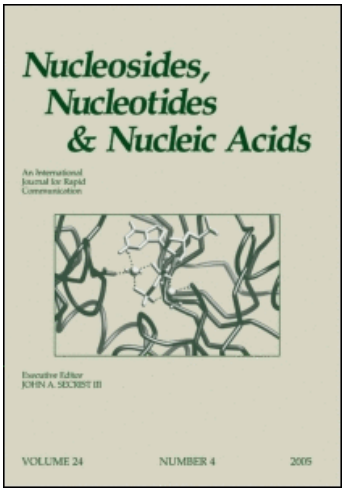

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LIGAND BINDING PROPERTIES OF BOVINE LIVER ADENOSINE KINASE.

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Abstract: Bovine liver adenosine kinase displays a characteristic intrinsic fluorescence due to 3 tryptophans/mol. This fluorescence is very sensitive to ligand binding and was used to characterize the ligand binding sites of the enzyme. ADP or ATP showed a monophasic saturation curve consistent with the existence of one binding site. In contrast, adenosine and AMP gave biphasic saturation curves suggesting the existence of at least two binding sites with high and low affinity. These binding sites were further characterized by studying the complexation of adenosine kinase with O-(N-methylanthraniloyl)adenosine nucleoside or nucleotide analogues.

Adenosine kinase (EC 2.7.1.20) (AK) catalyses the transfer of the ATP γ-phosphoryl group to adenosine. It has been purified from several sources especially mammalian (human erythrocytes[1], placenta [2] and liver [3]), and bovine or rat liver[4] ,heart [5] and brain [6]. The enzyme is monomeric, with a molecular mass in the range of 38-56kDa. In most cases, the accepted mechanism is an ordered sequential process in which adenosine is the first substrate to bind and AMP the last product to dissociate from the enzyme [1,7]. The structure of the enzyme is not known and the information about the active site is scarce. However, the occurrence of substrate inhibition of the enzyme and the dual character of some adenosine analogues (inhibitors or activators depending on the conditions) suggest the existence of two adenosine binding sites for adenosine kinase : a catalytic site and a regulatory site [1,5,8].

To study the activation of adenosine analogues potentially active against HIV, we have purified bovine liver adenosine kinase [9] and investigated its substrate properties and its ligand binding properties based on intrinsic or extrinsic fluorescence changes. Substrate properties and inhibition of bovine liver AK with various adenosine analogues have been examined using a spectrometric method [4] and HPLC to identify the reaction

TABLE 1 : Substrate properties of bovine liver adenosine kinase

Compounds	Substrates (S) or non substrates (NS)	Km (μM)	Concentration above which substrate inhibition was observed (μM)
2'-Deoxyadenosine	S	(b)	-
Cordycepine	S	27	(c)
α-Adenosine	NS	-	-
8-Azidoadenosine	S	4	~5
2-Chloroadenosine	S	(b)	-
8-Bromoadenosine	S	8.5	-
6-Methylmercaptopurine riboside	S	18	~35
Tubercidin	S	11	~12
ß-D-Arabinofuranosyl adenine	S	(b)	-
ß-4'-Thioadenosine [10]	S	22	(c)
α-4'-Thioadenosine [10]	NS	-	-

(a) Evidence for phosphorylation of substrates was obtained by HPLC analysis of a reaction medium (pH 7.4) containing the adenosine analogue (100μM), ATP(1mM), $MgCl_2$ (0.5mM) and adenosine kinase (16μg. ml-1) after 1 h of incubation at 37°C.
(b) In conditions of kinetic studies (ATP 1mM, $MgCl_2$ 0.5mM, and adenosine kinase 16 $ng.ml^{-1}$), the reaction was too slow for Km determination.
(c)No inhibition was observed up to a 100 μM concentration of substrate in kinetic studies.

products. As in the case of adenosine[11], some analogues induced substrate inhibition at concentration values generally higher than the Km (Table 1). Using adenosine as substrate, (bis)adenylyltetraphosphate (Ap_4A), (bis)adenylpentaphosphate (Ap_5A), and 5'-amino-5'-deoxyadenosine were inhibitors of the enzyme. The corresponding Ki values were : 0.48μM (non competitive), 0.6μM (non competitive) and 0.7μM (competitive), respectively.

