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Adenosine Kinase from Human Erythrocytes: Kinetic Studies and Characterization of Adenosine Binding Sites†

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ABSTRACT: The reaction catalyzed by adenosine kinase purified from human erythrocytes proceeds via a classical ordered sequential mechanism in which adenosine is the first substrate to bind to and AMP is the last product to dissociate from the enzyme. However, the interpretation of the steady-state kinetic data is complicated by the finding that while AMP acts as a classical product inhibitor at concentrations greater than 5 mM, at lower concentrations AMP can act as an apparent activator of the enzyme under certain conditions. This apparent activation by AMP is proposed to be due to AMP allowing the enzyme mechanism to proceed via an alternative reaction pathway that avoids substrate inhibition by adenosine. Quantitative studies of the protection of the enzyme afforded by adenosine against both spontaneous and 5,5′-dithiobis(2-nitrobenzoic acid)-mediated oxidation of thiol groups yielded "protection" constants (equivalent to enzyme–adenosine dissociation constant) of 12.8 μM and 12.6 μM, respectively, values that are more than an order of magnitude greater than the dissociation constant (K_{ia} = 0.53 μM) for the "catalytic" enzyme–adenosine complex. These results suggest that adenosine kinase has at least two adenosine binding sites, one at the catalytic center and another quite distinct site at which binding of adenosine protects the reactive thiol group(s). This "protection" site appears to be separate from the nucleoside triphosphate binding site, and it also appears to be the site that is responsible for the substrate inhibition caused by adenosine.

Although adenosine kinase (EC 2.7.1.20) has been partially purified from several different sources (Caputto, 1951; Lindberg et al., 1967; Schnebli et al., 1967; Murray, 1968; Divekar & Hakala, 1971; Henderson et al., 1972; Shimizu et al., 1972; Namm & Leader, 1974; DeJong, 1977; Andres & Fox, 1979) and purified to apparent homogeneity from brewers' yeast (Leibach et al., 1971), rabbit liver (Miller et al., 1979), rat brain (Yamada et al., 1980), murine leukemia L1210 cells (Chang et al., 1980) and human liver (Yamada et al., 1981) and many of its properties have been studied, there remains some uncertainty regarding the mechanism of the reaction catalyzed by this enzyme. Kinetic studies of the enzyme from human placenta (Palella et al., 1980) and from Ehrlich ascites tumor cells (Henderson et al., 1972) originally indicated that the reaction sequence proceeded via an ordered sequential mechanism, but these two studies disagreed on the order of binding of adenosine and $MgATP^{2-}$ to the enzyme. In a more recent study, Chang et al. (1983) have suggested that adenosine kinase purified from murine leukemia L1210 cells catalyzes the phosphorylation of adenosine via a two-site ping-pong mechanism. While it is possible that the enzyme from different sources may catalyze the kinase reaction via different reaction mechanisms, it is much more likely that these differences result from difficulties in the interpretation of the kinetic data. These difficulties arise largely from the properties of the enzyme such as its lability in dilute solution, the substrate inhibition caused by adenosine, the inhibition caused by excess free magnesium ion, and the interrelationships between some of these properties and the pH of the assay.

The lability of adenosine kinase was first reported by Caputto (1951) during initial attempts to purify the enzyme from

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Table I: Purification of Adenosine Kinase from Human Erythrocytes

step	vol (mL)	protein (mg)	sp act. (μmol·min^{-1}·mg^{-1})	recovery (%)
lysate	310	31 900	0.000 16	100
membrane-free lysate	290	27 300	0.000 18	98
CM-cellulose	300	2010	0.002	80
DEAE-Sephacel	83	660	0.002 8	36
ATP–agarose	84	490	0.003 7	36
AMP–agarose concentrate	1.2	0.25	1.6	8

yeast and rabbit liver. Leibach et al. (1971) then showed that adenosine could stabilize the enzyme during its purification from yeast and that adenosine could also protect the enzyme against heat inactivation. Subsequent reports (Neudecker & Hartmann, 1972; Neudecker & Hartmann, 1978) showed that the yeast enzyme possesses a highly reactive thiol group, which is essential for enzyme activity. These results suggest that the protection against inactivation afforded by adenosine is due to the formation of a specific enzyme–adenosine complex. We report here further studies of the protection of the human erythrocyte enzyme by adenosine, which, when taken together with the kinetic data, suggest that there are two distinct adenosine binding sites on the enzyme.

