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Ligand Binding to the Adenine Analogue Binding Protein of the Rabbit Erythrocyte†

R. A. Olsson

ABSTRACT: Adenine analogue binding protein of rabbit erythrocytes reversibly binds [^{3}H]adenosine with a K_D of 5.3 $\times 10^{-9}$ M, an association rate constant of 1.4×10^{-12} M^{-1} min^{-1} and a dissociation rate constant of 7.5×10^{-3} min^{-1}, as estimated by a nonlinear curve-fitting program applied to data on the time course of the binding reaction. Independent estimates of K_D by Scatchard plots and of the dissociation rate constant by dilution or adsorption of free [^{3}H]adenosine on charcoal or by the addition of excess adenosine agreed closely with the estimates from the curve-fitting program. Inhibition of [^{3}H]adenosine binding by a series of 77 adenosine analogues was used to define the factors determining the binding affinity of this nucleoside. These are: (1) the size and aromaticity of the purine base; (2) a glycosylic torsion angle of approximately $-120°$; (3) the ribo configuration of the 2′- and 3′-hydroxyls and also the 5′-hydroxyl. Bulky substituents in the region of C-2′ and to a lesser extent in the region of C-3′ reduce binding affinity.

In the course of studies of the cAMP[1]-dependent protein kinases of rabbit erythrocytes, Yuh and Tao (1974) purified two proteins, both of which bound cAMP and adenosine but neither of which had a regulatory effect on the catalytic subunits of rabbit erythrocyte protein kinases I, IIa, or IIb. Detailed studies on the more abundant of the two proteins showed that adenosine in equimolar concentrations inhibited cAMP binding but not vice versa. Because the binding affinity (K_m) of adenosine was similar to that of cAMP, 10^{-7} and 3×10^{-7} M, respectively, but the binding capacity for adenosine was at least three times greater than for cAMP, they concluded that the protein possesses two types of binding sites. The more abundant site accommodates only adenosine, the other either adenosine or cAMP.

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[1] Abbreviations used: AABP, adenine analogue binding protein; BSA, bovine serum albumin; cAMP, cyclic adenosine 3′,5′-monophosphate; Hepes, *N*-2-hydroxyethylpiperazino-*N*′-2-ethanesulfonic acid; SEM, standard error of the mean; Tris, tris(hydroxymethyl)aminomethane; PEI, polyethylenimine.

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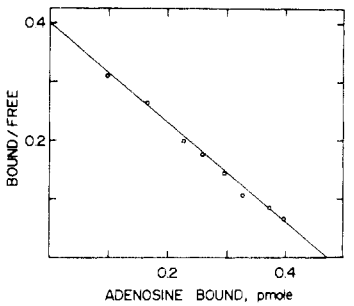


FIGURE 1: Scatchard plot of the binding of [^{3}H]adenosine to AABP. Tubes containing 7 μg of AABP, 0.73 pmol of [^{3}H]adenosine, 5×10^{-6} mol of Tris-HCl (pH 7.5), and 0–6.4 pmol of [^{12}C]adenosine in 0.1 mL were incubated 4 h at 0 °C. Free adenosine was separated with charcoal as described in the text and aliquots of the supernatant were counted. Each point is the mean of triplicate estimates.

More recently Sugden and Corbin (1976) isolated a similar protein from rat and bovine liver and, on the basis of its ability to bind a variety of 6-aminopurine derivatives, proposed the name adenine analogue binding protein (AABP). They showed that this protein differs from the regulatory subunit of protein kinases in molecular weight and susceptibility to inactivation by aging, trypsin, and heat. While a number of adenine derivatives displaced bound cAMP from AABP, they found that only adenosine and adenine inhibited the binding of [^{3}H]-adenosine.

This paper describes studies of the characteristics of [^{3}H]adenosine binding to rabbit erythrocyte AABP and of the determinants of binding affinity.

Experimental Procedure

Materials. Rabbit erythrocytes obtained from Pel-Freeze (Rogers, AR) were used to prepare AABP through the hydroxylapatite step exactly as described by Yuh and Tao (1974). The protein thus obtained was precipitated by adding $(NH_4)_2SO_4$ to 50% saturation at 0 °C. It was then redissolved in 0.05 M $KHPO_4$ (pH 6.5) containing 1 mM dithiothreitol, dialyzed against this buffer overnight, and stored at −80 °C. New England Nuclear supplied [2,8-^{3}H]adenosine, [2-^{3}H]-adenine, and [G-^{3}H]cAMP; [U-^{14}C]adenosine, [U-^{14}C]-5′-AMP, and [U-^{14}C]cAMP were from Amersham/Searle. The various sources of adenine analogues are listed in Table II. Prior to use cAMP solutions were purified by chromatography on 0.5 × 1 cm columns of PEI cellulose (Magnusson et al., 1976). The LiCl used to elute the cAMP was removed by chromatography on a column of Sephadex G-10 suspended in and eluted with water. This procedure quantitatively separates adenine and adenosine from cAMP.

Adenosine Binding Assay. The standard assay was performed in a total volume of 0.1 mL containing approximately 50 000 dpm of [2,8-^{3}H]adenosine (sp act. of various lots 28.2–32.6 Ci/mmol) in 50 mM Tris-HCl (pH 7.5). The optimum amount of AABP was determined in preliminary titrations by adding increasing amounts of protein to 50 000 dpm of [^{3}H]adenosine. The amount of protein capable of binding 70% of the maximum under these conditions, 4.5–10 μg in different lots of AABP, was added to each assay tube. After incubation for 4 h at 0 °C, the reaction mixture was diluted with 1 mL of ice-cold buffer, and then free and bound [^{3}H]-adenosines were separated by adsorption of the AABP-[^{3}H]adenosine complex on cellulose acetate membranes or by adsorbing unbound [^{3}H]adenosine on charcoal. When membrane filtration was employed, membranes having 0.22-μm pores were positioned in a multiport filter manifold and moistened with buffer. The reaction mixture was diluted with 1 mL of buffer and applied to the filter, followed by two 2-mL washes of the assay tube. The filters were then dissolved in 10 mL of Bray's solution for counting. Reproducibility depends critically on a filtration rate ≤5 mL/h. Free and bound [^{3}H]adenosine were also separated by diluting the reaction mixture with 1 mL of buffer and adding 25 μL of a mixture of (per 100 mL): 10 g of Norit A charcoal, 2.5 g of BSA, and 0.1 g of Pharmacia Dextran 250, pH adjusted to 7.5. After centrifugation at 700g for 30 min at 40 °C, an 0.7-mL aliquot of supernatant was counted.

cAMP Binding Assay. cAMP binding was assayed as described by Yuh and Tao (1974), the reaction mixture containing 70 μg of AABP, 160 000 dpm of [^{3}H]cAMP (sp act. 40 Ci/mmol), and unlabeled cAMP in 0.1 mL of 1 mM Tris-HCl (pH 7.5). After incubation at 37 °C for 1 h, the reaction mixture was cooled in ice water. Free and bound [^{3}H]cAMP in an 0.095-mL aliquot were separated at 2 °C on an 0.5 × 20 cm column of Sephadex G-50 equilibrated with 1 mM Tris-HCl (pH 7.5). The ^{3}H activity in 20-drop fractions was then counted.

