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HUMAN RIBONUCLEOTIDE REDUCTASE

ACTIVATION AND INHIBITION BY ANALOGS OF ATP

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Abstract—Sixteen ATP analogs were studied as activators of CDP reduction catalyzed by human ribonucleotide reductase. Activation constants were determined. Three analogs, 3-deazaATP, 5′-adenylimidodiphosphate, and 3′-dATP, activated approximately as efficiently as ATP. Four analogs were partial activators. These seven activators were also accessory activators of GDP reduction. Furthermore, two other analogs, adenosine-5′-*O*-(1-thiotriphosphate) and 8-bromoATP, selectively stimulated GDP reduction. Ten analogs, at equal molar concentrations with ATP, inhibited ATP-dependent activation of CDP reduction and/or accessory activation of GDP reduction by greater than 45%. No analog inhibited as potently as 2′-dATP, which had an IC_{50} of 30–50 μM versus the stimulation of CDP and GDP reduction by 2.0 mM ATP.

Mammalian ribonucleotide reductase catalyzes the reduction of nucleoside diphosphates in the rate-limiting step for DNA synthesis [1–3]. The enzyme is allosterically activated and inhibited by nucleoside triphosphates [1–3]. ATP plays two important roles in this regulation. It is an essential activator of CDP and UDP reduction (i.e. no reduction occurs in its absence) as well as an accessory activator of GDP and ADP reduction [4–6]. As an accessory activator, ATP increases the efficiency of the essential activator by decreasing the concentration of activator (K_{act}) required to produce one-half maximum activation, and by increasing the velocity obtained at maximum activation (V_{max}). The essential activators of GDP and ADP reduction are dTTP and dGTP, respectively [1–6]. Thus, in either role, ATP serves to stimulate the reduction of all four substrates.

Cory *et al.* [7] recently showed that formycin A-TP† can substitute for ATP as an activator of CDP reduction. That study with a murine ribonucleotide reductase prompted the present investigation of the effects of sixteen ATP analogs on human ribonucleotide reductase. The analogs were evaluated as essential activators of CDP reduction, as accessory activators of GDP reduction, and as antagonists of ATP.

MATERIALS AND METHODS

Nucleoside triphosphates. ATP and ultrapure deoxynucleoside triphosphates were obtained from Pharmacia Biochemicals; ATP-α-*S* was from Boehringer Mannheim Biochemical Corp. and formycin A-TP from Calbiochem. APP-TP, acycloATP, and C^3ATP were synthesized by unpublished procedures and provided by D. J. Nelson and W. H. Miller of these laboratories. The remaining analogs were purchased from the Sigma Chemical Co.

Ribonucleotide reductase. Human ribonucleotide reductase was purified from Detroit 98 cells (human sternal marrow) by a method described previously [8]. Product formation was linear with respect to time for at least 30 min at 37°. The enzyme preparations had an average ratio of ribonucleotide reductase to nucleoside diphosphate kinase of greater than 5:1. Less than 3% of the substrate was diverted to the triphosphate form during the reactions.

Other materials. [U-^{14}C]CDP (499–517 Ci/mol) was purchased from New England Nuclear, and [8-^{14}C]GDP (50 Ci/mol) from Moravek Biochemicals, Inc. *Escherichia coli* alkaline phosphatase and phosphoenolpyruvate were from Sigma, and pyruvate kinase was from the Boehringer Mannheim Biochemical Corp.

Purification of nucleoside triphosphate analogs. ThioITP, 3′-NH_2-3′-dATP, and APP-TP were less than 90% homogeneous by HPLC analysis. The major contaminant, the corresponding nucleoside diphosphate, was converted to the respective triphosphate form by the following procedure. The nucleotides (6–10 mM) were incubated for 2 hr at

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† Abbreviations: formycin A-TP, 7-amino-3-(β-D-ribofuranosyl)pyrazolo [4,3-*d*]-pyrimidine 5′-triphosphate; APP-TP, 4-amino-1-(β-D-ribofuranosyl)pyrazolo [3,4-*d*]-pyrimidine 5′-triphosphate; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; 8-bromoATP, 8-bromoadenosine 5′-triphosphate; ATP-α-*S*, adenosine-5′-*O*-(1-thiotriphosphate), *S*-isomer; thioITP, 6-mercaptopurine riboside 5′-triphosphate; 8-azidoATP, 8-azidoadenosine 5′-triphosphate; AMP-PNP, 5′-adenylimidodiphosphate; 3′-NH_2-3′-dATP, 3′-amino-3′-deoxyadenosine 5′-triphosphate; 3′-dATP, 3′-deoxyadenosine 5′-triphosphate; AMP-CPP, α,β-methyleneadenosine 5′-triphosphate; araATP, 9-β-D-arabinofuranoyladenine 5′-triphosphate; C^3ATP, 3-deazaadenosine 5′-triphosphate; 2′,3′-dideoxyATP, 2′,3′-dideoxyadenosine 5′-triphosphate; 2′-dATP, 2′-deoxyadenosine 5′-triphosphate; acycloATP, 9-((2-hydroxyethoxy)methyl) adenine triphosphate; DTT, dithiothreitol; TEAB, triethylamineammonium bicarbonate; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