Experimental Procedures

Materials. All ^{14}C-labeled substrates were obtained from the Radiochemical Centre, Amersham, England. AMP–agarose[1] and ATP–agarose were obtained from Sigma. Co$(NH_3)_4$ATP was provided by G. W. Smithers (University of New South Wales). All other chemicals were of analytical reagent grade. Units of fresh whole human blood or the equivalent in packed cells were provided by the New South Wales Red Cross Transfusion Service.

Purification of Adenosine Kinase from Human Erythrocytes. The enzyme was purified from human erythrocytes essentially as previously described by Miller et al. (1979) with minor modifications (Table I). The purified enzyme had a molecular weight and other properties similar to those of the kinase purified from other sources (Arch & Newsholme, 1978; Andres & Fox, 1979; Miller et al., 1979, 1981; Chang et al., 1980; Yamada et al., 1980, 1981, 1982; Fisher & Newsholme, 1984; Rotllan & Miras-Portugal, 1985). The purified enzyme contained at least one DTNB-reactive thiol group that was essential for enzyme activity. This thiol group could be protected against both DTNB-mediated and spontaneous oxidation by adenosine.

Adenosine Kinase Assay. A detailed study of the conditions required to assay adenosine kinase activity in crude hemolysates has been published from this laboratory (Kyd & Bagnara, 1980). The standard assay of the purified enzyme was performed at 37 °C and contained 100 mM Na-PIPES (pH 7.4) or Na-HEPES (pH 8.0), 0.5 mM $MgCl_2$, 1 mM ATP, 0.5 μM [8-^{14}C]adenosine (59 mCi/mmol), and 10 μL of enzyme solution (in 1% bovine serum albumin) in a total volume of 100 μL. Initial velocities were determined by withdrawing 10-μL samples at various time intervals and spotting them immediately onto the origin of PEI–cellulose chromatograms (Macherey-Nagel Co., West Germany), which had been previously spotted with 10–20 nmol of adenosine and AMP. Adenosine was separated from AMP on 5 cm long strips by development in methanol–water (1:1 v/v). The adenosine and AMP spots were cut out for liquid scintillation counting, and initial velocities were calculated as the percentage of total dpm found in AMP and expressed as picomoles per minute. Reaction rates were linear for at least 15 min.

For many of the experiments described here, $MgCl_2$ was added in concentrations 1 mM in excess of those of ATP to decrease the contribution of other species such as ATP^{4-} and $HATP^{3-}$ to a minimum. A 1 mM excess of free magnesium ion over total nucleotide concentration was also used when ADP was added as a product inhibitor. The initial velocity data were analyzed with the following factors taken into consideration: (1) $MgATP^{2-}$ was the only significant ATP species utilized by the enzyme; (2) $MgADP^-$ was assumed to be one of the product inhibitors; (3) while AMP^{2-} is probably the second product inhibitor, significant amounts of MgAMP and $NaAMP^-$ were present under some conditions, and inhibitor concentrations were treated as total AMP concentration. The nomenclature and data presentation for the kinetic studies presented here are those of Cleland (1963a,b) unless otherwise stated.

Concentrations of Metal–Nucleotide Species. Calculation of the concentrations of free and complexed species in the metal–nucleotide system for the various kinetic studies was performed as described by Storer and Cornish-Bowden (1976) and O'Sullivan and Smithers (1979).

