Substrate Properties of Cycloadenosines with Adenosine Aminohydrolase as Evidence for the Conformation of Enzyme-Bound Adenosine†

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ABSTRACT: Chemical removal of the isopropylidene group of 2′,3′-*O*-isopropylidene-8,5′-cycloadenosine has yielded a mixture of the two 5′ epimers of 8,5′-cycloadenosine. The stereochemically homogeneous allo and talo epimers of 5′-*C*-methyl and 5′-*C*-carboxamidoadenosine were also obtained from their respective isopropylidene derivatives. With calf intestinal adenosine aminohydrolase (EC 3.5.4.4), Michaelis constants and V_{max} values relative to adenosine were: adenosine, 55 μM, 100%; 8,2′-anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine, 180 μM, 1.7%; 8,3′-anhydro-8-hydroxy-9-β-D-xylofuranosyladenine was not a substrate; 8,5′-cycloadenosine (two 5′ epimers), 100 and 105 μM, 10.2 and 0.10%, respectively; 9-(6-deoxy-β-D-allofuranosyl)adenine, 78 μM, 0.36%; 9-(6-deoxy-α-L-talofuranosyl)adenine, 21 μM, 28%; 9-(β-D-allofuranuronamide)adenine was not a substrate; 9-(α-L-talofuranuronamide)adenine, 25 μM, 0.03%. From the substrate properties of various deoxyadenosines and 9-β-D-xylosyl- and arabinosyladenines, and from evidence that the adenine ring and the 5′-hydroxyl interact directly with the enzyme in the enzyme–adenosine complex, it is reasoned that enzyme-bound adenosine has an anti conformation in which H-8 is oriented most probably in the area above C-4′ and the furanose ring oxygen, and that the 5′-hydroxyl is probably located between H-3′ and H-4′. The lack of substrate activity of the 8,3′-anhydrocycloadenosine is ascribed to inability of the 5′-hydroxyl to reach its enzymic binding site as a consequence of prevention by O-3′ and C-8 of rotation of O-5′ about the 4′,5′ bond. The substrate properties of the talo and allo epimers of 5′-*C*-methyladenosine suggests the possibility that in the adenosine–enzyme complex the enzyme may closely approach the vicinity of H-4′, H-5′, and/or the furanose ring oxygen of adenosine.

Knowledge of the conformation of adenosine, both in aqueous solution and when bound to adenosine-utilizing enzymes, is clearly important for understanding the functioning of the biologically ubiquitous adenine nucleotides and nucleotide coenzymes. In addition, antibiotic properties are possessed by many adenine nucleosides (Suhadolnik, 1970). The major potential conformational variations in adenosine (Figure 1) are associated with modes of puckering of the ribofuranose ring and with rotation around the 9,1′ (glycosidic) and 4′,5′ bonds. A variety of physical analyses of purine nucleoside conformation in solution have been performed (Ts'o, 1970; Schirmer *et al.*, 1972, and references therein; Ikehara *et al.*, 1972) and the results have most frequently been interpreted to indicate low glycosidic rotational barriers with the population of anti rotamers probably in excess of the syn rotamers. It has been suggested that calf intestinal adenosine aminohydrolase (EC 3.5.4.4) utilizes an anti form of adenosine because a fixed rotamer with partial anti character, 8,2′-anhydro-8-mercapto-9-β-D-arabinofuranosylpurine, was found to be a substrate (Ogilvie *et al.*, 1971). The present report examines in more detail the question of the conformation of the aminohydrolase-bound adenosine with the aid of three cycloadenosines which possess varying degrees of fixed anti character. Two of these are the known 8,3′- and 8,2′-*O*-anhydrocyclonucleosides II and III (Ikehara, 1969). We recently described synthesis of 2′,3′-*O*-isopropylidene-8,5′-cycloadenosine (Harper and Hampton, 1972) and now report that chemical removal of the isopropylidene group furnishes 8,5′-cycloadenosine, I (Figure 2). This compound is of interest because its glycosidic torsion angle has a high degree of anti character; moreover, it was obtained as a mixture of two 5′ epimers which differed considerably from one another in their substrate properties and thereby served to furnish information regarding the 4′,5′ torsion angle of enzyme-bound adenosine.

The stereochemistry of the catalytic site of adenosine aminohydrolase has been further studied with the aid of two pairs (IV and V) of stereochemically homogeneous 5′ epimers which differ structurally from adenosine only by substitution of a single methyl or carboxamido group at the 5′ position.

