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SHORT COMMUNICATIONS

Substrate specificity of adenosine deaminase: the role of the substituents at the 2′- and 3′-carbons of adenine nucleosides, of their configuration and of the conformation of the furanose ring

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Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) plays an important regulatory role in purine metabolism, affecting adenine nucleotide levels [1]. Adenine nucleoside analogues of chemotherapeutic interest, particularly those modified in the carbohydrate moiety, such as ara-Ade, 3′dAdo, xylo-Ade, are rapidly deaminated by adenosine deaminase to inactive or considerably less active hypoxanthine derivatives [2]. The search of new analogues with low susceptibility to deamination is one of the ways for improvement on biological activity. A great deal of work has been carried out concerning the substrate specificity of adenosine deaminase and a comprehensive review of early investigations has appeared [3]. A number of structural and conformational requirements necessary for compounds to bind to the enzyme were clarified [4–9]. It is interesting to note that there are contradictory data concerning the role of the substituents at C-2′ and C-3′ in the carbohydrate moiety of adenine nucleosides and of their configuration on the binding of substrates to the enzyme; compare, e.g., the data of [10–13] with that of [4, 14, 15]. The aim of this study is an examination in more detail of the role of the various C-2′ and C-3′ substituents of adenine nucleosides, their configuration and the pentofuranose ring conformation in the adenosine deaminase-catalyzed deamination.

Adenosine deaminase (adenosine aminohyrolase, EC 3.5.4.4) from calf intestinal mucosa, specific activity 200 units/mg protein, solution in 50% glycerol, Ado and 2′dAdo were purchased from Boehringer Mannheim (FRG). Cordycepin was obtained from Sigma Chemical Co. (USA), and ara-Ade from Serva Feinbiochemica (Heidelberg, FRG). The preparation of the other compounds studied has been previously described [16–18].

The deamination of all compounds was followed by measuring the change of optical density at 265 nm resulting from the conversion of the adenine chromophore to hypoxanthine [19]. Assays were carried out at 22 ± 2° with a digital spectrophotometer Zeiss PMQ-II (FRG) and/or a recording spectrophotometer Shimadzu UV-200 (Japan) using 1.0 ml cuvettes with 1.0 cm path lengths. Initial solutions of the adenine nucleosides were made in 0.05 M phosphate buffer, pH 7.4, at 60–70 μM concentrations and were then successively diluted by the same buffer to a 10 μM concentration. We have observed that adenosine deaminase, 0.01 unit/ml, is inhibited by adenosine at concentrations higher than 70 μM. Adenosine deaminase was diluted to a concentration of 5 μg/ml in 0.05 M phosphate buffer, pH 7.4, 20 μl (0.02 unit of the enzyme) of this solution were added directly into the cuvette to a given amount of substrate in 1 ml of the same buffer and readings were made every 10 sec. The molar absorbancy for adenosine and analogues employed in these studies was assumed to be 8600 $M^{-1} \cdot cm^{-1}$ [20]. The initial rate values were used for calculation of K_m and V_{max} according to the method of Lineweaver and Burk [21]. The absolute value for V_{max} of 288 μmoles $\cdot$ min^{-1} per mg enzyme [22] or of 1.44 μmoles $\cdot$ min^{-1} per unit of enzyme was found for adenosine in the range of concentrations of 10–50 μM and 0.01 unit/ml of the enzyme.

The kinetic parameters, K_m and V_{max}, of 27 compounds studied with adenosine deaminase are presented in Table 1. The configuration of the substituents at C-2′ and C-3′ of Ado, xylo-Ade and ara-Ade form the parent compounds of the three groups of analogues examined. 2′,3′an-Ado and 2′,3′an-lyxo-Ade also are depicted (Fig. 1). Some of the compounds have been examined earlier [3] but are included in this study for comparative purposes. Our attention was focused on those analogues with activity comparable to the natural substrate. Therefore, for analogues which were hydrolyzed at very slow rates, the V_{max} values have not been determined, as would be possible using a large excess of the enzyme. We have observed that a substrate inhibition occurred at adenosine concentration higher than 70 μM and 0.01 unit/ml of adenosine deaminase (cf., e.g., [4] and [14]).