Protection by Substrates and Products against Enzyme Inactivation. Incubations for determining the rate of enzyme inactivation were performed at 37 °C in a volume of 100 μL in stoppered tubes. Each incubation contained 100 mM PIPES (pH 7.4), 0.6% (w/v) bovine serum albumin, approximately 0.25 μg of purified enzyme, and the given concentrations of ligand. Where inactivation was caused by DTNB, this reagent was added at zero time to a final concentration of 0.1 mM. Enzyme activity remaining after various time intervals was determined by removing 5-μL aliquots for assay in a 100-μL adenosine kinase standard assay. When various concentrations of adenosine were used as the protecting ligand, the assay mixture was modified to contain sufficient adenosine such that all assays were carried out at a final concentration of 10.5 μM adenosine.

Results

Initial Velocity Studies. Initial velocity studies performed with a range of substrate concentrations gave primary plots that are indicative of a sequential binding of substrates to form a ternary enzyme–substrate complex (Figure 1). The pattern was the same regardless of whether experiments were carried out at pH 7.4 with 0.4 mM excess $MgCl_2$ or at pH 8.0 with 1 mM excess $MgCl_2$. To determine whether the addition of substrates and the release of products in the adenosine kinase reaction were random or ordered, product inhibition studies were carried out.

Product inhibition by $MgADP^-$ was found to be linear noncompetitive with respect to both $MgATP^{2-}$ and adenosine. However, product inhibition studies with AMP were complicated by the ability of AMP to cause an apparent activation rather than inhibition of the enzyme under certain conditions. Figure 2 shows the inhibition pattern caused by AMP in the presence of 5 μM adenosine with the $MgCl_2$ concentration fixed as a 1 mM excess over the ATP concentration only. The apparent activation of the enzyme by AMP concentrations

[1] Abbreviations: AMP–agarose, agarose–hexane–adenosine 5′-monophosphate linkage through the N^6-amino group; ATP–agarose, agarose–hexane–adenosine 5′-triphosphate linkage through ribose hydroxyl groups; PIPES, 1,4-piperazinediethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CM-cellulose, (carboxylmethyl)cellulose; DEAE-Sephacel, diethylaminoethyl-Sephacel; EDTA, ethylenediaminetetraacetic acid disodium salt; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PEI, poly(ethylene imine).