Results

Characterization of AABP. Chromatography of an aliquot of the AABP preparation on an 0.5 × 20 cm column of QAE-Sephadex A-50 as described by Yuh and Tao (1974) revealed that the [^{3}H]adenosine binding activity emerged as a single peak at a KCl concentration of 0.12 M. cAMP binding activity coincided with and was limited to this peak. Thus, this preparation consisted entirely of receptor I. Aliquots of AABP (7 μg) were incubated with 50 μM solutions of [U-^{14}C]adenosine, [U-^{14}C]-5′-AMP, or [U-^{14}C]cAMP in 50 mM Tris-HCl (pH 7.5) for 4 h at 37 °C. Chromatography of 10-μL aliquots on thin-layer sheets of silica gel developed with 1-butanol:water:concentrated NH_4OH (86:14:1) by volume) and on sheets of PEI-cellulose developed in 1 M NaCl showed that all the ^{14}C activity was recovered as starting material. Thus, although this AABP was not purified to homogeneity, it did not contain enzymes which could degrade adenosine, 5′-AMP or cAMP under the conditions of the binding assays.

Binding of [^{3}H]Adenosine to AABP. Binding was essentially constant in 50 mM potassium phosphate buffer containing 0.5% BSA between pH 6.0 and 8.0, was 30% less in 20 mM phosphate–BSA, and was no greater in 100 mM phosphate–BSA. Binding was 70% greater in 50 mM Tris-HCl (pH 7.5) than in equimolar phosphate–BSA. Scatchard plots (Figure 1) revealed a single species of binding sites having a K_D (mean ± SEM) of $8.9 \pm 0.04 \times 10^{-9}$ M and an apparent binding capacity averaging 68.6 ± 1.3 pmol/mg of protein (12 experiments). At 0 °C, binding equilibrium was attained after 4 h and was essentially constant up to 6 h (Figure 2) but the amount of bound adenosine fell by about a third if incubation was extended to 18 h, probably reflecting a combination of AABP degradation as well as a decrease in the specific activity of the [^{3}H]adenosine by exchange of 8-^{3}H with solvent water (Elvidge et al., 1971; Tomasz et al., 1972). At 23 °C binding equilibrium was attained in 45 min and the subsequent decrease in activity began at 90 min and proceeded more rapidly than at 0 °C.

Analysis of the time course of [^{3}H]adenosine binding by means of a nonlinear curve fitting program (Rodbard and Weiss, 1973) afforded an independent estimate of the association, dissociation, and equilibrium constants for the reaction

$$\text{adenosine} + \text{AABP} \underset{k_b}{\overset{k_a}{\rightleftharpoons}} [\text{AABP-adenosine}];\ K_D = \frac{k_a}{k_b}$$

TABLE I: Parameters of the Binding of [^{3}H]Adenosine to Rabbit Erythrocyte AABP.

Method	Binding site concn (nM)	Association rate constant, $k_a \times 10^{12}$ (M^{-1} min^{-1})	Dissociation rate constant, $k_b \times 10^3$ (min^{-1})	Equilibrium constant, $k_b/k_a \times 10^{-9}$ (M)
Nonlinear curve fitting	5.07 ± 0.40	1.42 ± 0.17	7.54 ± 2.18	5.32 ± 1.42
Scatchard	5.63 ± 0.23			8.93 ± 0.97
Charcoal			3.58 ± 0.82	
0.23 mM adenosine			8.08 ± 1.12	
Dilution			6.35 ± 0.57	

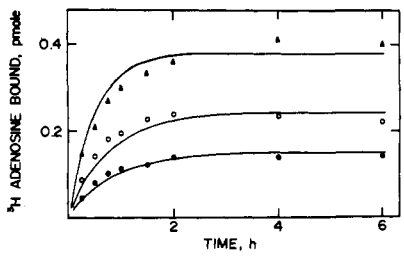


FIGURE 2: Binding of [^{3}H]adenosine to AABP. Mixtures containing (per 0.1 mL) 7 μg of AABP, 5×10^{-6} mol of Tris-HCl (pH 7.5), and 0.37 (●), 0.73 (○), or 1.93 (▲) pmol of [^{3}H]adenosine were incubated at 0 °C. At intervals aliquots were treated with charcoal as described in the text. Data on [^{3}H]adenosine bound, at all three concentrations represented in this figure as the mean of triplicates, were analyzed by a nonlinear curve-fitting program to obtain estimates of binding parameters. The curves depicted by solid lines are predicted from these parameters.

The results of this analysis are summarized in Table I, together with the estimate of K_D obtained from Scatchard plots of equilibrium binding data and estimates of the rate of dissociation of the AABP-adenosine complex by three different techniques, lowering the concentration of free ligand by dilution, by adsorption of [^{3}H]adenosine on charcoal, or by lowering the specific activity of free ligand by the addition of an excess of unlabelled adenosine. These estimates were made by incubating a mixture of [^{3}H]adenosine and AABP in 50 mM Tris-HCl (pH 7.5) for 4 h at 0 °C to achieve binding equilibrium. The concentration of free ligand was reduced in one portion of this mixture by diluting 250-fold with cold buffer. At hourly intervals for 9 h, duplicate aliquots were filtered through cellulose acetate membranes and the adsorbed AABP-[^{3}H]adenosine complex counted. A second portion of the reaction mixture was diluted with 10 volumes of buffer and 0.5 volume of charcoal-BSA dextran suspension and was stirred magnetically at 0 °C. Aliquots removed at hourly intervals were centrifuged and the supernatant counted. A third portion of the reaction mixture was mixed with 0.1 volume of 2 mM adenosine. Aliquots withdrawn hourly were diluted with buffer and treated with charcoal and the supernatant was measured. A fourth portion of the reaction mixture served as a control to account for the loss of bound ^{3}H activity observed on prolonged incubation. Aliquots of this solution were withdrawn hourly, diluted with buffer, and treated with charcoal and aliquots counted. These various estimates of the dissociation constant obtained by experimental techniques were internally consistent. That the dissociation of the [^{3}H]adenosine-AABP complex was unchanged in the presence of an excess of unlabeled adenosine is evidence against negative cooperativity between binding sites (De Meyts et al., 1973).

