Eur. J. Biochem. 86, 27–34 (1978)

An Adenosine 3′:5′-Monophosphate/Adenosine Binding Protein from Mouse Liver

A Study of Its Interaction with Synthetic and Naturally Occurring Adenosine Derivatives

Per Magne UELAND

Cell Biology Research Group, Preclinical Institutes, University of Bergen

(Received November 2, 1977)

Synthetic and naturally occurring adenosine derivatives were tested for their ability to inhibit the binding of cyclic [^{3}H]AMP and [^{3}H]adenosine to the activated cyclic AMP/adenosine binding protein from mouse liver [Ueland, P. M. and Døskeland, S. O. (1977) *J. Biol. Chem. 252*, 677–689]. The structural requirements for inhibition of the binding of the two ligands were different, as demonstrated by the existence of adenosine derivatives that inhibited the binding of [^{3}H]adenosine but not the binding of cyclic [^{3}H]AMP and vice versa. The interaction of the binding protein with AMP, ADP and adenine was investigated in more detail. The binding of radioactive AMP and ADP to the protein was determined under equilibrium conditions. Competition studies indicate that AMP binds to the cyclic AMP binding site with an apparent dissociation constant of 2×10^{-6} M and to a low-affinity site. The double-reciprocal plot for the binding of ADP deviated slightly from linearity suggesting the possibility of heterogeneity also for the ADP binding sites. Competition studies indicate that this nucleotide binds mainly to the cyclic AMP binding site.

Cyclic AMP has previously been shown to activate its own sites under certain conditions (30 °C, in the presence of KCl and Mg^{2+}). This phenomenon has been termed homologous activation of binding sites [Ueland, P. M. and Døskeland, S. O. (1978) *J. Biol. Chem.*, in the press]. Similar results were obtained with AMP and ADP.

Displacement of [^{3}H]adenosine binding from the sites specific for this adenine derivative was observed with unlabelled adenosine, adenine, AMP and ADP with decreasing efficiency in the order mentioned. This indicates that adenine, AMP and possibly ADP interact with the adenosine binding sites. Adenine and AMP inhibited the activation of the cyclic AMP binding capacity by ATP as previously shown for adenosine [Ueland, P. M. and Døskeland, S. O. (1978) *Arch. Biochem. Biophys. 185*, 195–203]. The inhibitory power closely parallelled the displacement of [^{3}H]adenosine binding. Adenine was also demonstrated to inhibit the homologous activation of the cyclic AMP binding site.

Binding factors for cyclic AMP not related to protein kinase have been isolated from various sources [1–8]. Interest has mainly been focused on their interaction with cyclic AMP. A few reports have appeared reporting that some of these binding factors also interact with adenosine [3, 6, 7].

We have purified to apparent homogeneity a cyclic AMP/adenosine binding protein from mouse liver [7]. This protein has been named according to its binding characteristics. The binding protein has a site which preferentially interacts with cyclic AMP, as judged from competition studies. In addition, there are sites which bind adenosine with high specificity [7].

The protein is isolated in a form characterized by low binding capacity for cyclic AMP relative to the amount of adenosine that binds at saturating concentrations of this ligand. The binding protein could be transformed by treatment with ATP into a state which has high binding capacity for cyclic AMP and high apparent affinity ($K_d = 2 \times 10^{-7}$ M) for adenosine. The binding capacity for adenosine and the apparent affinity for cyclic AMP ($K_d = 1.5 \times 10^{-7}$ M) are

Abbreviations. Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-Morpholino)ethanesulfonic acid; QAE-Sephadex, quaternary diethyl-(2-hydroxypropyl)aminoethyl-Sephadex.

Enzyme. Adenosine deaminase or adenosine aminohydrolase (EC 3.5.4.4).

unaffected by this treatment. This process has tentatively been termed activation of the binding protein by ATP [7,9]. When the non-activated binding protein is incubated in the presence of cyclic AMP at 30 °C, the binding increases slowly, because cyclic AMP activates its own sites [10]. This phenomenon has been termed homologous activation to distinguish it from the activation of the binding protein by ATP (i.e. heterologous activation). Both the heterologous and homologous activation are promoted by Mg^+ and K^+ and inhibited by adenosine [9,10].

Competition studies indicate that the protein also interacts with adenine derivatives other than cyclic AMP and adenosine, although cyclic AMP and adenosine seem to have the highest affinity towards the binding protein [7]. In this paper the effect of adenosine analogues was tested for three main purposes.

a) To elucidate further the specificity of and the structural requirements for binding of ligands to the cyclic AMP/adenosine binding protein.

b) Various adenosine analogues are known to affect the cyclic AMP level in tissues [11–16] and 2-chloroadenosine affects the adenylate cyclase in cell-free systems [12,13,17]. By testing these analogues clues to the functional role of the cyclic AMP/adenosine binding protein may be obtained.

c) The interaction between the binding protein and naturally occurring adenosine derivatives was investigated in some detail to see if the data obtained point to a role of the protein in the metabolism of purines other than cyclic AMP and adenosine.