Materials and Methods

Enzyme and Assay. Adenosine aminohydrolase of calf intestinal mucosa was crystalline material (type I) supplied by the Sigma Chemical Co. Initial velocity studies were carried out at 20° in a final volume of 1 ml by addition of the enzyme to 0.05 M Tris-HCl buffer (pH 7.6), followed by the substrate. The deamination was followed by measuring the decrease in optical density at 265 nm with a Cary Model 15 spectrophotometer. Michaelis constants and maximal velocity values were obtained from double-reciprocal substrate-initial velocity plots using four or more substrate concentrations. The enzyme concentration varied from 10^{-2} to 10 μg per ml of assay solution according to the substrate activity of the compound studied.

Adenosine Derivatives. Solvent systems for thin-layer chromatography (tlc, on cellulose) were: (A) 1-butanol–acetic acid–water (4:1:5), (B) isopropyl alcohol–ammonia–water

† From The Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111. *Received August 14, 1972.* This work was supported by U. S. Public Health Service Grant CA-11196, American Cancer Society Grant T-490, an award from the Pennsylvania Science and Engineering Fund, and by grants to The Institute for Cancer Research (U. S. Public Health Grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania).

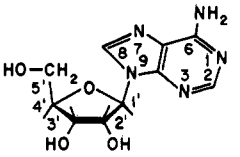

FIGURE 1: Structure of adenosine (illustrated as an anti-type conformer).

(7:1:2), (C) ethanol–1 M aqueous ammonium acetate (7:3), and (D) isoamyl alcohol–5% aqueous K_2HPO_4 (2:3).

The preparation of IVa and Va has been previously described (Howgate and Hampton, 1972). Compounds II and III were gifts from Dr. M. Ikehara and were crystallized from water immediately prior to use; both products ran as a single spot upon tlc on silica gel in methanol–chloroform (3:7). The melting points and ultraviolet spectra of the samples agreed with those reported (Ikehara *et al.*, 1968; Kaneko, 1970). At 265 nm, $\Delta\epsilon$ for III was 6,600.

The nucleoside IVb was prepared by heating 10 mg of 2′,3′-*O*-isopropylidene-9-(α-L-talofuranuronamide)adenine (A. Hampton and P. J. Harper, 1972, unpublished data) in aqueous acetic acid (pH 2.5) at 90° until tlc (system D) showed no starting material (*ca.* 1 hr). Removal of volatiles *in vacuo* at 25° gave a white crystalline powder showing one spot in systems A, B, C, and D with R_F values 0.12, 0.41, 0.65, and 0.85, respectively, and ultraviolet maxima at 256 nm (ε 14,900) (pH 2) and 259 nm (ε 15,200) (pH 7). Nucleoside Vb was prepared in the same manner from 2′,3′-*O*-isopropylidene-9-(β-D-allofuranuronamide)adenine (A. Hampton and P. J. Harper, 1972, unpublished data) and obtained as a solid which showed one spot on tlc in systems A, B, C, and D with R_F values 0.08, 0.35, 0.55, and 0.90, respectively, and ultraviolet maxima at 256 nm (ε 15,000) at pH 2 and at 258 nm (ε 15,600) at pH 7.

8,5′-Cycloadenosine (compound I) was obtained from its 2′,3′-*O*-isopropylidene derivative (Harper and Hampton, 1972) by an acidic treatment identical with the foregoing. This gave material which showed two spots on tlc. The material of higher R_F, which corresponded to 8,5′-cycloadenosine, was isolated in 50% yield by preparative tlc in system B. It had R_F values of 0.40, 0.68, 0.78, and 0.75 in systems A, B, C, and D, respectively, reacted positively toward a periodate spray for *cis*-α-glycols, and had ultraviolet maxima (pH 2) at 263 nm (ε 14,600) and (pH 7) 267 nm (ε 14,300).

Results

All the double-reciprocal substrate–initial velocity plots were linear. The Michaelis constants and maximal velocity values obtained from these plots are given in Table I. In the case of 8,5′-cycloadenosine the kinetic constants for the more rapidly reacting 5′ diastereoisomer were obtained with 0.01 μg of enzyme/ml of assay mixture, while the constants of the other diastereoisomer were obtained with 0.1 μg of enzyme after allowing sufficient time for all the other isomer to be deaminated. The deamination of 8,2′-anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine (2 μmoles) by 10 μg of enzyme in 100 μl of 0.05 M Tris buffer, pH 7.6 (*i.e.*, a 10-fold higher enzyme level than used in the spectrophotometric assay), was followed by tlc on silica gel with methanol–chloroform (3:7, v/v) as solvent; after 2 hr at 37°, the substrate (R_F 0.40; absorption maximum 255 nm at pH 7.6) was replaced by material of R_F 0.26 which in pH 7.6 buffer showed an absorption maximum at 250 nm (shoulder at 270 nm). Under the same conditions, 8,3′-anhydro-8-hydroxy-9-β-D-xylofuranosyladenine was unaffected.