The possibility that ligand binding would be activated by MgATP was evaluated by incubating 0.1 mL (0.35 mg) of AABP with 0.1 mL of a mixture of 18 mM ATP, 20 mM $MgCl_2$, and 0.3 M KCl in 30 mM Hepes-HCl (pH 7.0) for 30 min at 30 °C. A second mixture containing no ATP served as a control. The mixtures were cooled in ice/water and run at 2 °C through a 0.5 × 20 cm column of Sephadex G-50 equilibrated and eluted with 15 mM Hepes-HCl (pH 7.5) containing 20% glycerol and 1 mM dithiothreitol. The protein emerged in a volume of 2.3 mL in the void volume (4.5 mL). Adenosine and cAMP binding was assayed as described above at ligand concentrations of 5×10^{-8} and 10^{-6} M. The amounts of adenosine and cAMP bound by the AABP treated with Mg-ATP did not differ from control.

Scatchard plots of [^{3}H]cAMP binding data obtained over a concentration range between 0.05 and 100 μM indicated a single class of binding sites having a K_D of 9.09×10^{-9} M and a binding capacity of 82 pmol of cAMP/mg of protein. The equilibrium constant for the binding of [^{3}H]adenosine to AABP was estimated from Scatchard plots as 7.9×10^{-8} to 10^7 M and the adenine-binding capacity was 74 pmol/mg of protein. The differences in binding affinity estimated from the equilibrium constants of adenosine and adenine are similar to those estimated from the K_i of the inhibition of [^{3}H]adenosine binding (Table II). The latter technique assumes that adenine and adenosine bind to the same site on AABP and that one purine inhibits the binding of the other in a purely competitive manner. However, the K_i of competitive displacement equals the K_D of binding only when the concentration of ligand is substantially lower than the true K_D. At ligand concentrations higher than about 0.1 K_D, which was the case in these experiments, K_i will be larger than K_D (Jacobs et al., 1975). However, the K_i estimated from competitive displacement experiments can serve as an index by which to compare the *relative* affinities of a series of compounds. As shown in Table II, the binding of [^{3}H]adenosine was inhibited by adenosine with a K_i of 2.5×10^{-8} M, by adenine with a K_i of 5.9×10^{-7} M and cAMP with a K_i of 1.2×10^{-4} M, thus confirming that adenosine and adenine but not cAMP bind to the adenosine-binding site.

Determinants of Adenosine Binding to AABP. A series of adenosine analogues was tested for the ability to inhibit [^{3}H]adenosine binding to AABP as a means of defining the factors which determine binding affinity. As described in Table II, 77 analogues were surveyed by incubating analogue and AABP with two different concentrations of [^{3}H]adenosine. The percentage inhibition exerted by a compound was invariably lower at the higher concentration of [^{3}H]adenosine and the values of K_i calculated from the degree of inhibition at the two concentrations of [^{3}H]adenosine agreed closely, which is consistent with inhibition of the competitive type. This conclusion was confirmed for selected analogues by additional studies in which the concentration of [^{3}H]adenosine was held constant and the concentration of analogue was varied (Figure 3). Because few of these analogues were available in radiolabeled form, the inhibitor constant, K_i, was used as an index of

TABLE II: Inhibition of [^{3}H]Adenosine Binding to AABP by Adenine Analogues.[a]

No.	R	Name	K_i (M × 10^{-6})[b]	Source[c,d]
1	NH_2	Adenine	0.51 ± 0.19	A
2	H	Purine	>100	A
3	O	Hypoxanthine	>100	S
4	S	6-Mercaptopurine	>100	CB
5	Cl	6-Chloropurine	>100	S
6	Br	6-Bromopurine	>100	S
7	I	6-Iodopurine	>100	S
8	CN	6-Cyanopurine	>100	S

No.	Name	K_i	Source
9	4-Amino-7-β-D-ribofuranosylimidazo[4,5-*d*]-*v*-triazine	0.10 ± 0.17	JM
10	7-Amino-3-β-D-ribofuranosyl-*v*-triazolo[4,5-*d*]pyrimidine	5.0 ± 1.3	HCC
11	3-β-D-Ribofuranosylimidazo[2,1-*i*]purine	0.65 ± 0.09	PL
12	8-Amino-3-β-D-ribofuranosylimidazo[4,5-*g*]quinazoline	0.23 ± 0.09	NJL
13	5-Amino-1-β-D-ribofuranosylimidazole-4-carboxamide	>100	S
14	6-Amino-3-β-D-ribofuranosylpurine	0.10 ± 0.05	NJL
15	4-Amino-3-β-D-ribofuranosylpyrrolo[2,3-*d*]pyrimidine	15 ± 9.0	AMDP
16	7-Amino-3-β-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidine	13.2 ± 6.1	AMDP

No.	R	Name	K_i	Source
17	O	Adenosine N^1-oxide	1.2 ± 0.18	S
18	CH_3	N^1-Methyladenosine	16 ± 0.05	S
19	F	2-Fluoroadenosine	0.43 ± 0.03	CCNSC, SRI
20	Cl	2-Chloroadenosine	80 ± 9.1	S, CCNSC, ICN
21	O	2-Oxyadenosine	19 ± 3.5	CCNSC
22	NH_2	2,6-Diaminopurine riboside	1.74 ± 0.21	HCC
23	Br	8-Bromoadenosine	0.24 ● 0.02	S
24	I	8-Iodoadenosine	0.18 ± 0.14	MI
25	S	8-Mercaptoadenosine	0.47 ± 0.05	(1)
26	$NHCH_3$	N^6-Methyladenosine	3.0 ± 0.9	S
27	$N(CH_3)_2$	N^6-Dimethyladenosine	71 ± 4.4	S
28	Cl	6-Chloropurine riboside	0.19 ± 0.03	S
29	H	Purine riboside	9.2 ± 4.4	HCC
30	S	6-Mercaptopurine riboside	>100	CB
31	SCH_3	6-Methylmercaptopurine riboside	25 ± 16	CB