MATERIALS AND METHODS

Chemicals

Hepes, AMP, ADP, ATP, cyclic AMP, adenine, adenosine, 8-bromoadenosine, 2′-deoxyadenosine, 6-chloropurine riboside, tubercidin (7-deaza-adenosine), cordycepin (3′-deoxyadenosine), 2-chloroadenosine, dihydronicotinamide-adenine dinucleotide (β-NADH), and adenosine deaminase (type I from calf intestine) were obtained from Sigma Chemical Co. (St Louis, U.S.A.). N^6-Phenylisopropyladenosine, N^6-cyclohexylallyladenosine and erythro-9-(2-hydroxy-3-nonyl)adenine · HCl were a gift from Dr Bertil Fredholm, Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden, who had obtained them from Dr H. Storck, Boehringer, Mannheim. QAE-Sephadex, DEAE-Sephadex were from Pharmacia, Sweden and Ultrogel AcA 34 from LKB, Sweden. Polyethyleneimine-impregnated cellulose thin-layer sheets (Polygram Cel 300 PEI, 0.1 mm) were purchased from Macherey Nagel & Co, Germany. [2-^{3}H]adenosine (27 Ci/mmol), [2-^{3}H]adenosine 5′-monophosphate (21 Ci/mmol), [2-^{3}H]adenosine 5′-diphosphate (20 Ci/mmol) and cyclic [8-^{3}H]AMP (21 Ci/mmol) were products from The Radiochemical Centre (Amersham), and the purity checked by thin-layer chromatography [7].

Purification of Binding Protein

This was carried out essentially as described [7] except that Ultrogel AcA 34 was used instead of Sephadex G-150 in step five of the purification scheme. The binding protein was homogenous as judged by polyacrylamide gel electrophoresis in the absence and presence of dodecylsulfate [7].

Activation of the Binding Protein by ATP

Binding protein (4 mg/ml) was incubated in the presence of 7.5 mM ATP, 10 mM Mg^{2+}, and 150 mM KCl in 15 mM Mes buffer pH = 6. The incubation was run for 30 min at 30 °C and stopped by cooling (0 °C). Samples of 60 µl from the incubate were applied to a Sephadex G-25 column (0.4 × 6 cm) equilibrated with the buffer in which the binding of radioactive ligand was measured, and eluted in the same buffer. The temperature was 0 °C. The binding protein treated in this way is referred to as activated binding protein throughout this paper [9].

Purification of Nucleotides

AMP was purified as follows. The nucleotide was dissolved in distilled water to a final concentration of 3 mM and pH adjusted to 7.5 by adding 0.1 M NaOH. 6 ml of this solution was applied to a QAE-Sephadex column (1.5 × 3 cm) equilibrated with 0.05 M triethylamine carbonate buffer, pH = 7.5. Adenosine and adenine emerged in the flow-through, whereas AMP was eluted with a linear gradient (200 ml) from 0.05 M to 0.9 M triethylamine carbonate buffer clearly separated from ADP and ATP, which were eluted at a higher concentration of buffer than AMP. Fractions of 2 ml were collected. Absorbance at 260 nm was measured in the separate fractions and those corresponding to the main ultraviolet-absorbing peak were pooled (20 ml), lyophilized and redissolved in distilled water three times and pH finally adjusted to 7.0 by adding 0.1 M NaOH. The identity and purity of the nucleotide were determined by thin-layer chromatography on polyethyleneimine-cellulose sheets developed either in 1.2 M LiCl or 0.5 M ammonium acetate/96% ethanol (5/2, v/v). ADP and ATP were purified by a similar procedure but DEAE-Sephadex was used instead of QAE-Sephadex. Adenosine, adenine and AMP emerged in the flow-through and ADP was eluted with a linear gradient of 0.05 M to 0.9 M triethylamine carbonate buffer before ATP.

Measurement of the Binding of ^{3}H-Labelled Nucleotides and Nucleosides

Binding protein was incubated in the presence of radioactive ligand under conditions given in the legends to the separate figures. The incubations were terminated by adding 90 µl from the incubate into 2 ml ice-cold 80% saturated ammonium sulfate containing unlabelled cyclic AMP and adenosine (0.1 mM). The protein precipitate was collected on Millipore filters and the radioactivity determined as previously described for the measurement of cyclic [^{3}H]AMP and [^{3}H]adenosine binding [7]. Data suggesting that this method gives a true estimate of protein-bound ligand have been published [7, 10].