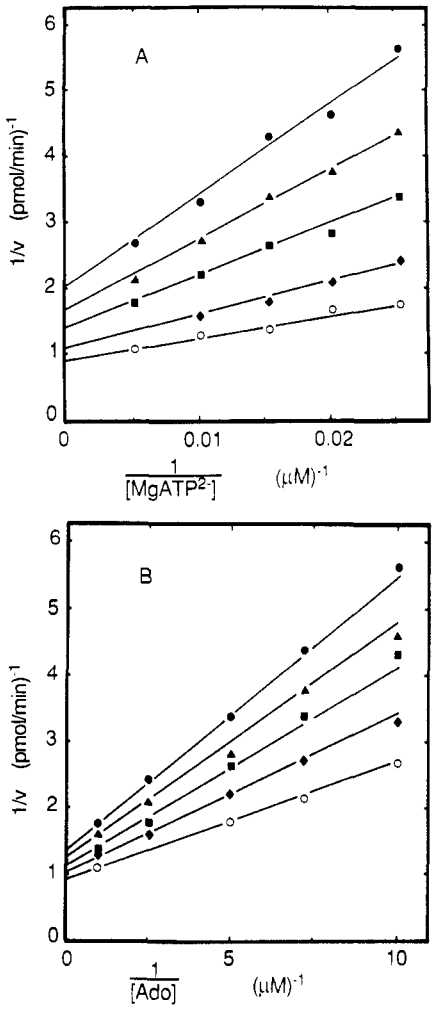


FIGURE 1: Double-reciprocal plots of initial velocity against $MgATP^{2-}$ concentration (A) and adenosine concentration (B). Assays were performed at pH 8.0, and $MgCl_2$ was 1 mM in excess of ATP in all cases. In (A), adenosine concentrations were 0.1 (●), 0.138 (▲), 0.2 (■), 0.4 (◆), and I μM (○). In (B), $MgATP^{2-}$ concentrations were 39 (●), 49 (▲), 64 (■), 99 (◆), and 197 μM (○). Values for the following kinetic constants were obtained by fitting the data to the rate equation for sequential reactions with the SEQUEN computer program of Cleland (1979): K_{ia} = 0.54 ± 0.12 μM; K_m(adenosine) = 0.16 ± 0.02 μM; $K_m(MgATP^{2-})$ = 27 ± 4 μM.

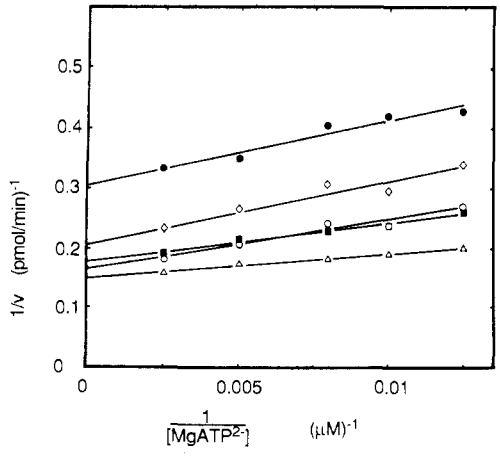


FIGURE 2: Product inhibition by AMP with varying $MgATP^{2-}$. Assays were performed at pH 8.0, and $MgCl_2$ was 1 mM in excess of ATP. Adenosine concentration was 5 μM. The AMP concentrations were 0(○), 2.5 (△), 5 (■), 7.5 (◇), and 10 mM (●).

below about 5 mM cannot be explained by the chelation of excess free magnesium ion by AMP as the same pattern of

Table II: Product Inhibition of Adenosine Kinase[a]

product	variable substrate	saturating substrate	type of inhibition	inhibition constants
$MgADP^-$	$MgATP^{2-}$	adenosine	noncompetitive	K_{ii} = 534 μM K_{is} = 30 μM
$MgADP^-$	adenosine	$MgATP^{2-}$	noncompetitive	K_{ii} = 428 μM K_{is} = 362 μM
AMP	$MgATP^{2-}$	adenosine	noncompetitive[b]	
AMP	adenosine	$MgATP^{2-}$	competitive	K_{is} = 1840 μM

[a] All assays were performed at pH 8.0 with a calculated free magnesium ion concentration of 1.0 mM. [b] Only when 5–10 mM AMP was used (see Figure 2).

Table III: Effect of AMP and $MgCl_2$ on Substrate Inhibition by Adenosine[a]

expt	[AMP] (mM)	$[Mg^{2+}]_{free}$ (mM)	V_{max} (pmol·min⁻¹)[b]	K_m (μM)[b]	K_i (μM)[b]
a	0	1.00	2.19 (0.06)[c]	0.71 (0.04)[c]	51 (10)[c]
	1.0	0.94	3.09 (0.06)	1.18 (0.05)	75 (15)
	2.5	0.86	2.81 (0.09)	1.75 (0.11)	238 (212)
b		0	2.4 (0.3)	3.91 (0.79)	45 (14)
		0.03	3.8 (0.2)	0.35 (0.04)	15 (2.0)
		0.17	3.7 (0.1)	0.41 (0.02)	4.6 (0.2)
		4.01	0.72 (0.06)	0.40 (0.06)	5.9 (1.1)

[a] Assays were performed at pH 8.0 with 0.08 mM ATP (a) or at pH 7.4 with 1 mM ATP (b). [b] Kinetic parameters were obtained from the SUBIN computer program of Cleland (1979). [c] Values in parentheses are the standard error of estimation.

activation was observed when the experiment was repeated with a constant concentration of 1 mM free magnesium ion. However, this apparent activation of the enzyme by low concentrations of AMP was found to be dependent on the adenosine concentration, and the activation was also enhanced when the free magnesium ion concentration was increased from 1 to 5 mM. In essence, provided that the concentrations of adenosine and free magnesium ion were maintained below 1 μM and 1 mM, respectively, AMP tended toward classical product inhibition, showing noncompetitive inhibition with respect to $MgATP^{2-}$ (at the higher AMP concentrations) and competitve inhibition with respect to adenosine. The product inhibition data are summarized in Table II.