No.	R	R′	Name	K_i	Source
32	O		Inosine	>100	CB
33	OCH_3		6-Methoxypurine riboside	9.8 ± 0.1	S
34	CH_3		6-Methylpurine riboside	2.0 ± 0.11	S
35	Cl	NH_2	6-Chloroguanosine	11.3 ± 5.2	S
36	H	NH_2	2-Aminopurine riboside	33 ± 4.4	HCC
37	OCH_3	NH_2	6-Methoxyguanosine	41 ± 7.0	S
38	O	NH_2	Guanosine	>100	PL
39	S	NH_2	6-Mercaptoguanosine	>100	A
40	O	O	Xanthosine	>100	A
41			*erythro*-9-(2-Hydroxyl-3-nonyl)adenine	>100	D

No.	X	Name	K_i	Source
42	O	8,2′-Anhydro-8-oxyarabinofuranosyladenine	8.6 ± 2.1	MI
43	S	8,2′-Anhydro-8-mercaptoarabinofuranosyladenine	25 ± 4.4	(2)
44	O	8,3′-Anhydro-8-oxyxylofuranosyladenine	>100	MI
45	O	8,5′-Anhydro-8-oxyribofuranosyladenine	>100	MI
46	S	8,5′-Anhydro-8-mercaptoribofuranosyladenine	>100	(3)

No.	R	R′	Name	K_i	Source
47			Psicofuranine	14 ± 3.5	U
48	H	H	2′-Deoxyadenosine	1.3 ± 0.03	ICN
49	H	OH	Adenine β-D-arabinofuranoside	22 ± 2.2	ICN
50	OCH_3	H	2′-*O*-Methyladenosine	44 ± 2.6	PL
51	H	H	3′-Deoxyadenosine	1.9 ± 0.70	S
52	H	OH	Adenine β-D-xylofuranoside	5.8 ± 0.35	(4)
53	OCH_2	H	3′-*O*-Methyladenosine	62 ± 3.5	PL
54	NH_3	H	3′-Amino-3′-deoxyadenosine	0.14 ± 0.03	CCNSC

No.	R	Name	K_i	Source
55		9-(α-L-Lyxofuranosyl)adenine	0.31 ± 0.03	TM
56		9-(5-Deoxy-β-D-*erythro*-pent-4-enofuranosyl)adenine	4.3 ± 1.3	JGM
57	H	5′-Deoxyadenosine	3.4 ± 1.5	TM
58	Cl	5′-Chloro-5′-deoxyadenosine	5.1 ± 2.1	(5)
59	Br	5′-Bromo-5′-deoxyadenosine	0.74 ± 0.10	TM

TABLE II (Continued)

No.	R	Name	K_i	Source
60	I	5′-Deoxy-5′-iodoadenosine	11 ± 1.4	S
61	OCH_3	5′-*O*-Methyladenosine	5.8 ± 0.35	DS
62	SCH_3	5′-Deoxy-5′-methylthioadenosine	33 ± 70	WJ
63	SC_2H_5	5′-Deoxy-5′-ethylthioadenosine	14 ± 1.7	WJ
64	NH_2	5′-Amino-5′-deoxyadenosine	0.67 ± 0.50	WJ
65	N_3	5′-Azido-5′-deoxyadenosine	0.86 ± 0.03	WJ
66	$SOC_2H_5^+$	5′-Deoxy-5′-ethylsulfoxyadenosine	62 ± 3.5	WJ
67	$SOC_2H_5^-$	5′-Deoxy-5′-ethylsulfoxyadenosine	11 ± 5.2	WJ
68	NO_2	Adenosine 5′-nitrate	0.18 ± 0.10	(6)
69	OOH	Adenine ribofuranuronic acid	19 ± 9.6	(7)
70	PO_4	Adenosine 5′-phosphate	29 ± 7.8	CB
71	$SO_2C_6H_4CH_3$	5′-*O*-Toluenesulfonyladenosine	55 ± 7.8	A

No.	Name	K_i	Source
72	α-(Hydroxymethyl)-α′-(6-aminopurin-9-yl)diglycolaldehyde	0.53 ± 0.10	JGC
73	1-(1,3-Dihydroxy-2-propyl)-1-(adenin-9-yl)-1(*R*),2-ethanediol	1.2 ± 0.0	JGC
74	2-*O*-[1(*s*)-(9-Adenyl)-2-(hydroxyl)ethyl]ethanediol	16 ± 7.8	JGC
75	Cyclic adenosine 3′,5′-monophosphate	118 ± 10	PL
76	Cyclic adenosine 2′,3′-monophosphate	>100	S
77	2′,3′-*O*-Isopropylideneadenosine	>100	A

[a] Partially purified AABP (7 μg, binding capacity 0.48 pmol) was incubated with either 0.73 or 2.92 pmol of [2,8-^{3}H]adenosine (sp act. 31.0 Ci/mmol), 10^{-9} mol of adenosine analogue and 5×10^{-6} mol of Tris-HCl (pH 7.5) in a final volume of 0.1 mL for 4 h at 0 °C. The reaction mixture was then treated with charcoal as described in the text and an aliquot of the supernatant was counted employing the external standard ratio method of quench correction. Under these conditions, 0.066 and 0.17 pmol of [^{3}H]adenosine were bound in the absence of inhibitor. Compounds which inhibited adenosine binding by ≥90% were retested at 10^{-7} M. The apparent K_i of the inhibitor was calculated as described in the text and the mean and SEM were calculated by the formula: $\bar{x} = \Sigma x/N$; SEM = $[\Sigma(x - \bar{x})^2/N(N-1)]^{0.5}$. When only one concentration of inhibitor was tested, SEM = standard deviation. Under these conditions the apparent K_i of adenosine was $2.5 \pm 0.07 \times 10^{-8}$ M. [b] The K_i of a compound which inhibits the binding of 7.3×10^{-9} M [^{3}H]adenosine ≤5% is reported as $>100 \times 10^{-6}$ M. [c] Abbreviations used to designate the commercial sources and the investigators who generously furnished samples of these compounds are: (A) Aldrich Chemical Co., Milwaukee, Wis.; (CB) Calbiochem, LaJolla, Calif.; (CCNSC) Dr. Harry B. Wood, Jr., Cancer Chemotherapy National Service Center, Bethesda, Md.; (D) Dr. James Downey, University of South Alabama, Mobile, Ala.; (DS) Professor David Shugar, Department of Biophysics, University of Warsaw, Poland; (HCC) Het-Chem Co., Harrisonville, Mo.; (ICN) International Chemical and Nuclear, Irvine, Calif.; (JGC) Professor Joseph Cory, University of South Florida, Tampa, Fla.; (JM) Dr. John Montgomery, Southern Research Institute, Birmingham, Ala.; (JGM) Dr. John. G. Moffatt, Syntex Institute of Molecular Biology, Palo Alto, Calif.; (MI) Professor Morio Ikehara, Osaka University, Osaka, Japan; (NJL) Professor Nelson J. Leonard, University of Illinois, Champlain, Ill.; (PL) P-L Biochemicals, Milwaukee, Wis.; (S) Sigma Chemical Co., St. Louis, Mo.; (SRI) Southern Research Institute, Birmingham, Ala.; (TM) Terra-Marine Biochemicals, LaJolla, Calif.; (U) Dr. R. G. Whitfield, Upjohn Co., Kalamazoo, Mich.; and (WJ) Professor Werner Jahn, Max Planck Institute for Medical Research, Heidelberg, West Germany. [d] Numbers in parentheses refer to the methods used to synthesize these compounds: (1) (1964) *J. Am. Chem. Soc. 86*, 1242; (2) (1975) *Tetrahedron 31*, 1369-1372; (3) (1970) *Tetrahedron 26*, 5757-5762; (4) (1966) *Cancer Res. 26*, 893-897; (5) (1971) *Tetrahedron Lett.*, 88-90; (6) (1974) *J. Carbohydr. Nucleosides Nucleotides 1*, 411-430.