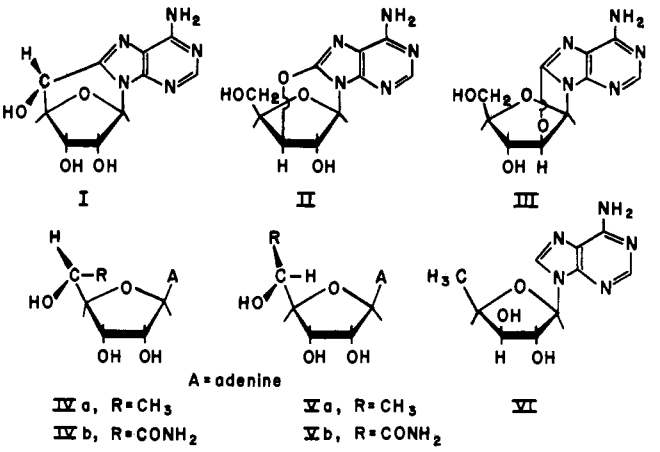

FIGURE 2: Structures of adenine nucleosides examined as substrates of adenosine aminohydrolase. The compounds are named in Table I, except for VI, 5′-deoxy-9-β-D-xylofuranosyladenine. Compound I is depicted as the epimer thought to be preferentially bound by the enzyme.

Discussion

The synthetic route to 8,5′-cycloadenosine comprised borohydride reduction of 5′-keto-2′,3′-*O*-isopropylidene-8,5′-cycloadenosine to 2′,3′-*O*-isopropylidene-8,5′-cycloadenosine (Harper and Hampton, 1972) (creating a new asymmetric center at C-5′), followed by removal of the isopropylidene group. As discussed elsewhere (Hampton *et al.*, 1972) the reduction is capable of proceeding stereoselectively. This

TABLE I: Adenosine Derivatives as Substrates of Adenosine Deaminase of Calf Intestine.

Compound	K_M (μM)	Rel V_{max}
Adenosine	55	100
8,2′-Anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine (III)	180	1.7
8,3′-Anhydro-8-hydroxy-9-β-D-xylofuranosyladenine (II)	*a*	<0.0001[c]
8,5′-Cycloadenosine (two 5′ epimers) (I)	100 105	10.2 0.10
9-(6-Deoxy-β-D-allofuranosyl)-adenine (Va)	78	0.36
9-(6-Deoxy-α-L-talofuranosyl)-adenine (IVa)	21	28.0
9-(β-D-Allofuranuronamide)-adenine (Vb)	*b*	<0.001[c]
9-(α-L-Talofuranuronamide)-adenine (IVb)	25	0.03

[a] Deamination was too slow to allow determination of K_M: with 10 μg of enzyme the optical density of a 2.2 mM solution decreased by only 0.015 in 30 min. [b] No detectable substrate activity. [c] Calculated from the assumption that the K_M value was the same as that of adenosine.

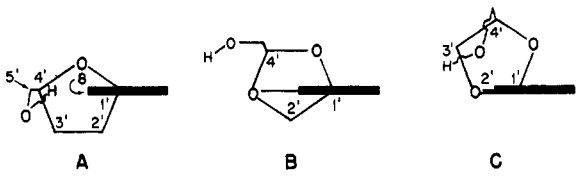

FIGURE 3: Diagrams of enzyme-bound adenosine aminohydrolase substrates. The adenine ring is shown edge-on with C-8 located as indicated. (A) Adenosine, (B) 8,3′-anhydro-8-hydroxy-9-β-D-xylofuranosyladenine (II), and (C) 8,2′-anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine (III).

was reflected in the finding that the ratio of the 5′ epimers of 8,5′-cycloadenosine, as determined with the adenosine aminohydrolase, varied widely from one preparation to another despite attempts to standardize reaction conditions. Attempts at separation of the epimers by crystallization or partition chromatographic procedures were not successful.