Substrate Inhibition by Adenosine. The activation of the enzyme by low concentrations of AMP was caused, at least in part, by decreasing the substrate inhibition by adenosine. When the adenosine concentration was varied over a broad range (0.2–10 μM), the K_i for adenosine acting as a substrate inhibitor was increased by AMP while the K_m for adenosine was also increased by the AMP acting as a product inhibitor (Table III, experiment a). The activation by AMP was also proportionately higher in assays performed with increased free magnesium ion concentration.

Substrate inhibition by adenosine was influenced both by pH and free magnesium ion concentration. The substrate inhibition by adenosine was most severe at pH 7.4, with less inhibition at pH 8.0 and no inhibition at pH 6.5 (C. F. Hawkins and A. S. Bagnara, unpublished observations). Free magnesium ion enhanced substrate inhibition by adenosine (Table III, experiment b) with the adenosine inhibition being noncompetitive with respect to $MgATP^{2-}$.

DTNB-Mediated and Spontaneous Inactivation of Adenosine Kinase. For an enzyme that can undergo either spontaneous or chemically-induced inactivation and where the enzyme may be protected against these inactivations by the binding of one of its substrates, a "protection" constant analogous to a K_m can be calculated for the enzyme–substrate (ES) complex (Burton, 1951; Chuang & Bell, 1972). Various

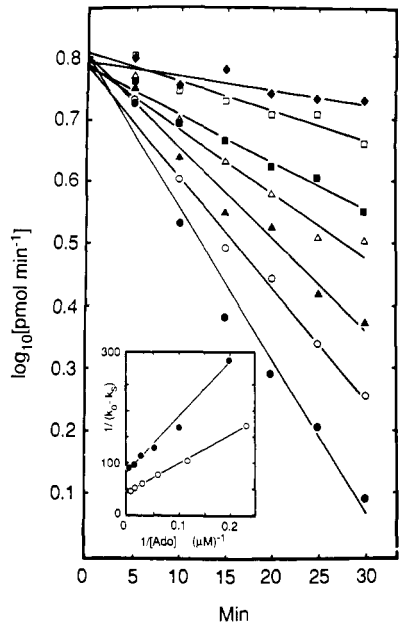


FIGURE 3: Protection by adenosine against DTNB-mediated inactivation of adenosine kinase. Adenosine concentrations were 0 (●), 4.3 (○), 8.6 (▲), 17.2 (△), 34.5 (■), 69 (□), and 172 μM (◆). (Inset) Determination of dissociation constants for adenosine from the primary data shown above. Plots are of DTNB-mediated (○) and spontaneous (●) inactivation of the enzyme.

workers have since shown that this protection constant is equal to the dissociation constant of the ES complex (Scrutton & Utter, 1965; O'Sullivan & Cohn, 1966).

Rate constants were measured for the inactivation of the enzyme in the presence of 0.1 mM DTNB at 37 °C at a series of adenosine concentrations over the range 0–200 μM (Figure 3). These data show an adenosine concentration dependent protection of the enzyme against inactivation by DTNB. The plot of $1/(k_0 - k_s)$ against 1/[adenosine] was linear and gave a value for K_s of 12.6 ± 0.40 μM (see inset to Figure 3). Rate constants were also measured for the protection afforded by adenosine against the spontaneous inactivation of adenosine kinase at 37 °C; these results gave a value for K_s of 12.8 ± 1.7 μM.