affinity for AABP. This was calculated from the survey data by the formula

$$K_i = I(1 - i)/i(1 + [\text{Ado}]/K_D)$$

The amount of [^{3}H]adenosine bound in the presence and absence of analogue is represented by v_i and v, respectively, i is $1 - (v_i/v)$, I and [Ado] are the respective concentrations of analogue and adenosine, and K_D is the equilibrium constant of the formation of the AABP–adenosine complex, 5.3×10^{-9} M.

Because adenosine is chemically complex, analogues were chosen to include those chemical features which were of potential importance to binding affinity: the purine base and the furanose ring, the chemical nature and spatial disposition of their respective exocyclic substituents, and the spatial relationship between purine and sugar as defined by the glycosylic torsion angle. The contribution of the more subtle determinants such as the distribution of purine π electrons or puckering of the furanose ring seem beyond current interpretation. Nonetheless, certain structure–activity rules emerge from the data in Table II.

Purine Base. Of the purine bases, only adenine inhibited adenosine binding, but with a K_i 21 times higher than that of adenosine. Although the ribosides of purine (compound 2) and 6-chloropurine (compound 5) were relatively good inhibitors,

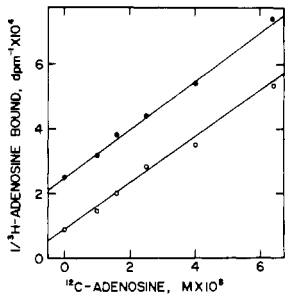
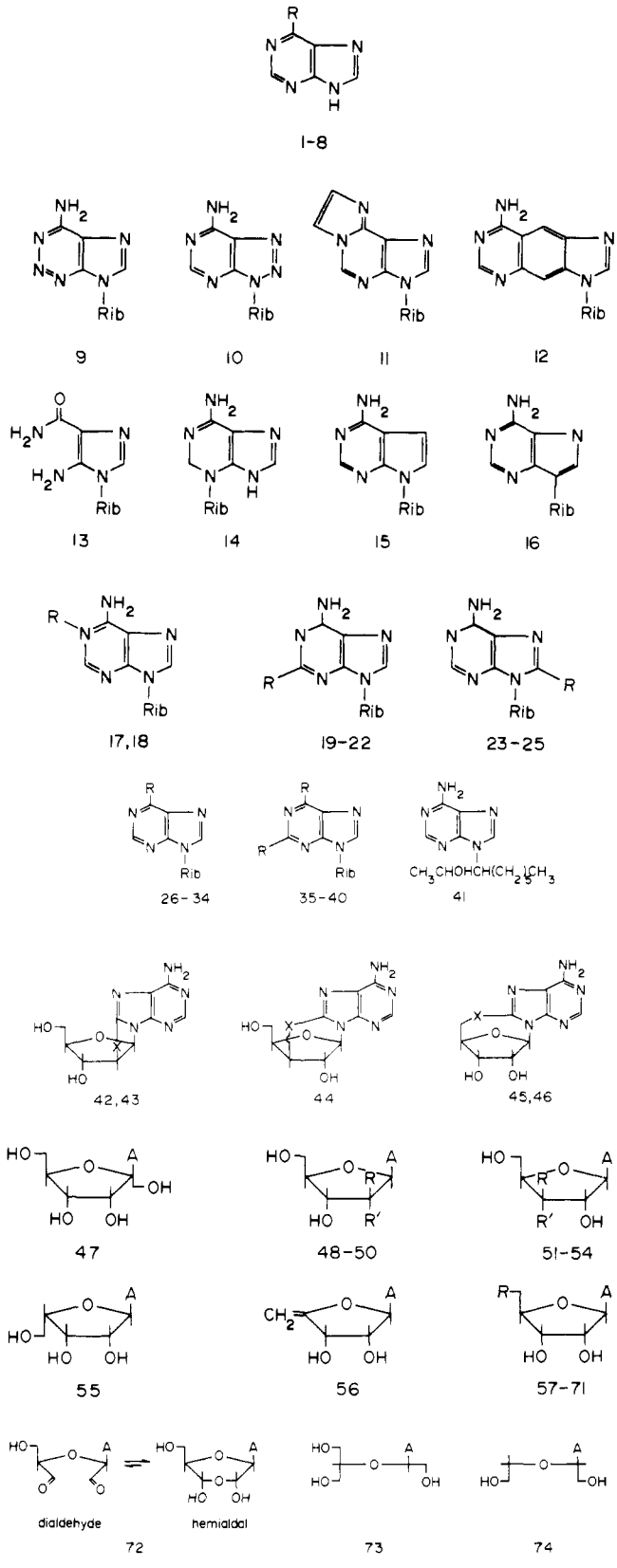


FIGURE 3: Inhibition of [^{3}H]adenosine binding by 6-chloropurine riboside. A series of tubes containing (per 0.1 mL) 7 μg of AABP, 0.73 pmol of [^{3}H]adenosine, 5×10^{-6} mol of Tris-HCl (pH 7.5), and 0–6.4 pmol of [^{12}C]adenosine was incubated 4 h at 0 °C. A second series of tubes identical with the first contained, in addition, 10^{-6} M 6-chloropurine riboside. Bound ^{3}H activity was estimated by the charcoal method described in the text. (○) No inhibitor; (●) 10^{-6} M 6-chloropurine riboside. The slopes of the two lines calculated by weighted least-squares regression did not differ significantly ($p > 0.5$), indicating competitive inhibition.

neither base was active, indicating that the ribose is a very important determinant of affinity.