Ribo series. The nature of the substituents at the C-2′ and the C-3′ carbons of the sugar moiety of adenosine nucleosides substantially affects their kinetic parameters in the adenosine deaminase reaction. Substitution of a hydrogen atom for the hydroxyl group at C-2′ does not lead to an essential alteration of the K_m, but the V_{max} of 2′dAdo is 1.85-fold greater than that of Ado. The K_m value increases upon going from Ado to 3′dAdo (about 1.5-fold) and the V_{max} value also increases. Practically identical K_m values have been found for 2′dAdo and 2′Cl-2′dAdo. In contrast the K_m value of 3′Cl-3′dAdo is about 4-fold higher than that of Ado, whereas the V_{max} value is 2-fold lower. Remarkably, the K_m value of 3′NH_2-3′dAdo is 7-fold greater than that of Ado perhaps because amino group and hydroxyl group differ considerably in participating in hydrogen bonds [23, 24]. The enzyme–substrate affinity is substantially enhanced upon replacement of the 2′ hydroxyl group by an azido group. Comparing 3′N_3-3′dAdo with 3′Cl-3′dAdo it is clear that an azido group enhances the affinity of the substrate to the enzyme, K_m, and the breakdown of the enzyme–substrate complex, V_{max}.

Xylo series. A change in the 3′hydroxyl group configuration from *ribo* to *xylo* impairs the binding of the substrate to the enzyme about 2-fold. An additional replacement of the 2′hydroxyl group by a hydrogen atom results in about a 7-fold reduction of the affinity of the substrate, 2′d-xylo-Ade, to the enzyme. A bromine atom at C-2′ leads to a marked improvement of the binding of 2′Br-xylo-Ade with the enzyme as compared to 2′d-xylo-Ade, and with an azido group at C-2′, 2′N_3-xylo-Ade, the affinity is similar to that of adenosine. A hydroxyl group at C-3′ of xylo-Ade is apparently important for binding since a chlorine atom, having approximately the same spatial size as a hydroxyl group, cannot assume the function of this group. With an increasing bulk of the substituents at C-3′ in the *xylo* configurations as, e.g., in the case of the 3′Br- and 3′I-xylo-Ade, the substrate activity was practically abolished, indicating that the enzyme–substrate complex cannot tolerate a large group at the C-3′ *xylo* position. The presence

Table 1. Kinetic parameters, K_m and V_{max}, of adenine nucleosides for adenosine deaminase