25° with 15 mM phosphoenolpyruvate, 10 mM $MgCl_2$, 10 mM KCl, 50 mM potassium Hepes, pH 7.7, and 12 units/mL of pyruvate kinase. Pyruvate kinase was then removed with a centrifree micropartition filter (Amicon). The filtrate was loaded on a QAE A25 column equilibrated with 20 mM triethylamineammonium bicarbonate (TEAB). Phosphoenolpyruvate was eluted with 200 mM TEAB, and the triphosphate analogs with 1 M TEAB. TEAB was removed under vacuum using a Speed Vac concentrator. HPLC analysis revealed the triphosphate content of the treated samples to be >97% of the UV absorbing material.

Ribonucleotide reductase assays. Assays for CDP [9] and GDP [10] reduction were performed as previously described. Since iron stimulates the activity of the enzyme [11–13], 5 μM $FeSO_4$ was included in all assays to avoid differences in the degree of stimulation that could be caused by the extraneous iron associated with ATP [13] or the analogs.

Radiolabeled CDP and GDP were purified by ion exchange chromatography, and the reactions were terminated with 100 mM hydroxyurea and 60 mM EDTA to maintain very low blank rates [10, 14]. Between 0.1 and 10% of the substrates were converted to products during the reactions. Velocities are expressed as nanomoles per hour per unit enzyme. One unit of ribonucleotide reductase converts 1 nmol of CDP to dCDP/hr under standard assay conditions. Sets of six reactions were assayed simultaneously as described elsewhere [15].

The standard assay components for CDP reduction were 100 mM sodium Hepes, pH 7.4, 5 mM dithiothreitol (DTT), 2.4 mM $MgCl_2$, 5 μM $FeSO_4$, 50 μM [^{14}C]CDP, and 2 mM ATP. Duplicate reaction mixtures were incubated for 30 min at 37°. Potential antagonists of activation by ATP were assayed in reaction vials containing these standard components (with ATP) plus 0.5 or 2 mM analog and a total of 2.9 or 4.4 mM $MgCl_2$, respectively.

The standard assay components for GDP reduction were 100 mM sodium Hepes, pH 7.4, 5 mM DTT, 3.5 mM $MgCl_2$, 5 μM $FeSO_4$, 50 μM [^{14}C]GDP, 0.2 mM dTTP, and 2 mM ATP. The concentration of $MgCl_2$ was 0.5 mM in reactions without ATP. Duplicate reaction mixtures were incubated for 20 min at 37°. Potential antagonists of accessory activation by ATP were assayed in reaction vials containing these standard components (with ATP) plus 0.5 or 2 mM analog and a total of 4 or 5.5 mM $MgCl_2$, respectively.

Miscellaneous methods. Kinetic constants, K_{act}, the concentration of activator required to produce one-half maximum activation, and V_{max}, the velocity obtained at maximum activation, were calculated by fitting the initial, linear velocity data to a hyperbola using the computer program described by Cleland [16]. The concentrations of free and complexed ATP and Mg^{2+} were calculated by the method of Storer and Cornish-Bowden [17]. The analog triphosphates and dTTP were considered to be equivalent to ATP with respect to their binding constant for Mg^{2+} [18]. Since free ATP is a more potent inhibitor of human ribonucleotide reductase than is free Mg^{2+} [10], the concentration of $MgCl_2$ was adjusted to minimize the formation of free nucleoside triphosphate. The resultant concentrations of 0.4 to 0.7 mM free Mg^{2+} and 0.01 to 0.16 mM free triphosphate for CDP reduction, 1.3 mM free Mg^{2+} and 0.02 to 0.05 mM free triphosphate for GDP reduction with ATP present, and 0.3 mM free Mg^{2+} and 0.01 mM free triphosphate for GDP reduction with ATP absent were not inhibitory.