Isosteric substitution of purine nitrogens and carbons (compounds 9–11, 15, 16) or additions of an imidazole (compound 11) or benzene ring (compound 12) to the purine ring



system had a relatively small effect on affinity. Attachment of ribose to N-3 instead of N-9 likewise had little effect; the K_i of compound 14 was only 4.4 times that of adenosine. Substitutions in or additions to the pyrimidine moiety generally had less effect on affinity than substitutions in the imidazole moiety. An imidazole nucleoside (compound 12) had no inhibitory activity, nor did cytosine, uridine or thymidine (data not shown). These data suggest that binding may depend on a base having minimum dimensions greater than those of pyrimidine, but that above this minimum the size and shape of the base may be relatively unimportant. In addition to its size, the aromaticity of the purine base may also contribute to binding. The aromatic character of purines resides in the pyrimidine moiety, but is of a rather low degree (Cavelieri and Bendich, 1950). These authors point out that purines may not participate at all in reactions such as sulfonylation and nitrosation, which are characteristic of aromatic compounds, or else they may give atypical reaction products, e.g., nitrous acid converts aminopurines and pyrimidines to hydroxy derivatives rather than diazonium salts. Substitutions which preserve or restore aromaticity to the purine base seem to conserve binding affinity for AABP. Thus, alkylation at N-1 (compound 18) or N-6 (compound 26) results in nucleosides of K_i 640 and 120 times that of adenosine, but the K_i of compound 11, in which both nitrogens are alkylated but are also incorporated into an aromatic ring, is only 26 times that of adenosine. Evidence presented below establishes that binding affinity depends on both the base and sugar moieties. Ribosides of a number of bases can bind to AABP, but, among the bases, binding was limited to adenine alone, suggesting a preponderant contribution by the sugar.

Exocyclic purine substituents also have an important influence on binding affinity. Substitutions at N-1 decreased affinity by at least 50-fold. The K_i values of 2-amino-, 2-chloro-, and 2-oxoadenosine were at least 70 times greater than that of adenosine. However, the K_i of 2-fluoroadenosine was only 17 times higher. Repeat testing with 2-fluoroadenosine from two lots (CCNSC and custom synthesis by SRI) and 2-chloroadenosine from three sources (Sigma, ICN and CCNSC) gave essentially the same results, leaving the exceptional behavior of 2-fluoroadenosine unexplained.

An amino group at C-2 reduced the affinity of adenosine as well as that of other 6-substituted purine ribosides. Thus, the K_i values of adenosine and compounds 38, 39, and 33 were lower than those of the corresponding 2-aminonucleosides, compounds 22, 35, 36, and 37, respectively. Since inosine, 6-mercaptopurine riboside and their 2-amino derivatives were all inactive, they neither support nor refute this rule.

Substitutions at C-8 had less effect on binding affinity than substitutions at any other purine position. Thus, the K_i values of 8-iodo-, 8-bromo-, and 8-mercaptoadenosine exceeded that of adenosine by no more than 20-fold.

Substitution of a chlorine atom for the 6-amino group increased the K_i less than an order of magnitude. The other 6-substituted purine ribosides had affinities lower than adenosine by two orders of magnitude or more, and inosine was completely inactive. However, methylation of the 6-oxo or 6-mercapto function of inosine and 6-mercaptopurine restored inhibitory activity and 6-methylpurine was a somewhat better inhibitor than purine riboside. An amino group was an exception to this enhancement of affinity by methylation; N-6 methyladenosine had a K_i 120 times and N-6 dimethyladenosine a K_i almost 2900 times higher than that of adenosine.

Glycosylic Torsion Angle. The shape of the adenosine molecule, particularly the relationship of the planar purine base to the quasi-planar ribofuranose ring, is an important determinant of binding affinity. The energy barriers to the rotation of the base around the N-9–C-1′ glycosylic bond are not large (Teng et al., 1971). While certain conformations are statistically more likely, one cannot infer which one describes that of bound adenosine. Anhydronucleosides in which C-8 is covalently linked to a sugar carbon via an oxygen or sulfur are useful probes for this purpose because the glycosylic torsion

angle is fixed. Of the five such compounds studied, only the 8,2′-anhydronucleosides (compounds 42 and 43) inhibited adenosine binding, suggesting that a glycosylic torsion in the range of −120° may be optimal for binding. An alternate explanation, that this pattern of activity is due to changes in the sugar moiety, is unlikely. Comparisons of the relative activities of these anhydronucleosides with the appropriate deoxy and epimeric nucleosides assesses the individual contributions of these changes. Hampton and Sasaki (1973) have pointed out that 8,2′- and 8,3′-*O*-anhydronucleosides differ from adenosine in three respects: (1) the 2′- or 3′-hydroxyl is replaced by a hydrogen atom; (2) the 2′- or 3′-hydroxyl is inverted, resulting in a sugar with an arabino or a xylo configuration; and (3) there is a covalent linkage between C-8 and the particular sugar carbon. In the present series compounds 43 and 46 are further modified by the substitution of sulfur for oxygen in the anhydro linkage. The differences between the three types of anhydronucleosides cannot be explained by loss of a critical hydroxyl since 2′-deoxyadenosine, 3′-deoxyadenosine, and 5′-deoxyadenosine (compounds 48, 51, and 57) had K_i values which were similar but nearly two orders of magnitude higher than of adenosine, indicating that each hydroxyl is important for binding but that none is absolutely required or even preeminent. Similarly, epimerization of either the 2′- or the 3′-hydroxyl (compounds 49 and 52) resulted in nucleosides with K_i values over two orders of magnitude higher than adenosine but not greatly different from each other. Thus, the capacity to inhibit [^{3}H]adenosine binding unique to the 8,2′-anhydronucleosides can be explained only in terms of a decisive influence of the glycosylic torsion angle. The diminished binding affinity of the 8,2′-*O*-anhydronucleoside relative to that of adenosine seems adequately accounted for by epimerization of the 2′-hydroxyl. The higher K_i of the 8,2′-*S*-nucleoside may reflect a somewhat less optimal torsion angle due to the bulkier sulfur atom.