Compound	Abbreviation	K_m(μM)	V_{max} (rel.)
Ribo series			
Adenosine	Ado	31	100
2′-Deoxyadenosine	2′dAdo	34	185
3′-Deoxyadenosine (cordycepin)	3′dAdo	43	120
3′-Chloro-3′-deoxyadenosine	3′Cl-3′dAdo	118	45
2′-Chloro-2′-deoxyadenosine	2′Cl-2′dAdo	35	146
3′-Azido-3′-deoxyadenosine	3′N_3-3′dAdo	52	81
2′-Azido-2′-deoxyadenosine	2′N_3-2′dAdo	19	27
3′-Amino-3′-deoxyadenosine	3′NH_2-3′dAdo	218	148
2′-Amino-2′-deoxyadenosine	2′NH_2-2′dAdo	208	40
2′,3′-Anhydroadenosine	2′,3′an-Ado	555	20
Xylo series			
9-β-D-Xylofuranosyladenine	xylo-Ade	73	55
2′-Deoxy-9-β-D-xylofuranosyladenine	2′d-xylo-Ade	208	63
3′-Chloro-3′-deoxy-9-β-D-xylofuranosyladenine	3′Cl-xylo-Ade	333	55
3′-Bromo-3′-deoxy-9-β-D-xylofuranosyladenine	3′Br-xylo-Ade	*	*
2′-Bromo-2′-deoxy-9-β-D-xylofuranosyladenine	2′Br-xylo-Ade	118	80
3′-Iodo-3′-deoxy-9-β-D-xylofuranosyladenine	3′I-xylo-Ade	*	*
3′-Azido-3′-deoxy-9-β-D-xylofuranosyladenine	3′N_3-xylo-Ade	416	16
2′-Azido-2′-deoxy-9-β-D-xylofuranosyladenine	2′N_3-xylo-Ade	32	126
Arabino series			
9-β-D-Arabinofuranosyladenine	ara-Ade	142	53
3′-Deoxy-9-β-D-arabinofuranosyladenine	3′d-ara-Ade	†	†
3′-Chloro-3′-deoxy-9-β-D-arabinofuranosyladenine	3′Cl-ara-Ade	†	†
2′-Chloro-2′-deoxy-9-β-D-arabinofuranosyladenine	2′Cl-ara-Ade	149	20
3′-Bromo-3′-deoxy-9-β-D-arabinofuranosyladenine	3′Br-ara-Ade	*	*
3′-Iodo-3′-deoxy-9-β-D-arabinofuranosyladenine	3′I-ara-Ade	*	*
2′-Iodo-2′-deoxy-9-β-D-arabinofuranosyladenine	2′I-ara-Ade	†	†
3′-Azido-3′-deoxy-9-β-D-arabinofuranosyladenine	3′N_3-ara-Ade	91	9
2′,3′-Anhydro-9-β-D-lyxofuranosyladenine	2′,3′an-lyxo-Ade	530	98

* K_m value was so high that an exact evaluation became difficult; compound was deaminated very slowly.
† Compound is no substrate of the enzyme.

of an azido group at the C-3′ in the *xylo* configuration, 3′-N_3-xylo-Ade, reduces the affinity compared with xylo-Ade.

Arabino series. In the *arabino* series the presence of a hydroxyl or an azido group at C-3′ is an important requirement for the substrate activity. The *arabino* hydroxyl group at C-2′ does not apparently participate in the binding as judged by comparison of the K_m values for ara-Ade and 2′Cl-ara-Ade, and on the basis of the inability of 3′d- and 3′Cl-ara-Ade to act as substrates. The size of a substituent at C-2′ probably plays an important role as suggested by the absence of binding of 2′I-ara-Ade to the enzyme.

Anhydro compounds. An anhydro ring in the *ribo* and *lyxo* configurations of 2′,3′an-Ado and 2′,3′an-lyxo-Ade, respectively, impairs to a great extent the substrate activity. It is interesting to note that such a type of modification in the sugar as in the case of 2′,3′an-lyxo-Ade does not affect the processing of the enzyme–substrate complex to products, i.e. V_{max}, whereas the K_m is 17-fold greater than that of Ado.

Adenosine deaminase-catalyzed deamination of adenine-β-D-pentofuranosides at rates comparable to that of adenosine needs an *anti* conformation [7, 9] and a 4′ CH_2OH group [4, 5] in these compounds.* All compounds studied by us have a 4′CH_2OH group† and an *anti* conformation is highly probable [25, 26]. The substitutents at C-2′ and C-3′ of the analogues play a substantial role in the enzyme–substrate binding. However, not only the partial contribution of atoms and groups but also the conformational states of the furanose ring have to be considered. The carbohydrate moiety of the β-D-ribofuranosides exists mainly in the two ranges of the pseudorotational cycle centered at C-2′ *endo* and C-3′ *endo* conformations [25]. The population of the latter leads to an *anti* conformation about the glycosidic bond. Displacement of the C-3′ *endo* ⇌ C-2′ *endo* equilibrium towards the latter conformer causes an increase of the population of the *syn* state of the base [27]. There are considerably fewer conformational data for nucleosides with a *xylo* and an *arabino* configuration. From ara-Ade [28] and pyrimidine-β-arabinofuranosides it was concluded that there is a wider range of sugar pucker conformations available to *arabino* nucleosides [29]. Ara-Ade in aqueous medium is in the C-3′ *endo*/*anti* conformation [30]. A thorough conformational analysis of xylo-Ade, 3′Cl-xylo-Ade and 2′Cl-ara-Ade has pointed to a C-3′ *endo* ⇌ C-2′ *endo* equilibrium of the sugar fragments and to the *anti* conformation as the most populated state [26].