Measurement of Adenosine Deaminase Activity

This was performed by the radioisotope technique described previously [7].

Determination of Protein

Protein was estimated by the method of Klungsøyr [18] using bovine serum albumin as standard.

RESULTS

Effect of Adenine Analogues on the Binding of Cyclic [^{3}H]AMP and [^{3}H]Adenosine

The binding of cyclic [^{3}H]AMP and [^{3}H]adenosine to the activated cyclic AMP/adenosine binding protein was measured at two concentrations of radioactive ligand (0.1 µM and 5 µM) in the presence of various adenosine derivatives (Table 1). The inhibition by the substances listed in Table 1 seems to be of the competitive type as inhibition was decreased by increasing the concentration of radioactive ligand.

A role of the ribose moiety of a ligand in its interaction with the cyclic AMP binding site and to a less degree with the adenosine binding site(s) is suggested by absence of inhibition of cyclic [^{3}H]AMP binding by adenine and erythro-9-(2-hydroxy-3-nonyl)adenine and only a slight inhibition of both radioactive cyclic AMP and adenosine by 2′-deoxyadenosine and 3′-deoxyadenosine. The NH_2 group of adenosine seems to play a significant role in the binding of ligands to both sites, as judged by near absence of inhibition of cyclic [^{3}H]AMP and [^{3}H]adenosine binding by 6-chloropurine riboside, N^6-cyclohexylallyladenosine, N^6-

Table 1. *Inhibition of cyclic [^{3}H]AMP and [^{3}H]adenosine binding by purine derivatives*
Activated binding protein (100 µg/ml) was incubated either in the presence of 0.1 µM cyclic [^{3}H]AMP (column A) or 5 µM cyclic [^{3}H]AMP (column B) or 0.1 µM [^{3}H]adenosine (column C) or 5 µM [^{3}H]adenosine (column D) in the presence of inhibitors indicated in the table. The binding of cyclic [^{3}H]AMP (420 Ci/mol) was measured by incubation in 30 mM Hepes, pH = 8.0, containing 20% glycerol, whereas for the binding of [^{3}H]adenosine (420 Ci/mol) 30 mM Hepes, pH = 7.0, containing 20% glycerol and 0.1 mM unlabelled cyclic AMP, was used [9]. The binding of [^{3}H]adenosine was measured in the presence of unlabelled cyclic AMP to inhibit the binding of [^{3}H]adenosine to the cyclic AMP binding site [7]. The incubation was run for 18 h at 0 °C. The adenosine derivatives are divided into four groups according to their inhibitory power towards the binding of cyclic [^{3}H]AMP and [^{3}H]adenosine. Group I: analogues that significantly inhibit cyclic [^{3}H]AMP binding only. Group II: analogues that inhibit the binding of [^{3}H]adenosine but not cyclic [^{3}H]AMP. Group III: analogues that inhibit the binding of both ligands to a significant extent. Group IV: analogues that do not inhibit or inhibit only to a small extent the binding of either ligand

Group	Inhibitor	Concn	Cyclic [^{3}H]AMP bound		[^{3}H]Adenosine bound	
			A	B	C	D
		µM	pmol/µg protein			
	no addition		1.42	6.28	0.70	6.48
	adenosine	10	0.06	2.90	0.24	5.50
I	7-deaza-adenosine (tubercidin)	10	0.02	0.83	0.72	6.50
	ADP	10	0.14	5.32	0.73	6.45
	3′-deoxyadenosine	10	0.72	4.97	0.60	6.20
	β-NADH	10	0.88	5.57	0.70	6.54
	2-chloroadenosine	30	1.07	6.06	0.76	6.00
	6-chloropurine riboside	10	1.21	6.25	0.68	6.47
II	adenine	10	1.41	6.32	0.42	6.02
	erythro-9-(2-hydroxy-3-nonyl)adenine	30	1.47	6.34	0.35	6.09
III	8′-bromoadenosine	10	0.07	2.11	0.40	5.92
	2′-deoxyadenosine	10	0.70	4.82	0.48	6.04
	AMP	10	0.10	4.91	0.56	6.39
IV	N^6-phenylisopropyladenosine	10	1.44	6.25	0.74	6.45
	N^6-cyclohexylallyladenosine	30	1.50	6.39	0.54	6.30