Various ionic and molecular species (other than adenosine) that are present during the adenosine kinase assay (viz., ATP^{4-}, $MgATP^{2-}$, ADP^{3-}, $MgADP^{-}$, AMP^{2-}, and free magnesium ion) were also tested for their ability to protect adenosine kinase against inactivation by DTNB. All these species showed varying abilities to protect the enzyme against inactivation, but such protection was achieved only at concentrations approximately 100-fold higher than was required for protection of the enzyme by adenosine (Table IV). The various species mentioned above as well as $Co(NH_3)_4ATP$, β,γ-methylene-ATP, 2′-deoxyadenosine, adenine, and guanosine were then tested in the presence of a low, protecting concentration of adenosine (Table IV). The presence of adenosine did not enhance the protection given by any other species. Such combination experiments therefore gave no evidence to suggest that a protection site was induced by the binding of adenosine to the "catalytic" adenosine binding site. While 1 mM concentrations of ADP^{3-}, AMP^{2-} and $MgCl_2$ all gave protection equivalent to that achieved by 10 μM adenosine, they were antagonistic to the protection afforded by the adenosine. The low level of protection given by both 2′-deoxyadenosine and adenine when added separately or together was found to be additive when in the presence of adenosine. Guanosine neither gave protection nor was it antagonistic toward adenosine protection.

Table IV: Protection of Adenosine Kinase against Inactivation by 0.1 mM DTNB

protecting agent	concn (mM)	% activity remaining after 20 min[a]
adenosine	0.2	89
adenosine	0.01	37
nil		11
ATP	1.0	44
$Co(NH_3)_4ATP$	1.0	44
ADP	1.0	43
AMP	1.0	40
$MgCl_2$/β,γ-methylene-ATP	1.0/1.0	33
adenine	1.0	27
2′-deoxyadenosine	1.0	25
β,γ-methylene-ATP	1.0	15
guanosine	1.0	13
adenosine (0.01 mM) plus		
2′-deoxyadenosine	1.0	48
$Co(NH_3)_4ATP$	1.0	44
adenine	1.0	42
$MgCl_2$/β,γ-methylene-ATP	1.0/1.0	37
β,γ-methylene-ATP	1.0	37
$MgCl_2$	1.0	35
ADP	1.0	32
AMP	1.0	22

[a] See Experimental Procedures for details of the assay.

DISCUSSION

The reaction catalyzed by human erythrocyte adenosine kinase appears to proceed via an ordered sequential mechanism. Product inhibition data, in the absence of substrate inhibition by adenosine, are consistent with an ordered bi-bi addition of substrates and release of products in which adenosine is the first substrate to bind to and AMP is the last product to dissociate from the enzyme. These results are in agreement with the mechanism proposed for adenosine kinase from human placenta (Palella et al., 1980) and differ, in the order of substrate binding, from the mechanism proposed for the enzyme from Ehrlich ascites tumor cells (Henderson et al., 1972). More recently, in a report on kinetic studies of adenosine kinase from murine leukemia L1210 cells, Chang et al. (1983) proposed a two-site ping-pong mechanism for the enzyme with a phosphorylated enzyme as an obligatory intermediate. All other data, however, provide evidence for a sequential reaction mechanism catalyzed by adenosine kinase from several sources. This evidence includes not only the kinetic data reviewed above but also evidence that the adenosine kinase reaction results in the stereochemical inversion of the terminal phosphoryl group of ATP when it is transferred to the 5′-hydroxyl group of adenosine (Richard et al., 1980).