Ribofuranose. As indicated above, the ribose moiety makes a very important contribution to the affinity of adenosine for AABP. Binding appears to depend on all three hydroxyls and especially on the ribo configuration of the secondary hydroxyls. That compound 42 completely lacks the capacity to inhibit [^{3}H]adenosine binding to AABP suggests that a single hydroxyl group is unable to effect binding. Derivatization (compounds 50 and 53), epimerization (compounds 49 and 52), or absence of any one hydroxyl (compounds 48, 51, and 57) reduces but does not abolish affinity. Of these three classes of compounds, the deoxynucleosides have the least loss of affinity, followed by the epimeric nucleosides and finally the 2′- and 3′-*O*-methyl ethers. The very low inhibitory potency of the *O*-methyl nucleosides suggests that not only does the methyl group block a hydroxyl, but its bulk may also impair the ability of the vicinal hydroxyl to interact with AABP. The relative inhibitory potencies of adenine arabinoside and xyloside (compounds 49 and 52) seem better explained in terms of the effect of the transposed hydroxyl on the glycosylic torsion angle rather than any bulk intolerance of AABP. The 2′-hydroxyl of the arabinoside probably prevents C-8 from lying directly over C-2′, which the studies of anhydronucleosides indicate may be the structure of bound adenosine. The 3′-hydroxyl of adenine xyloside does not hinder rotation to the proper torsion angle; accordingly, the K_i of this nucleoside is essentially identical with that of 3′-deoxyadenosine. Its binding affinity can be accounted for simply by the loss of the contribution of the 3′-hydroxyl. The K_i of 3′-amino-3′-deoxyadenosine (compound 54) was less than six times higher than that of adenosine, indicating that an amino group can substitute for a hydroxyl group in the interaction with AABP. The K_i of psicofuranine (compound 47), intermediate between those of 2′-deoxyadenosine and 2-*O*-methyladenosine, is additional evidence for limited bulk tolerance in the region of C-2′ and probably can be accounted for in terms of the steric hinderance exerted by the C-1′ hydroxymethyl group opposed by retention of the 3′-hydroxyl and its capacity to interact with AABP (the 3′-hydroxyl of this ketohexoside is analogous to the 2′-hydroxyl of adenosine). If this interpretation is correct, the relatively low K_i of adenine α-L-lyxoside (compound 55) indicates that there is relatively more bulk tolerance in the region of the C-3′ hydroxyl than of the C-2′. This nucleoside is the 4′ epimer of adenosine; the 5′-hydroxymethyl group is transposed to lie near the 3′-hydroxyl and like the C-1′ hydroxymethyl of psicofuranine might sterically hinder its interaction with AABP. Instead, the K_i of this nucleoside is only 13 times higher than that of adenosine and lower than that of either 3′- or 5′-deoxyadenosine, suggesting that not only does this alteration not sterically hinder the 3′-hydroxyl but that the 5′-hydroxyl may continue to contribute to binding. Introduction of a double bond between C-4′ and C-5′ and reduction of the 5′-hydroxyl (compound 56) results in a nucleoside having a K_i very similar to that of 5′-deoxyadenosine.

All of the 5′-substituted adenosines (compounds 57–71) inhibited [^{3}H]adenosine binding with values for K_i ranging between 0.21 to 71 × 10^{-6} M. The most potent inhibitor in this series was 5′-amino-5′-deoxyadenosine, confirming the inference drawn from the relatively low K_i of its 3′ isomer that an amino group can substitute for a hydroxyl with only a modest loss of activity. The oxidation of 5′-deoxy-5′-ethylthioadenosine results in two diastereoisomers. The K_i of the levorotatory member of the pair (compound 67) was 5.5 times lower than that of the dextrorotatory member (compound 66), suggesting an effect by additional factors such as intramolecular interactions between the substituents of C-8 and C-5′ which stabilize an anti conformation (Follman and Gremals, 1974). These workers interpret NMR evidence in terms of an intramolecular interaction which increases according to the 5′ substituent as follows: hydrogen < hydroxyl ≅ amino < thio < halogen < phosphate < carboxylate. However, the binding activities of the other 5′-substituted adenosines in this study are not ordered in a way which would support this hypothesis.

Dialdehyde and Trialcohol Nucleosides. Because of their *cis*-glycol configuration, the secondary hydroxyls of adenosine are easily oxidized by periodate to yield a dialdehyde (compound 72) which in solution exists primarily as the hemialdal (Uziel, 1975). This nucleoside dialdehyde had a K_i only 20 times higher than that of adenosine. Borohydride reduction of this aldehyde (Lerner, 1970) yields a trialcohol (compound 73) whose K_i is about twice that of the hemialdal. It appears that, although the positions of the three hydroxyls of the hemialdal or the trialcohol may be somewhat different than those of adenosine, the resulting compounds retain substantial inhibitory potency. The relatively lower affinity of compound 74, the nucleoside dialcohol produced by oxidation and reduction of adenine α-D-arabinopyranoside (Lerner and Rossi, 1972) could be due simply to the participation of only two rather than three hydroxyls in binding.

Compounds 75–77 were included in this series to evaluate the effect of substituting two of the ribose hydroxyls. None inhibited [^{3}H]adenosine binding at 10^{-5} M, and additional studies with 10^{-3} M cAMP yielded an estimate of K_i of 0.12 mM.

Discussion

This study confirms the affinity of AABP for a number of

adenine derivatives and adenosine analogues and provides a detailed description of the steric and chemical factors which determine the binding of ligands to the adenosine-binding site. These results are consistent with the original description of this protein by Yuh and Tao (1974); i.e., it possesses two types of binding sites, one accommodating only adenosine, the other both adenosine and cAMP. The linearity of Scatchard plots of adenosine binding data indicates that the affinity of the cAMP site for this nucleotide is less than an order of magnitude lower.

The AABP of rabbit erythrocytes differs from those of liver in several respects. The erythrocyte protein appears to be larger, having a molecular weight of 240 000 (Yuh and Tao, 1974) vs. 180 000 for the proteins of mouse, rat, and bovine liver (Ueland and Døskeland, 1977; Sugden and Corbin, 1976). Adenosine and cAMP binding to erythrocyte AABP is essentially constant between pH 6 and 8 whereas binding to the rat liver protein is optimum at pH 5 (Sugden and Corbin, 1976). Mouse liver AABP has both high and low affinity adenosine binding sites, and binding of adenosine and cAMP is activated by the binding of ATP to a site other than the cAMP and adenosine binding sites (Ueland and Døskeland, 1977). There is only a single class of adenosine-binding sites on the erythrocyte protein, and purine binding is not enhanced by ATP. The significance of these differences awaits the elucidation of the role of AABP in cellular purine metabolism.