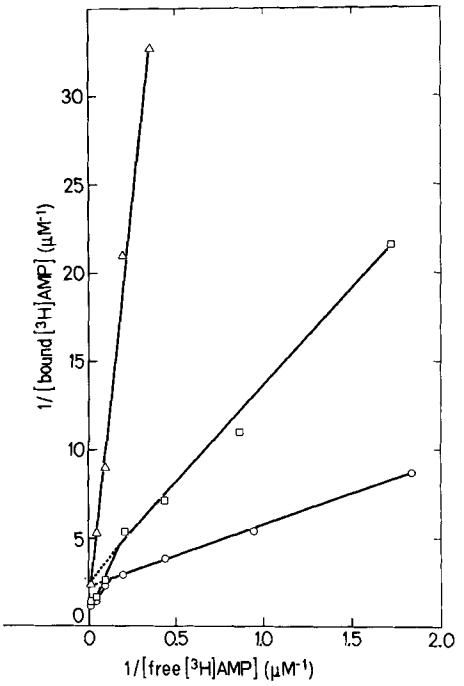

Fig. 1. *Double-reciprocal plot for the binding of [^{3}H]AMP.* The activated binding protein (100 μg/ml) was incubated for 18 h at 0 °C in 30 mM Hepes/NaOH, pH = 8.0, containing 20% glycerol and various concentrations of [^{3}H]AMP (420 Ci/mol), 10 μM cyclic AMP (△——△) or 10 μM ADP (□——□) or no addition (○——○). The amount of [^{3}H]AMP bound was determined as described under Materials and Methods

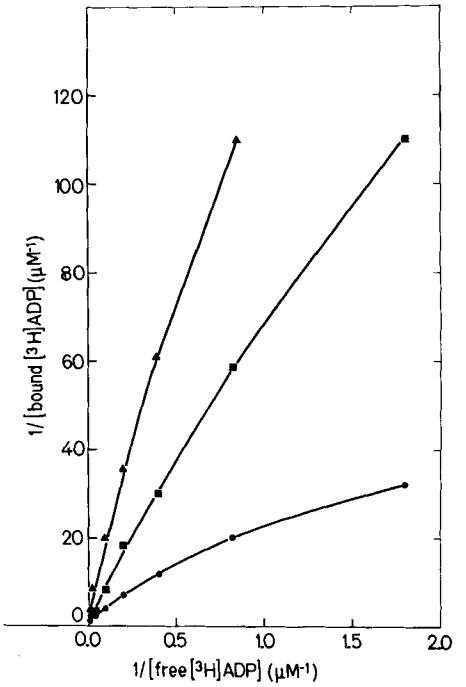

Fig. 2. *Double-reciprocal plot for the binding of [^{3}H]ADP.* The incubation conditions were the same as those given in legend to Fig. 1 except that the protein was incubated in the presence of various concentrations of [^{3}H]ADP (instead of [^{3}H]AMP) and 10 μM cyclic AMP (▲——▲) or 10 μM AMP (■——■) or no addition (●——●)

phenylisopropyladenosine and inosine [7]. The results obtained with 7-deaza-adenosine indicate that interaction with the adenosine binding sites but not the cyclic AMP site depends on intactness of the purine ring.

Equilibrium Binding of [2-^{3}H]Adenosine 5'-Monophosphate and [2-^{3}H]Adenosine 5'-Diphosphate

The equilibrium binding of [^{3}H]AMP and [^{3}H]ADP to the activated binding protein was determined in the presence of 10 μM cyclic AMP or 10 μM AMP or 10 μM ADP or no addition. The double-reciprocal plot for the binding of [^{3}H]AMP to the activated binding protein (Fig. 1) is non-linear corresponding to a high-affinity site with an apparent dissociation constant of 1.5×10^{-6} M and to a low-affinity site. The following nucleotides seem to inhibit competitively the binding of [^{3}H]AMP to the high-affinity site with decreasing efficiency in the order mentioned: cyclic AMP, AMP (data not shown for AMP) and ADP. These results indicate that AMP binds to the site which preferentially interacts with cyclic AMP.

The double-reciprocal plot for the binding of [^{3}H]ADP (Fig. 2) deviates slightly from linearity. This may indicate heterogeneity of the ADP binding sites. Cyclic AMP, AMP and ADP (data not shown for ADP) inhibit the binding of [^{3}H]ADP with decreasing efficiency in the order mentioned. The inhibition seems to be of a competitive type. These results indicate that ADP interacts with the cyclic AMP binding site with an apparent dissociation constant of about 5×10^{-6} M.

Data presented in Table 2 show that the binding capacity for cyclic [^{3}H]AMP, [^{3}H]AMP and [^{3}H]ADP increases upon activation of the binding protein. The term binding capacity refers to the amount of nucleotides bound within the concentration range tested. At high concentration (0.5 mM) of nucleotides the amount of [^{3}H]AMP bound to the activated protein is about twice the amount of cyclic [^{3}H]AMP and [^{3}H]ADP bound. A part (about 50%) of the binding capacity for ADP and AMP is present in the non-activated form of the protein. This part is relatively more pronounced at high concentrations of nucleotide. This could be attributed to AMP and possibly ADP binding to low-affinity site(s) in addition to the cyclic AMP binding site. Only the binding site interacting with cyclic AMP is activated by treatment with ATP. The data presented in Fig. 1 and 2 and Table 2 thus confirm the suggestion, based on competition studies published previously [7], that AMP and ADP

