reaction, one can prepare DPN labeled with ^{13}C in nicotinamide and with ^{15}N in the exocyclic amino group of adenosine. One also prepares DPN containing ^{14}N (that is, nitrogen with an ^{15}N content reduced an order of magnitude or more below natural abundance) in the exocyclic amino group of adenosine, but no ^{13}C label. These two species are mixed to produce material with the natural abundance ^{15}N ratio of 0.36% in the exocyclic amino group. Isotopic discrimination caused by ^{13}C will change the mass ratio in the exocyclic amino group of product or residual substrate. By degrading residual substrate or product with nucleotide pyrophosphatase, alkaline phosphatase, and adenosine deaminase, one obtains the exocyclic amino group as ammonia, since adenosine deaminase catalyzes the elimination of ammonia to give inosine. The ammonia is then converted to N_2 for isotope ratio mass spectrometry. Primary and secondary ^{18}O isotope effects for phosphoryl-transfer reactions can also be determined in this way, with appropriately labeled ATP.

These techniques are currently being developed in this laboratory, and the present study was originally undertaken in order to know how quantitative the conversion of adenosine to ammonia had to be to avoid isotopic fractionation. The low value for the ^{15}N isotope effect with adenosine (0.4%) means that incomplete deamination will not introduce large errors in such experiments.

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Registry No. Adenosine deaminase, 9026-93-1; adenosine, 58-61-7; 7,8-dihydro-8-oxoadenosine, 29851-57-8; 7,8-dihydro-8-oxoinosine, 63699-77-4; cadmium, 7440-43-9.

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The Rat Liver Insulin Receptor†

Klaus Hofmann,* Hana Romovacek, Gail Titus, Kevin Ridge, John A. Raffensperger, and Frances M. Finn

Protein Research Laboratory and Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

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ABSTRACT: Using insulin affinity chromatography, we have isolated highly purified insulin receptor from rat liver. When evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, the rat liver receptor contained the M_r 125 000 α-subunit, the M_r 90 000 β-subunit, and varying proportions of the M_r 45 000 β'-subunit. The specific insulin binding of the purified receptor was 25–30 μg of ^{125}I-insulin/mg of protein, and the receptor underwent insulin-dependent autophosphorylation. Rat liver and human placental receptors differ from each other in several functional aspects: (1) the adsorption–desorption behavior from four insulin affinity columns indicated that the rat liver receptor binds less firmly to immobilized ligands; (2) the ^{125}I-insulin binding affinity of the rat liver receptor is lower than that of the placental receptor; (3) partial reduction of the rat liver receptor with dithiothreitol increases its insulin binding affinity whereas the binding affinity of the placental receptor is unchanged; (4) at optimal insulin concentration, rat liver receptor autophosphorylation is stimulated 25–50-fold whereas the placental receptor is stimulated only 4–6-fold. Conversion of the β-subunit to β' by proteolysis is a major problem that occurs during exposure of the receptor to the pH 5.0 buffer used to elute the insulin affinity column. The rat receptor is particularly subject to destruction. Frequently, we have obtained receptor preparations that did not contain intact β-subunit. These preparations failed to undergo autophosphorylation, but their insulin binding capacity and binding isotherms were identical with those of receptor containing β-subunit. Proteolytic destruction and the accompanying loss of insulin-dependent autophosphorylation can be substantially reduced by proteolysis inhibitors. In summary, rat liver and human placental receptors differ functionally in both α- and β-subunits. Insulin binding to the α-subunit of the purified rat liver receptor communicates a signal that activates the β-subunit; however, major proteolytic destruction of the β-subunit does not affect insulin binding to the α-subunit. This suggests that communication does not occur in the reverse direction, i.e., $\beta \rightarrow \alpha$.

Jacobs et al. (1977) reported the first isolation of an insulin receptor preparation, i.e., that of rat liver. Their material exhibited a specific activity of 2.4 μg of ^{125}I-insulin bound/mg of protein. The scheme employed in this pioneering investigation, which provided the blueprint for all subsequent isolations of insulin receptors, involved (1) preparation of a crude membrane fraction, (2) solubilization of the receptor with Triton X-100, (3) chromatography on DEAE-cellulose, and (4) affinity chromatography on insulin–agarose resins. Although a number of studies characterizing the rat liver insulin receptor have appeared, we are not aware of studies aimed at isolation and characterization of homogeneous rat liver receptor. This paper deals with this subject.

In a previous paper (Finn et al., 1984) we have described an affinity resin for the routine isolation of human placental insulin receptor that was prepared by noncovalently attaching ligand II (Figure 1) to succinoylavidin–Sepharose. Having available a reliable affinity resin for the isolation of insulin receptors from human placenta, we reasoned that it should be a simple matter to isolate insulin receptor from rat liver by the same procedure. This, however, proved not to be the case. We were surprised to find that wheat germ lectin purified rat insulin receptor failed to bind to the above-mentioned affinity resin under conditions that were employed for the isolation of the human placental receptor. This observation suggested that placenta and rat liver receptors were different and prompted a systematic comparison of the adsorption–desorption characteristics of the two receptors with a series of affinity resins containing different ligands. In connection with these studies, we have employed two new ligands (compounds III and IV, Figure 1), whose syntheses are described.

Using an affinity resin based on ligand IV, we succeeded in isolating purified rat liver insulin receptor in good yield essentially by the procedure described for the isolation of the human placental receptor. Unfortunately, many of our

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