The data on analogue inhibition of [^{3}H]adenosine binding furnish evidence about the adenosine but not the cAMP-binding site because these studies were performed under nonsaturating conditions (binding site concentration 5×10^{-9} M, [^{3}H]adenosine concentration 7×10^{-9} M). The displacement of any [^{3}H]adenosine bound to the cAMP site would be obscured by a rise in the concentration of free [^{3}H]adenosine and, consequently, enhanced binding to the adenosine site. These results show that binding to the adenosine site is determined by the size and aromaticity of the adenine base, by the 6-amino group, by the three ribose hydroxyls and by a glycosylic torsion angle in the anti range, approximately $-120°$. Although these experiments do not directly examine the determinants of affinity for the cAMP site, they are probably different and perhaps less restrictive. This site may bind nucleosides and nucleotides in the syn conformation, the preponderant species of cAMP in solution, and may not depend on unsubstituted 3′- and 5′-hydroxyls.

It is interesting to compare the factors determining adenosine binding to AABP with those determining the binding of adenine derivatives to enzymes for which they are substrates. Calf intestinal adenosine deaminase, like AABP, had rather broad binding requirements; a number of 6-substituted and 2,6-disubstituted purine nucleosides are substrates and/or competitive inhibitors (Cory and Suhadolnik, 1965; Chassy and Suhadolnik, 1967; Wolfenden et al., 1969; Simon et al., 1970). Purine is not the only nitrogen heterocycle capable of binding to this enzyme; 2-aza-, 8-aza, 1-deaza-, *lin*-benzoadenosine, and 3-isoadenosine are substrates and/or inhibitors (Wolfenden et al., 1969; Ikehara and Fukui, 1974; Leonard et al., 1976). Coformycin, which has a seven-membered diazepin ring in place of the pyrimidine moiety, has one of the highest affinities for adenosine deaminase of any compound known, $K_i = 10^{-12}$ M (Cha et al., 1975). N-9 must be substituted in order for a purine to bind to adenosine deaminase, and, although the 5-hydroxyl is very nearly an absolute requirement for binding, the 2′- and 3′-hydroxyl groups are not. This enzyme deaminates 8,2-*O*-, -*S*-, and -*NH*-cycloadenosines, but not adenosines with bulky C-8 substituents. This is interpreted as evidence that the anti conformer serves as the substrate (Ikehara and Fukui, 1973). The relatively high affinity of 8-substituted adenosines for AABP is surprising since 8-bromoadenosine is neither a substrate nor an inhibitor of adenosine deaminese (Simon et al., 1970) nor are its nucleoside triphosphates incorporated into polynucleotides (Tavale and Sobel, 1970). The explanation given for this lack of substrate activity is that the bulk of the bromine atom restricts rotation about the glycocylic bond to the syn range and thereby prevents the fit of the nucleoside into a catalytic site (Ikehara and Fukui, 1973). This explanation does not seem appropriate here for two reasons: (1) examination of CPK models of adenosine shows that a bulky substituent does not absolutely prevent rotation in the anti range such that the C-8 substituent can lie above and between C-2′ and C-3′; (2) this explanation predicts the K_i should vary directly with the bulk of the substituent, whereas in these three compounds it varies inversely with the van der Waals radius of the C-8 substituent. It is likely, therefore, that the relative affinities of these nucleosides are better explained by electronic rather than by the steric effects of the C-8 substituent. That the *S*-enantiomer of 8,5′-cycloadenosine is a better adenosine deaminase substrate than the 8,2′-anhydronucleosides suggests that in the preferred conformation H-8 lies over C-4′ and the 5′-hydroxyl lies between H-3′ and C-4′ (Hampton et al., 1972a). Studies using AMP analogues as substrates and/or inhibitors reveal that the purine, ribose, and phosphate moieties of this nucleotide all participate in binding to AMP aminohydrolase, AMP kinase, and snake venom 5′-nucleotidase. The substrate activity of 8,5′-cyclonucleotides indicates that the anti conformer of AMP binds to these enzymes (Hampton et al., 1972b). The extensive literature on the structure–activity relationships of cAMP indicates that binding to an activation of certain cAMP-dependent protein kinases depends on a glycosylic torsion angle in the syn range and 2′- and 3′-hydroxyls in the ribo configuration (Simon et al., 1973). The adenosine kinases of liver (Lindberg et al., 1967) and tumor cells (Schnebli et al., 1967) have a high affinity for 2′- and 6′-substituted purine ribosides, but alterations of the sugar, particularly at C-2′, markedly diminish affinity. Thus, the factors which promote the binding of adenosine to AABP resemble but are not identical with those important in the binding of this nucleoside and its phosphates to other proteins.

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Active Centers of *Streptomyces griseus* Protease 1, *Streptomyces griseus* Protease 3, and α-Chymotrypsin: Enzyme–Substrate Interactions†

Carl-Axel Bauer

ABSTRACT: Kinetic constants for *Streptomyces griseus* protease 1 (SGP1) catalyzed hydrolysis of a number of peptides of increasing chain length have been determined and indicate that the active center of the enzyme extends over 6-7 subsites (21-25 Å). The rate of substrate hydrolysis is highly dependent on the peptide chain length, the rate increase being about 10^7-fold, or more, on going from a specific acetyl amino acid amide to an acetyl heptapeptide amide. This rate increase is largely due to an increase in the acylation rate. The specificity of the S_1 and S_1' subsites of SGP1 has been investigated with peptide substrates. New data on the specificity of these subsites in *Streptomyces griseus* protease 3 and α-chymotrypsin are also presented. Generally, the specificities of the S_1 subsites in the three enzymes are similar. However, there are important differences between the microbial and the pancreatic enzymes' ability to hydrolyze peptides with certain P_1 residues, notably Trp. The implications of the kinetic data for the structures of the S_1 subsites are discussed. Exchanging the P_1' NH_2 group of a tetrapeptide for a P_1' amino acid amide increases the hydrolysis rate in all three enzymes, the increase being as large as 100-fold in the most favorable case. The abilities of the enzymes to use strain in facilitating hydrolysis of the P_1' residue differ markedly. Available kinetic and crystallographic data are used to throw light on the role of the active center structure for the rate of peptide substrate hydrolysis in the above three enzymes and trypsin, elastase, and subtilisin BPN'.

Studies of homologous proteins have provided valuable information about several different aspects of protein chemistry such as functionally and structurally essential amino acid residues, the mechanism of action of certain enzyme families and the relationship between the evolution of species and protein structure. The "chymotrypsin-trypsin" family of serine proteases constitutes such a group of homologous proteins, which includes enzymes from microorganisms to mammals (de Haen et al., 1975).

Pronase, the extracellular enzyme mixture from *Streptomyces griseus,* contains at least three proteases homologous to chymotrypsin (Wählby and Engström, 1968). The finding

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