Table 2. *Binding of cyclic [^{3}H]AMP, [^{3}H]AMP and [^{3}H]ADP to the non-activated and activated binding protein*
Activated and non-activated binding protein (100 μg/ml) were incubated with cyclic [^{3}H]AMP (420 Ci/mol), [^{3}H]AMP (420 Ci/mol) or [^{3}H]ADP (400 Ci/mol) at concentrations indicated in the table. The incubation buffer was 30 mM Hepes, pH = 8.0, containing 20% glycerol. The incubation was run for 18 h at 0 °C

Nucleotide		Nucleotide bound to the non-activated binding protein	Nucleotide bound to the activated binding protein
	μM	pmol/μg protein	
Cyclic [^{3}H]AMP	5	0.33	6.70
	100	0.40	7.20
[^{3}H]AMP	100	4.60	11.17
	500	14.25	21.36
[^{3}H]ADP	100	2.05	6.40
	500	5.56	10.50

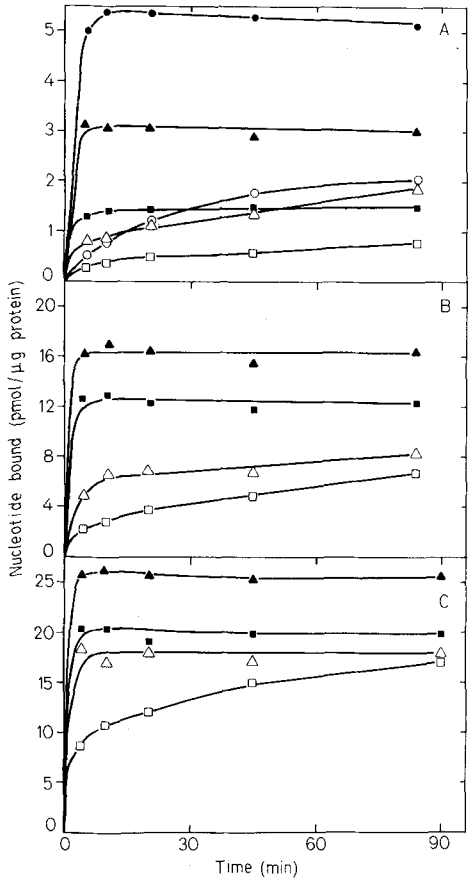

Fig. 3. *Time course of the binding of cyclic [^{3}H]AMP (○, ●) [^{3}H]AMP (△, ▲) and [^{3}H]ADP (□, ■) to the non-activated protein (○, △, □) and to the activated protein (●, ▲, ■).* (A) Binding protein (100 μg/ml) was incubated in 30 mM Hepes/NaOH, pH = 7.0, containing 0.2% bovine serum albumin, 10 mM Mg^{2+}, 150 mM KCl and 1 μM cyclic [^{3}H]AMP (420 Ci/mol), or [^{3}H]AMP (420 Ci/mol) or [^{3}H]ADP (400 Ci/mol). The incubation was run at 30 °C for the time indicated on the figure. (B) The concentration of nucleotides was 0.1 mM. The incubation conditions were as described in (A). (C) The concentration of nucleotides was 0.5 mM. The incubation conditions were as described in (A)

interact with the cyclic AMP binding site, but also indicate the presence of additional sites for these nucleotides, especially AMP.

Homologous Activation of Binding Sites by AMP and ADP

Cyclic AMP binding to the non-activated binding protein is characterized by a slow progressive binding (homologous activation), whereas the binding to the protein preincubated in the presence of ATP displays a rapid initial phase [10]. The time course of the binding of [^{3}H]AMP and [^{3}H]ADP to these two forms of the protein was investigated at increasing concentrations of nucleotides (Fig. 3).

At all concentrations tested, the nucleotide binding to the activated protein displayed a rapid initial phase, after which a plateau was reached, whereas the binding to the non-activated protein was characterized by a slow progressive phase. This slow progressive binding was, however, preceded by a rapid phase, which was relatively more pronounced at high concentrations of AMP and ADP, and more pronounced for AMP than ADP. This rapid initial phase was not followed by slow progressive binding at high concentration (0.5 mM) of AMP (Fig. 3C).

The activating potency for cyclic AMP, AMP and ADP, determined as nucleotide binding to the non-activated protein as a function of time, was compared at 1 μM nucleotide (Fig. 3A). It was highest for cyclic AMP followed by AMP and ADP. More cyclic AMP than AMP and ADP was bound to the activated and non-activated protein. By increasing the concentration of cyclic AMP, AMP and ADP to 10 μM, the amount of AMP bound to both forms of the protein approached the amount of cyclic AMP bound (data not shown). More AMP than ADP was bound to both activated and non-activated binding protein at 0.1 mM and 0.5 mM nucleotide (Fig. 3B and C).