We suggest that (a) the enzyme accepts a substrate in C-2′ *endo* or C-3′ *endo* conformation and that (b) population of the sugar conformations of the adenosine analogues centered at the C-4′ *exo* or C-1′ *exo* regions of the

* The data of 2,5′- and 4,5′-anhydroformycins [9] supported [7, 8] an *anti* conformation to be essential, and suggested that a 5′ hydroxyl is not critically essential. Adenine is deaminated at an extremely slow rate [34]; that *lin*-benzoadenine and *lin*-benzoadenosine might be good substrates appears therefore puzzling [35].

† A *xylo* substituent at C-3′ can affect the spatial arrangement of a 4′ CH_2OH group that can in turn impair the binding [36]. However, with conformationally mobile nucleosides this need not be of critical importance in preventing an interaction of a 5′ hydroxyl group with the enzyme.

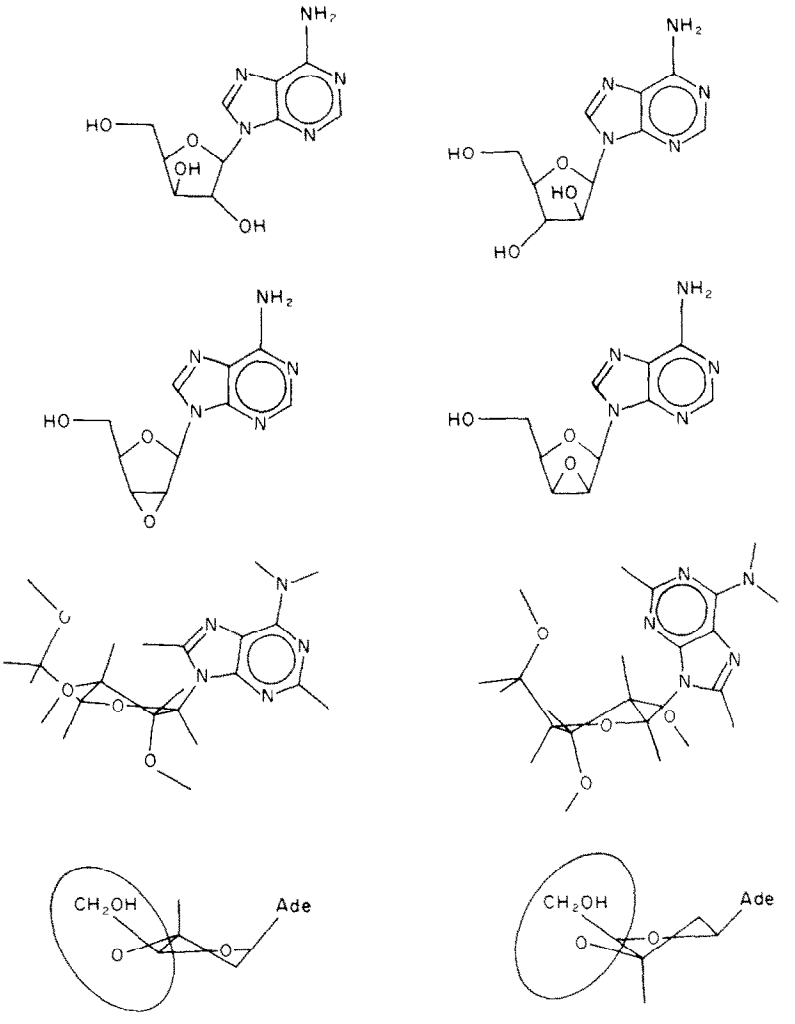

Fig. 1. Chemical formulae of 9-β-D-xylofuranosyladenine (above, left), 9-β-D-arabinofuranosyladenine (above, right), 2′,3′-anhydro-β-D-ribofuranosyladenine (below, left) and 2′,3′-anhydro-β-D-lyxofuranosyladenine (below, right), and stereochemical views of adenosine (above, left: C′-3′ *endo* (*N*) *anti*; above, right: C-2′ *endo* (*S*) *syn*; these are the most populated conformations) and of C-3′ *endo* ribonucleosides (below, left) and C-2′ *endo* xylonucleosides (below, right) with possible areas of hydrogen bond formation by the 3′-OH group.

pseudorotational cycle abolishes their substrate activity. We assume that (a) the 5′ and 3′ hydroxyl groups participate in the binding with the active centre of the enzyme by means of a hydrogen bond and that (b) the analogues having a *ribo* or *arabino* 3′ hydroxyl group bind to the enzyme in C-3′ *endo* conformation and those with a *xylo* 3′ hydroxyl group in C-2′ *endo* conformation (Fig. 1). It is of note that the hydrogen bond energy for an interaction of the R-OH. . .OR_2 type is 10–11 kJ·mole^{-1} and for the R-OH. . .NR_3 type is 12–13 kJ·mole^{-1} whereas for aliphatic amines of the R-NH_2. . .OR_2 type the energy does not exceed 1–3 