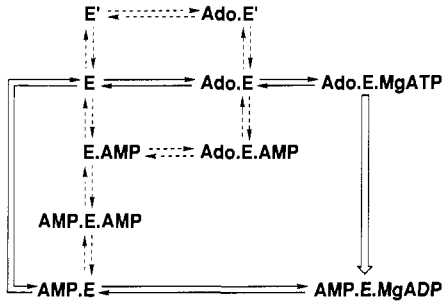
The apparent activation of the enzyme by low concentrations of AMP is likely to be due to a lowering of the high substrate inhibition caused by adenosine. This activation by AMP was dependent on the concentrations of both adenosine and free magnesium ion and occurred only when the enzyme would otherwise be inhibited by these two substrates. The expected pattern for any substrate inhibition in an ordered sequential mechanism is one of uncompetitive inhibition by the second substrate (Cleland, 1971), where a dead-end complex might be formed by combination, in this case, of $MgATP^{2-}$ with the enzyme–AMP complex. If such a complex were formed by adenosine combining with the enzyme–AMP complex, uncompetitive inhibition by adenosine would still be expected with respect to $MgATP^{2-}$ and the presence of AMP should enhance the substrate inhibition. The noncompetitive substrate inhibition by adenosine with respect to $MgATP^{2-}$ shows that adenosine does not inhibit by binding to the nucleotide binding site and suggests that the binding of adenosine as an inhibitor occurs before the binding of $MgATP^{2-}$ in the reaction se-

Table V: Kinetic and Dissociation Constants for Adenosine

constant[a]	value (μM)
K_{ia}	0.53 (0.22)[b]
K_s	
spontaneous	12.8 (1.71)
DTNB mediated	12.6 (0.40)

[a] Values for the various constants were obtained as follows: K_{ia}, by fitting the data of an experiment similar to that shown in Figure 1 (but performed at pH 7.4) to the rate equation for sequential reactions with the SEQUEN computer program of Cleland (1979); K_s, by fitting the data of Figure 3 (inset) with the HYPER computer program of Cleland (1979) modified to omit the weighting of velocities. [b] Values in parentheses are the standard errors of estimation.

Scheme I: Reaction Mechanism for Adenosine Kinase[a]



[a] Broken arrows denote alternative pathways that account for adenosine inhibition (via E') and AMP activation.

quence. The apparent decrease in this inhibition in the presence of AMP and the finding that free magnesium ion increases the substrate inhibition by adenosine suggest that adenosine binds as an inhibitor to the free enzyme via an adenosine binding site that is distinct from the catalytic center. The existence of a second distinct adenosine binding site is supported by the finding that the protection of the human red cell enzyme by adenosine against both spontaneous and DTNB-induced inactivation was characterized by a dissociation constant of 12–13 μM, a value more than 20-fold higher than the dissociation constant for adenosine binding at the catalytic center (Table V). The presence of more than one adenosine binding site on adenosine kinase has previously been noted for the enzyme from yeast (Neudecker & Hartmann, 1978), but these authors proposed that the second adenosine bound to the nucleoside triphosphate binding site.

Apart from the kinetic data discussed above, there is additional evidence that the adenosine binding site responsible for the protection of the essential thiol group(s) is distinct from the nucleotide binding site at the catalytic center. If the nucleoside "pocket" in the ATP binding site were the site for protective adenosine binding, then it might be expected that guanosine would also provide some protection when it is considered that GTP was a better phosphoryl donor than ATP (C. F. Hawkins and A. S. Bagnara, unpublished data). However, guanosine neither provided protection nor did it antagonize the protection afforded by adenosine.

A reaction mechanism that can account for the observed results is proposed whereby the apparent activation by AMP is due to an alternative reaction pathway that avoids substrate inhibition by adenosine (Scheme I). If AMP were to cause the apparent activation of the enzyme by binding to the protective adenosine binding site, one would expect that AMP would protect the enzyme against inactivation and that this protection would be additive with that afforded by adenosine. The finding that AMP causes antagonism toward adenosine protection argues against this possibility. In the scheme proposed, the binding of AMP to the enzyme as an "activator" occurs at the nucleoside triphosphate binding site.

The data we have presented here indicate that the kinase from human erythrocytes has at least two distinct binding sites for adenosine. These two sites have been characterized with respect to their binding constants, and a model has been put forward for the involvement of one of these sites in the expression of such properties as the high substrate inhibition by adenosine, the inhibition caused by excess free magnesium ion, and the protection afforded by adenosine against inactivation of the enzyme by DTNB.