Table 1. Activation of ribonucleotide reductase

Analogs	Relative activation* (%)†	
	CDP reduction	GDP reduction
ATP	100	100
C^3ATP	78	47
AMP-PNP	60	41
3′-dATP	50	48
AMP-PCP	30	25
8-azidoATP	12	45
Formycin A-TP	11	11
AMP-CPP	9	25
8-bromoATP	<6	21
ATP-α-S	<6	16
ITP	<6	<6
2′,3′-dideoxyATP	<6	<6
3′-NH_2-3′-dATP	<6	<6
ThiolTP	<6	<6
AcycloATP	<6	<6
APP-TP	<6	<6
AraATP	<6	<6

* Analogs were substituted for ATP as the activator of CDP reduction and as the accessory activator of GDP reduction (see Results). The amount of enzyme used was 2 to 8 units/mL in the various experiments measuring CDP reduction, and 5 to 10 units/mL (0.75 to 1.5 units/mL when assayed without ATP) for those measuring GDP reduction.

† The lower limit for detecting activation was 6% of the velocity obtained with ATP as the activator.

RESULTS

Activation of CDP reduction. The velocity of CDP reduction was measured in the presence of either ATP or each analog. The relative activation by the analogs is expressed in Table 1 as a percentage of the velocity obtained with ATP as the activator. No reduction was detected in the absence of ATP or a substitute activator.

Seven analogs activated the reduction of CDP. Saturation curves for these analogs are shown in Fig. 1. Their activation constants are summarized in Table 2.

Accessory activation of GDP reduction. The velocity of GDP reduction was measured in the presence of dTTP and either ATP or each analog. The relative accessory activation by the analogs is expressed in Table 1 as a percentage of the increase in velocity obtained with ATP as the accessory activator. Thus, these values represent the ability of the analogs to augment the essential activation by dTTP. ATP produced about a 5-fold increase in the

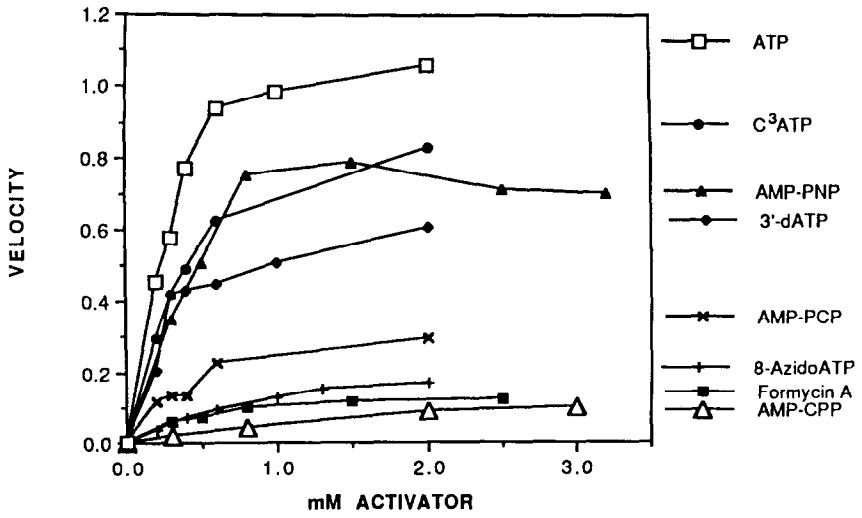

Fig. 1. Velocity of CDP reduction versus the concentration of ATP or analog. Reaction mixtures contained the standard components for the CDP reduction assay plus the indicated concentration of activator. The concentration of $MgCl_2$ was increased relative to the activator, such that the unbound Mg^{2+} remained between 0.4 and 0.7 mM (see Materials and Methods). A representative example of two experiments is shown. The apparent activation constants for the analogs are presented in Table 2.

Table 2. Apparent activation constants*

Sample	K_{act} (mM)	V_{max} (nmol/hr/unit)
ATP	0.30 ± 0.1	1.3 ± 0.1
C^3ATP	0.43 ± 0.04	1.0 ± 0.04
AMP-PNP	0.28 ± 0.1	0.84 ± 0.1
3'-dATP	0.29 ± 0.1	0.69 ± 0.1
AMP-PCP	0.57 ± 0.2	0.41 ± 0.1
8-azidoATP	1.2 ± 0.4	0.27 ± 0.02
Formycin A-TP	0.49 ± 0.1	0.15 ± 0.01
AMP-CPP	2.8 ± 0.6	0.20 ± 0.02

* See Fig. 1 for assay conditions. Values ± standard errors are presented.

rate of the dTTP-activated reduction of GDP. No velocity was detected in the absence of dTTP (with or without ATP).

Inhibition of CDP and GDP reduction. The analogs were studied as antagonists of ATP in its role as activator of CDP reduction and as accessory activator of GDP reduction. Analogs were studied at 0.5 and 2 mM in the presence of 2 mM ATP. To determine if ATP was "self" inhibitory at higher concentrations, 4 mM ATP was studied. Since the velocity at 4 mM ATP was only 21% less than that at 2 mM, "self" inhibition was not a significant factor. The results are presented in Table 3.