The rapid increase in binding of AMP and to a less degree of ADP to the non-activated protein could be explained by interaction with site(s) which are available to AMP and ADP without being activated by a slow time-dependent process. This phenomenon was observed especially at high concentrations of AMP and ADP indicating low-affinity binding to these sites.

Displacement of [^{3}H]Adenosine Binding

Displacement of [^{3}H]adenosine binding was chosen as an experimental approach to investigate further the interaction of adenine and adenine nucleotides with the binding protein. Unlabelled adenosine was the most efficient inhibitor to the binding of [^{3}H]adenosine followed by adenine. High concentrations of AMP were required to displace the binding of [^{3}H]adenosine

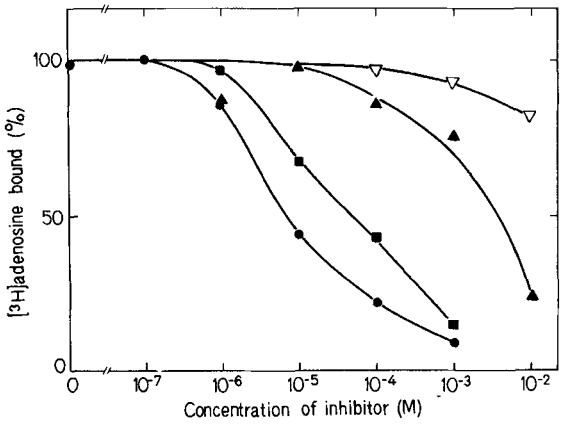

Fig. 4. *Displacement of [^{3}H]adenosine binding.* Activated binding protein (100 µg/ml) was incubated with 0.1 µM [^{3}H]adenosine (900 Ci/mol) in 30 mM Hepes/NaOH, pH = 7.0, containing 20% glycerol and increasing concentrations of the following potential inhibitors: unlabelled adenosine (●——●), adenine (■——■), AMP (▲——▲) or ADP (▽——▽). The incubation was run for 18 h at 0 °C. The binding of [^{3}H]adenosine is given as a percentage of the amount bound in the absence of inhibitor

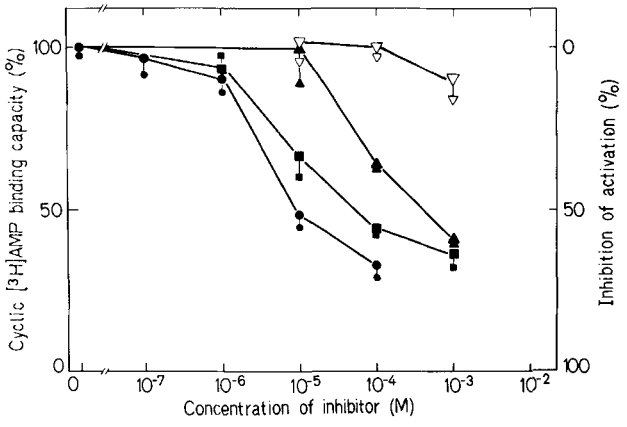

Fig. 5. *Activation of the binding protein by ATP in the presence of increasing concentrations of adenosine, adenine, AMP, and ADP.* The binding protein (2.5 mg/ml) was preincubated for 45 min at 30 °C in the presence of 5 mM ATP, 10 mM Mg^{2+}, 150 mM KCl in 30 mM Hepes, pH = 7.0, which contained adenosine (●——●), adenine (■——■), AMP (▲——▲) or ADP (▽——▽) at concentrations indicated on the figure. Samples of 30 µl were applied to Sephadex G-25 columns (0.4 × 6 cm) equilibrated with 30 mM Hepes pH = 8.0 containing 20% glycerol, and eluted (200 µl) in the same buffer. The temperature was 0–2 °C. The binding protein (100 µg/ml) was incubated in the equilibration buffer which contained either 5 µM or 10 µM cyclic [^{3}H]AMP (420 Ci/mol). The incubations were run for 18 h at 0 °C. The reason for incubating at two concentrations of cyclic [^{3}H]AMP is given in the text. The small symbols indicate the amount of cyclic AMP bound at 5 µM and the large symbols the amount bound at 10 µM of cyclic [^{3}H]AMP. The binding is given as a percentage of the amount of cyclic [^{3}H]AMP bound to the protein preincubated in the absence of adenosine, adenine, AMP and ADP

whereas ADP was almost inefficient (Fig. 4). These data suggest that adenosine, adenine and AMP bind to the adenosine site(s) with decreasing affinity in the order mentioned. The low-affinity site for AMP could thus represent binding to the site(s) which preferentially interact(s) with adenosine.