Registry No. AMP, 61-19-8; MgADP, 7384-99-8; MgATP, 1476-84-2; adenosine, 58-61-7; adenosine kinase, 9027-72-9.

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Effect of Drug–DNA Interactions upon Transcription Initiation at the *lac* Promoter†

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ABSTRACT: We have examined the effects of six DNA binding drugs upon initiation at the *lac* UV5 promoter by *Escherichia coli* RNA polymerase. Experiments were directed at determining the influence of added drug on open complex formation, open complex stability, initiation from the open complex, and stability of the resulting initiated complex. The narrow groove binding drugs distamycin and 4′,6-diamidino-2-phenylindole dihydrochloride were more effective in inhibiting initiation through their effect on the first three of these factors than were the intercalators ethidium bromide, daunomycin, and actinomycin. The bisintercalator bis(daunomycin) inhibited open complex formation better than its parent daunomycin. With the possible exception of actinomycin, the drugs tested were not able to disrupt preformed initiated complex, in contrast to their destabilizing effect upon the open complex. Combined with other results, the data suggest that the antitumor activity of daunomycin is unlikely to result from its effect on transcription. We compare the relative effectiveness of the drugs with the known physical properties of the corresponding drug–DNA interactions. The rate of open complex formation seems to be influenced by both the on and off rates of the drug, probably due to the relative slowness of open complex formation. This is in contrast to elongation, a much quicker process, which seems to be limited by the drug off rate alone; these considerations may possibly rationalize the difference in relative effect of particular drugs upon initiation and elongation. All drugs were able actively to disrupt open complex, although to substantially different extents; some possible mechanisms for this disruption, and the insensitivity of the initiated complex, are discussed.

DNA binding drugs have the potential for blocking DNA replication and transcription, which are vital for cell maintenance and proliferation. The effect of drugs upon transcription in vitro has been studied with a wide variety of templates including bulk bacterial and calf thymus DNA and copolymers such as poly(dA-dT) [see reviews by Gale et al. (1972) and Zimmer et al. (1975)]. Although studies on bulk DNA allow an overview on how a drug will affect transcription, this heterogeneous mix of genes or nonpromoter sequences cannot provide details upon the specific target of the drug action in this complex process. Recent developments in molecular genetic technology have encouraged us to reexamine longstanding problems, initially addressed many years ago, related to the specific biochemical targets of action of DNA binding drugs.

The importance of studying the target of drug action is seen in the differential inhibition of transcription from certain genes by a given drug (Bleyman et al., 1969; Pennman et al., 1968); target specificity could therefore be utilized for selective drug action. Knowledge of which sequences a drug will bind [see Niedle & Abraham (1984)], and the thermodynamic and kinetic characteristics of the interaction, provides a basis for predicting and analyzing the drug's effect upon each step of transcription from a given sequence. Several studies have focused upon the stages of transcription affected by drug bindng. Distamycin has been shown to inhibit transcription initiation but not elongation, on the basis of the resistance of transcription to drug added to DNA after polymerase preincubation (Puscendorf et al., 1976; Küpper et al., 1973). Also, actinomycin D has been shown to inhibit elongation rather than initiation (Richardson, 1966; Hyman & Davidson, 1970). The sites of drug-induced blockage of *Escherichia coli* RNA polymerase in the elongation stage have been studied on T7 phage DNA (Aivashahvilli & Beabeahashvilli, 1983) and on *lac* run-off transcripts (Phillips & Crothers, 1986), providing information on both sequence specificity of drug binding and the efficiency of blockage.

In this paper, we compare the effect of six DNA binding drugs upon the steps of transcription initiation at the *lac* UV5 promoter using *E. coli* RNA polymerase. We utilize native polyacrylamide gel electrophoresis of the intermediates in initiation; gel complexes representing open complex and a

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