kJ·mole^{-1} [23, 24]. Just for this reason 5′-amino-5′-deoxyadenosine is likely to be a very poor substrate for adenosine deaminase [4] and the affinity of 3′NH_2-3′dAdo to the enzyme is markedly reduced [10, 14]. The space in which hydrogen bond formation by a 3′ hydroxyl *ribo* or *arabino* group is possible in the C-3′ *endo* conformation overlaps considerably with that for a 3′ hydroxyl *xylo* group in C-2′ *endo* conformation. The absence of substrate activity of 3′*d*-ara-Ade [22] can be explained by the population of a C-4′ *exo* or C-1′ *exo* conformation. This explanation appears to be most likely in the case of the other compounds with a very low affinity towards the enzyme [6, 8, 27, 31, 32]. With bromine and iodine atoms the reason for the low affinity may be the bulk of the substituents or the conformational changes of the furanose ring mediated thereby. Compounds with an azido group at C-2′ in a *ribo* configuration show enhanced affinity and could potentially be used for affinity labelling. The low conformational mobility of 2′,3′an-Ado and 2′,3′an-lyxo-Ade possibly account for the low substrate activity of these analogues. It was earlier shown, that 2′,3′-didehydro-2′,3′-dideoxyadenosine is a poorer substrate for adenosine deaminase than is 2′,3′-dideoxyadenosine by reason of the lower mobility of the modified sugar ring [4]. *In vivo* biological activity of nucleosides is bound with their successive phosphorylation [33]. Bearing in mind the structural requirements of kinase-catalyzed reactions some of the compounds studied here can be considered as candidates of biological interest.

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Max-Planck-Institut für Experimentelle Medizin
Abteilung Chemie
D-3400 GÖTTINGEN
Hermann-Rein-Str. 3
Federal Republic of Germany

IGOR A. MIKHAILOPULO*
HARALD WIEDNER
FRIEDRICH CRAMER

* Alexander von Humboldt Foundation Fellow, permanent address: Institute of Biooganic Chemistry, Byelorussian SSR Academy of Sciences, 220600 Minsk, Leninsky prospect 68, U.S.S.R.

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