DISCUSSION

Our data show that human ribonucleotide reductase did not have a stringent specificity requirement for activation and accessory activation by analogs of ATP. In general, the enzyme tolerated a range of modifications of the ATP molecule, including nucleobase, sugar, and phosphate linkage changes. Seven of the sixteen analogs activated the enzyme with respect to CDP reduction. Three of these, C^3ATP, AMP-PNP, and 3'-dATP, had K_{act} and V_{max} values that were similar to those of ATP. The remaining four, AMP-PCP, 8-azidoATP, formycin A-TP and AMP-CPP, were partial activators.

The seven compounds that supported CDP reduction were also accessory activators of GDP reduction. However, ATP-α-*S* and 8-bromoATP selectively stimulated GDP reduction (Table 1), and thereby may differentiate between the two roles of ATP.

AMP-PNP, which activates vaccinia virus-induced [20] and murine [21] ribonucleotide reductases for CDP reduction, has a non-cleavable phosphate linkage. It was used to activate these enzymes in crude extracts because it does not support phosphorylation of CDP by kinases and it inhibits cleavage of CDP nucleotidases. Our study showed that AMP-PNP may also be useful as an accessory activator of GDP reduction in crude preparations.

Our data showing that formycin A-TP was a partial activator (about 12% the effect of ATP) of CDP reduction with human ribonucleotide reductase confirms the similar observation of Cory *et al.* [22] with a murine enzyme. We also showed that formycin A-TP is a partial accessory activator of GDP reduction.

Many of these ATP analogs are anabolites of pharmacologically active nucleoside analogs. Their net effect on human ribonucleotide reductase is represented by the inhibition data of Table 3, which reflects the balance between the ability of the analog

Table 3. Inhibition of ribonucleotide reductase

	% Inhibition*			
	CDP reduction (2 mM ATP)		GDP reduction (2 mM ATP)	
[Analog]	0.5 mM	2.0 mM	0.5 mM	2.0 mM
C^3ATP†‡	12 (1)§	26	13	62
AMP-PNP†‡	0 (5)	17	27	40
3′-dATP†‡	21 (7)	45	50	50
AMP-PCP†‡	41 (6)	60	22	48
8-azidoATP†‡	28 (4)	44	25	39
Formycin A-TP†‡	11 (10)	27	31	37
AMP-CPP†‡	45 (2)	62	14	29
8-bromoATP‡	7	30	13	37
ATP-α-S‡	58	80	32	52
ITP	21	46	27	34
2′,3′-dideoxyATP	50	92	76	91
3′-NH_2-3′-dATP	51	74	67	91
ThioITP	37	52	62	76
AcycloATP	44	83	38	79
APP-TP	34	51	37	76
AraATP	82	94	75	92
2′-dATP	$IC_{50} = 28 \pm 7$ μM		$IC_{50} = 49 \pm 2$ μM	

* Velocities of inhibited reactions are compared to those of the standard assays of CDP reduction and GDP reduction (see Materials and Methods). The amount of enzyme used was 1.5 to 3 units/mL in the various experiments measuring CDP reduction, and 2.5 to 5 units/mL for those measuring GDP reduction.

† Activator of CDP reduction.

‡ Accessory activator of GDP reduction.

§ Calculated value [19] predicted for the combined effects of ATP and the analog activator.

to either activate the enzyme or inhibit the activation of ATP at molar ratios of analog to ATP of 1:4 and 1:1.

The enzyme was susceptible to inhibition by a wide variety of analogs. Three non-activator analogs, AraATP, 2′,3′-dideoxyATP and 3′-NH_2-3′-dATP, were particularly potent inhibitors of both CDP and GDP reduction. AraATP and 2′,3′-dideoxyATP were identified previously as inhibitors of various ribonucleotide reductases [7, 16, 23, 24].

Since these inhibition assays measured the net combined activation of ATP and the analog, the inhibition by the efficient substitute activators was expected to be limited. For example, a partial competitive activator producing 40% of the activation of ATP would theoretically inhibit the activation by ATP by a maximum of 60%. The predicted percent inhibition of CDP reduction was calculated (parenthetical values of Table 3) for the analogs for which activation constants were obtained. Four of the analog activators, AMP-PNP, C^3ATP, 3′-dATP, and formycin A-TP, produced the expected weak inhibition (Table 3). However, the other three, AMP-PCP, AMP-CPP, and 8-azidoATP, were markedly more inhibitory than predicted. These analogs may be inhibiting at an additional site.

No analog inhibited as potently as 2′-dATP, which had an IC_{50} of 30–50 μM against CDP and GDP reduction. This nucleotide is the physiological negative effector that inhibits the reduction of all four substrates [1–6, 22]. Its roles are directly opposed to the roles of ATP.

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