Effect of Adenosine, Adenine, AMP and ADP on the Activation of the Binding Protein by ATP

The effect of adenosine or adenosine derivatives on the activation is measured by preincubating the binding protein in the presence of ATP and the substance to be tested and thereafter removing unbound ATP and the substance by gel filtration. The degree of activation determined as cyclic AMP binding capacity, was measured at two concentrations (5 µM and 10 µM) of cyclic [^{3}H]AMP to avoid interference from a competitive inhibitor carried over from the preincubation mixture [9].

In the experiments presented in Fig. 5, the effect of increasing concentrations of adenosine, adenine, AMP and ADP on the activation process was determined. Adenosine was the most efficient inhibitor followed by adenine and AMP in the order mentioned. Almost no inhibition of activation was observed with ADP. Thus, the inhibition of activation closely parallels the displacement of [^{3}H]adenosine binding (Fig. 4).

The following experiments were conducted to check that the inhibition observed with AMP was not affected by contamination with adenosine. The effect of adenosine and AMP on the activation was determined in the absence and presence of adenosine deaminase, which converts adenosine to inosine. AMP is not deaminated [19]. Adenosine deaminase (200 units/ml) completely abolished the effect of adenosine (10–100 µM) on the activation process whereas the inhibition observed with AMP (0.1–1 mM) was the same in the absence and presence of the enzyme. A similar result could be obtained if high concentrations of AMP inhibited adenosine deaminase. This possibility was excluded by measuring the hydrolysis of adenosine to inosine by adenosine deaminase in the absence and presence of 1 mM AMP showing no effect on the enzyme activity by the nucleotide (data not shown).

Effect of Adenine on the Homologous Action by Cyclic AMP

Adenine does not inhibit the binding of cyclic AMP (7, and Table 1). The experimental situation is, therefore, optimal for detection of a possible effect of adenine on the homologous activation by cyclic AMP. The homologous activation by cyclic AMP, determined as cyclic [^{3}H]AMP binding to the non-activated binding protein as a function of time, was determined in the absence and presence of adenine (50 µM). The incubation buffer was 30 mM Hepes, pH 7.0, containing 0.2% bovine serum albumin,

5 mM Mg^{2+} and 75 mM KCl; the temperature and the concentration of cyclic [^{3}H]AMP were 30 °C and 5 μM respectively. The slope of the cyclic [^{3}H]AMP binding versus time graph was reduced to about 80% in the presence of adenine. By addition of adenine after 15 min incubation, the curve acquired the same slope as in the presence of adenine from time 0 (data not shown). These data indicate that adenine inhibits the activation of cyclic AMP binding sites by cyclic AMP. The inhibition of activation by adenine is probably mediated by its interaction with adenosine binding site(s). The following experiment was conducted to investigate this possibility. The binding of cyclic [^{3}H]AMP to the non-activated protein was measured under the conditions depicted above in the presence of adenine (50 μM), adenosine (2.5 μM), adenine (50 μM) and adenosine (2.5 μM) in combination or no addition. The slope of the cyclic [^{3}H]AMP binding versus time curve was reduced to about 80% by the adenine, to about 65% by adenosine and to about 60% in the presence of adenine and adenosine in combination. Although part of the reduction obtained with adenosine could be explained by competitive inhibition of the cyclic [^{3}H]AMP binding by adenosine [10], the inhibition observed in the presence of both adenine and adenosine was less than that expected for an additive effect. This suggests that the adenine and adenosine effects are partly, at least, mediated through interaction with common site(s).

DISCUSSION

Data presented in this paper show that the requirements for the binding to the cyclic AMP binding site are different from those determining the interaction with the adenosine binding site(s). This is clearly demonstrated by the observation that some inhibitors to the binding of adenosine did not affect the binding of cyclic AMP and vice versa (Table 1). Furthermore, the cyclic AMP binding site displays less structural specificity than the adenosine binding site(s).

The inhibition of [^{3}H]adenosine binding by unlabelled adenosine is rather low. 10-fold and 100-fold excesses of unlabelled adenosine inhibit the binding by 15% and 60% respectively (Table 1, Fig. 4). This could be explained by heterogeneity of adenosine binding sites [7, 10] or by transformation of the protein by high concentrations of adenosine into a state possessing altered binding properties towards adenosine. It is, therefore, possible that interaction between the adenosine binding sites and an adenosine derivative would not be detected by measuring displacement of [^{3}H]adenosine binding.