Bovine liver adenosine kinase presented a characteristic intrinsic tryptophan fluorescence with maximal excitation at 284 nm and maximal emission at 335 nm. Tryptophan titration of the unfolded enzyme based on fluorescence emission at 350 nm,with free tryptophan as the standard, indicated that the enzyme contains 3.0±0.2 mol tryptophans/mol. Binding various ligands to the enzyme resulted in a significant quenching (6-20%) of the intrinsic fluorescence intensity without shifting the maximum of emission. From the saturation curves of the enzyme by addition of ligands, the dissociation constants Kd of the enzyme.ligand complexes could be calculated using already reported quadratic equations (1 binding site or 2) [11].

TABLE 2 : Interaction of bovine liver adenosine kinase with ligands. Determination of Kd constants from fluorescence measurements(a).

Ligands	Intrinsic fluorescence			Extrinsic fluorescence	
	Kd_1(μM)	Kd_2(μM)	Quenching (%)	Ligands	Kd(μM)
Adenosine (Ado)	0.69	122	7+12	2'(3')-*mant* Ado:	No binding
+$MgCl_2$ (0.5mM)	0.7	152	8+12		
+$MgCl_2$ (0.5mM) + ATP(100μM)	0.71		9	5'-*mant* Ado :	No binding
+$MgCl_2$ (0.5mM) +Ap5A (2μM)	5.1		8		
AMP	3	144	6+9	2'(3')-*mant* ADP:	28
+$MgCl_2$ (0.5mM)	3.8	210	6+10		
+$MgCl_2$ (0.5mM)+Ado(100μM)	-		<1	2'(3')-*mant* ATP:	8.5
+$MgCl_2$ (0.5mM)+ATP(100μM)	13		7		
ADP	109		14	2'(3')-*monomant* Ap5A :	1.6
+$MgCl_2$ (0.5mM)	76		13		
ATP	73		14		
+$MgCl_2$ (0.5mM)	38		16		
Ap5A	1.3		20		
+$MgCl_2$(0.5mM)	0.59		18		

(a) Fluorescence experiments were performed at 25°C in 0.7 ml 50 mM Tris/HCl pH 7.5, 1mM EDTA, 1mM dithiothreitol, and 10% glycerol in a quartz cuvette with a 5 mm optical pathlength.

In another set of experiments, fluorescent N-methylanthraniloyl (*mant*) derivatives of adenosine were used as ligands of AK to complete the information gained from intrinsic fluorescence experiments. The *mant* derivatives of adenosine, ADP, ATP and Ap5A were obtained as already published[12,13]. Upon excitation at 360 nm, these compounds showed emission spectra centered at 450 nm. In the presence of AK, the fluorescence emission maxima were 10 nm blue-shifted and their intensities increased about 3-fold. From titration experiments of the enzyme with the *mant* ligands, we obtained the Kd constants of the corresponding complexes using published calculation procedures [14] (Table 2) .

Titration of adenosine kinase with adenosine monitored by intrinsic fluorescence variation gave a biphasic curve suggesting the existence of a high affinity binding site (Kd_1 = 0.7 μM) and a low affinity site (Kd_2 = 122μM) for adenosine. The low affinity binding was affected by the presence of $MgCl_2$ and was no longer detectable in the presence of ATP, suggesting that adenosine binds at the ATP site. AMP gave similar results. Only one binding site was detected from enzyme titration curves with ATP or

ADP, and the affinity was increased about two fold in the presence of $MgCl_2$ (Table 2). Competition studies indicated that adenosine or AMP have low affinities for the ATP binding site. The titration curve of AK by the bisubstrate inhibitor Ap5A is monophasic, suggesting the existence of one binding site with high affinity (Kd 1.3 μM). This affinity was improved two-fold in the presence of $MgCl_2$, and competition experiments indicated that Ap5A binds to the enzyme, at least in part, at the ATP site.

Extrinsic fluorescence experiments with *mant* derivatives of ADP, ATP and Ap5A gave monophasic curves with Kd constants fairly different from the constants obtained in intrinsic fluorescence experiments. Whereas 2'(3')-*mant* or 5'-*mant* adenosine compounds did not bind to the enzyme at concentrations lower than 100 μM (Table 2), competition studies indicated that *mant* ADP and *mant* ATP bind to adenosine kinase at the same site as ATP with improved affinity, as already noted in the case of *ras* p21 and the F1–ATPase[15] . In contrast, *mant* Ap5A binds to the enzyme at the ATP site with reduced affinity compared to Ap5A. This suggests that the high affinity adenosine binding site is considerably more selective that the ATP binding site of the enzyme.

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