Studies with deoxy derivatives of adenosine have shown that the 5′-hydroxyl is required for substrate activity with adenosine aminohydrolase whereas the 2′- and 3′-hydroxyls are not (York and LePage, 1966; Bloch *et al.*, 1967; Cory and Suhadolnik, 1965; Chassy and Suhadolnik, 1967). Furthermore, the enzymatic deamination involves addition of water across the 1,6 bond from one specific side of the purine ring system (Wolfenden, 1972), showing that for deamination to occur the adenine portion of adenosine must be bound to the enzyme in one exclusive orientation. Interaction of the 5′-hydroxyl group with the enzyme presumably occurs simultaneously with adenine binding at some stage of catalysis, thereby severely restricting rotation about the 1′,9 and 4′,5′ bonds of the enzyme-bound adenosine. It is possible that the ribofuranose ring of adenosine may not interact with the enzyme (Baker, 1967) and, if so, this could permit a limited degree of synchronous rotation about the foregoing bonds without perturbing binding of the adenine nucleus or of the 5′-OH. That the V_{max} of one of the 5′ epimers of 8,5′-cycloadenosine (I) is 10% that of adenosine itself and 100-fold greater than that of the other 5′ epimer is consistent with such a view and indicates that during rate-determining catalytic events adenosine is bound to the enzyme with an anti-type sugar–base torsion angle and that O-5′ is oriented to either one side or the other of the plane in 8,5′-cycloadenosine defined by C-1′, C-4′, and C-5′. The preferred 4′,5′ rotamer of enzyme-bound adenosine probably resembles sterically the 5′ (*S*) configuration of 8,5′-cycloadenosine (depicted in Figure 2) in which O-5′ is situated between H-3′ and H-4′, because such a rotamer is stabilized by staggered 4′,5′ substituents and by approximate equalization of the distances between O-3′, O-4′, and O-5′. In particular, this assignment of the position of enzyme-bound O-5′ is in accord with the conclusion of Shah *et al.* (1965) that the binding site of the 5′-OH of adenosine is accessible to the 3′-OH of 5′-deoxyxylofuranosyladenine (VI), a compound which is a substrate of adenosine aminohydrolase (V_{max} 6% that of adenosine; York and LePage, 1966).

That enzyme-bound adenosine possesses an anti-type adenine–ribose torsion angle is further supported by the substrate activity of 8,2′-anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine (Figure 2, III). The V_{max} value of this cyclonucleoside was only 1.7% that of adenosine and considerably less than the values for 2′-deoxyadenosine (a better substrate than adenosine at the one level tested; Cory and Suhadolnik, 1965) and 9-β-D-arabinofuranosyladenine (V_{max} 26% that of adenosine; York and LePage, 1966) and the weak activity is hence not associated either with the absence of the 2′-OH of adenosine or with the presence of O-2′ of 9-β-D-arabinofuranosyladenine. That the V_{max} of this 8,2′-cyclonucleoside (III) was less than 20% that of 8,5′-cycloadenosine (I) tends to suggest that in enzyme-bound adenosine the anti conformation may more likely be such that H-8 is positioned over H-3′, H-4′, or O-4′ than over H-2′. Further discussion of the glycosidic torsion angle is presented later.

A significant observation was the absence of detectable substrate properties in 8,3′-anhydro-8-hydroxy-9-β-D-xylofuranosyladenine (Figure 2, II). This is presumably unrelated to the inversion in II of O-3′ of adenosine inasmuch as the V_{max} values of 3′-deoxyadenosine and of 9-β-D-xylofuranosyladenine are 55 and 58%, respectively, that of adenosine (York and LePage, 1966); it is also not attributable simply to an unfavorable sugar–base torsion angle in view of the substrate properties of cycloadenosines I and III. It was suggested above that H-8 of enzyme-bound adenosine is near C-4′ and that O-5′ is positioned between H-4′ and H-3′ in similar fashion to one of the 5′ epimers of 8,5′-cycloadenosine (see Figure 3, structure A). This would mean that the ribofuranose ring of the 8,3′-anhydronucleoside II would become displaced from its normal position within the enzyme site subsequent to specific binding of its adenine ring system, thereby requiring rotation of the 5′-OH toward C-3′ to enable it to interact with the 5′-OH site of the enzyme (Figure 3B). Examination of a Corey–Pauling–Koltun molecular model showed that this rotation is blocked by O-3′ and C-8, thus precluding enzymatic deamination for which participation of the 5′-OH of adenosine or another suitably positioned hydroxyl is mandatory. Further, the inertness of II implies that its O-3′ does not bind to the 5′-OH site, either because this may require participation of a hydroxylic hydrogen, or because the adjoining imidazole ring exerts steric interference, or because O-3′ of II is unable to rotate about either of its bonds. 9-β-D-Xylofuranosyladenine, although structurally similar to II, is a good substrate (York and LePage, 1966); in this case, however, the substrate is free to bind with a normal glycosidic torsion angle and the 5′-OH can interact with the site without interference from O-3′. In the case of 8,2′-anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine (III), O-2′ cannot prevent rotation of O-5′ around the 4′,5′ bond, and the substrate activity of this compound could result from the ability of the 5′-hydroxylic hydrogen to assume a similar spatial relationship (depicted in Figure 3C) to the adenine moiety as postulated for enzyme-bound adenosine (Figure 3A).