Adenosine has been shown to affect the cyclic AMP level in various tissues [11, 12, 16, 20, 21]. Adenylate cyclase in cell-free preparations from different tissues is activated by adenosine and 2-chloroadenosine [12, 17, 22]. Some N^6-monosubstituted adenosine derivatives affect the concentration of cyclic AMP in intact cells [14, 15, 22]. Adenosine receptors on the cell surface have been postulated to mediate some of these effects [11, 14, 17, 21]. Although the cyclic AMP/adenosine binding protein is isolated from the soluble fraction of liver homogenate, the possibility exists that the binding protein might be related to the adenosine receptor or perhaps could play a role in mediating the effect of adenosine on the adenylate cyclase and the cyclic AMP level in the cell. Therefore, 2-chloroadenosine [12, 13, 16, 17, 23], N^6-phenylisopropyladenosine [14, 15, 22, 23] and N^6-cyclohexylallyladenosine [22, 23] were tested for their inhibitory effect on the binding of cyclic AMP and adenosine. These analogues were not or only slightly efficient as inhibitors to the binding of cyclic AMP and adenosine to the binding protein. These data suggest that the cyclic AMP/adenosine binding protein is not related to the hypothetical adenosine receptor(s) mediating the effect on the cyclic AMP level in the cell.

Erythro-9-(2-hydroxy-3-nonyl)adenosine is a potent inhibitor of adenosine deaminase [24]. A high concentration of this substance (30 μM) is required to inhibit the binding of [^{3}H]adenosine to the cyclic AMP/adenosine binding protein. The inhibition constant (K_i) towards adenosine deaminase is 10^{-8} M [24]. This adenine derivative may, therefore, be a useful tool to inhibit adenosine deaminase and thus make possible measurement of adenosine binding in crude tissue extract [25].

The antibiotic and cytotoxic substance 7-deazaadenosine (tubercidin) structurally closely resembles adenosine [26]. This substance has been shown to inhibit a wide variety of enzymes [26, 27] and to be incorporated into DNA [26, 28]. Somewhat unexpectedly 7-deaza-adenosine did not affect the binding of [^{3}H]adenosine but only the binding of cyclic [^{3}H]-AMP (Table 1).

The increase in the binding capacity for cyclic AMP, after preincubation in the presence of ATP and cyclic AMP, has been described previously [7, 9, 10]. The possibility existed that activation, as measured by the method involving ammonium sulfate precipitation [7], corresponds to the conversion of the protein from a state possessing a high dissociation rate for cyclic AMP to another where the ligand is sequestered. The observation that the activation of the cyclic AMP binding capacity by ATP could be confirmed by an equilibrium binding method [10] does not support this suggestion. However, the assay used has not been evaluated using adenine derivatives other than cyclic AMP.

With the reservations made above, it is suggested that ligands interacting with the cyclic AMP binding site activate the cyclic AMP binding capacity. The

following substances bind to the cyclic AMP site as judged by competition studies [7] and binding of radioactive ligands (Fig. 1 and 2): cyclic AMP, adenosine, AMP, ADP and ATP. The apparent affinity decreases in the order mentioned

The interaction between a ligand and the other type of binding site(s), the so-called adenosine binding site(s), seems to inhibit the generation of the cyclic AMP sites. Adenosine is most efficient in this respect followed by adenine and AMP (Fig. 5).

Adenine is the only naturally occurring adenosine derivative studied that interacts with the adenosine site(s) but not the cyclic AMP site. Preliminary experiments using [^{3}H]adenine showed that adenine binds to the protein (Sæbø and Ueland, unpublished results). Extention of these studies was hampered by high background radioactivity, which probably could be explained by the retention of adenine on nitrocellulose filters as reported by Schneider and Guranowski [29].

The equilibrium binding studies using [^{3}H]AMP (Fig. 1) and the displacement of [^{3}H]adenosine binding by AMP are in favour of the interpretation that AMP has high affinity towards the cyclic AMP site and low affinity towards the adenosine site. The fact that AMP interacts with the adenosine binding site at high concentrations of the nucleotide may explain the curve obtained for AMP binding to the non-activated protein at 0.5 mM AMP (Fig. 3C). The rapid initial phase, which probably represents the interaction with the adenosine site, was not followed by a slow progressive phase. The high concentration of AMP probably inhibits the homologous activation by the nucleotide through interaction with the adenosine site.

Attention has previously been payed to the question whether the cyclic AMP/adenosine binding protein may function to sequester cyclic AMP [7, 10]. The data presented in this paper strongly suggest that adenine derivatives other than adenosine and cyclic AMP should be taken into account when discussing the possible physiological role of this binding protein.

This work was supported by the Norwegian Research Council for Science and the Humanities and by *Nordisk insulinfond*. The technical assistance of T. Ellingsen is highly appreciated.

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P. M. Ueland, Farmakologisk Institutt, Universitetet i Bergen, MFH-bygget, Haukeland Sykehus, N-5016 Bergen, Norway