Several lines of evidence tend to indicate that the glycosidic torsion angle of enzyme-bound adenosine is characterized by proximity of H-8 to either O-4′ or C-4′. An 8,2′-type torsion angle, for example, is contraindicated by the relatively weak substrate activity of III; in addition it should be noted that were an 8,2′ torsion angle to be postulated, then the lack of substrate properties of II could no longer be accounted for along the lines suggested above and would, in fact, become difficult to explain by other means.[1] The substrate effectiveness of 9-β-D-xylofuranosyladenine (V_{max} 58% that of adenosine; York and LePage, 1966) is essentially the same as that of 3′-deoxyadenosine (55%) to indicate that the two compounds are probably interacting with the enzyme in the same manner and that, in particular, O-3′ of the xylosyladenine manifests

[1] The inactivity of II would likewise be difficult to explain from the supposition that the 4′,5′ torsion angle of enzyme-bound adenosine resembled that of the 5′(*R*) configuration of 8,5′-cycloadenosine

effect on V_{max}. On this basis, if the glycosidic torsion angle enzyme-bound adenosine resembles that of the 8,3′-cyclo mpound II, then this latter compound should, like xylosyl-enine, be a good substrate. That II is essentially devoid of bstrate activity therefore argues strongly against an 8,3′-pe torsion angle and leads to the conclusion that H-8 is ost likely directed toward C-4′ or O-4′ in the adenosine–zyme complex. Studies with 8,5′-cyclo-AMP (Hampton *al.*, 1972) have indicated that AMP, when complexed with MP-aminohydrolase and several other enzymes, possesses ose–adenine and 4′,5′ torsion angles similar to those sug-sted here for adenosine.

The talo epimer of 5′-*C*-methyladenosine (IVa) exhibits onounced substrate activity, whereas its allo isomer (Va) 80-fold less effective. Corey–Pauling–Koltun space-filling odels in which O-5′ was positioned as proposed for enzyme-und adenosine (see Figure 2, IV and V) showed that the ethyl group of the talo isomer is more favorably situated an the methyl of the allo isomer to interfere with attainment an 8,5′-type torsion angle by interaction with H-8, although ch interference requires specific puckering modes of the ofuranose ring. An effect of this nature, however, predicts order of substrate effectiveness of IVa and Va opposite to at observed. The relatively weak activity of the allo epimer could, on the other hand, indicate that the enzyme–enosine complex possesses very limited bulk tolerance jacent to the 5′-hydrogen atom which is situated between -4′ and H-4′, and which is substituted by a methyl group the allo epimer, as depicted in Figure 2. A possibly related servation is that substitution of a methylene group for -4′ abolishes substrate activity, and it has been suggested at, among other reasons, this could be due to very limited lk tolerance near O-4′ (Schaeffer *et al.*, 1964). The good bstrate activity of the talo epimer IVa may derive from its nfigurational similarity, when enzyme bound, to the active imer of 8,5′-cycloadenosine. Thus, reference to Figure 2 ows that the methyl group of IVa, analogously to the 8,5′ nd of I, is directed toward C-8 and is centered above the ane defined by C-1′, C-4′, and C-5′. The moderately good bstrate properties of I suggests that this location of the ethyl group of IVa may represent a region of bulk tolerance the enzyme–substrate complex.

The feeble substrate activities of the epimeric 5′-carbox-nidoadenosines (IVb and Vb) could be associated with creased bulk of the 5′ substituent or with interference with ucial enzyme binding of the 5′-OH as a result of its tendency to intramolecularly hydrogen bond to the amide carbonyl group.

Acknowledgments

The authors thank Dr. Morio Ikehara of Osaka University for providing 8,2′-anhydro-8-hydroxy-9-β-D-arabinofuranosyl-adenine and 8,3′-anhydro-8-hydroxy-9-β-D-xylofuranosyl-adenine.

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