IRREVERSIBLE INHIBITION OF RIBONUCLEOTIDE REDUCTASE FROM EHRLICH TUMOR CELLS BY A MODULATOR ANALOG[1]

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Summary: Periodate-oxidized ATP (ATP-PI) was prepared and studied as a modulator analog of ATP in the ribonucleotide reductase system from Ehrlich tumor cells. ATP-PI could not replace ATP as an activator of CDP reduction, but was found to be an effective inhibitor of both CDP and ADP reduction. The inhibition was time dependent with 1 μM ATP-PI causing 100% inhibition after 20 hrs. The inhibition was shown to be irreversible by the Ackermann-Potter plot and enzyme activity was not restored by passage of the ATP-PI-treated enzyme over a Sephadex G-25 column. ^{14}C-ATP-PI eluted with the protein peak on Sephadex G-25 chromatography.

Ribonucleotide reductase is the enzyme which is uniquely responsible for the formation of the deoxyribonucleotides which are the precursors of DNA. Ribonucleotide reductase has been studied in bacterial and mammalian systems (1). One common feature of the reductase systems from bacteria and mammals is that they are subject to allosteric regulation by ribo- and deoxyribonucleoside triphosphates.

In this report we present data which show that periodate-treated ATP[3], presumably the dialdehyde derivative of ATP, cannot replace ATP as an activator of CDP reductase, but is effective as an irreversible inhibitor of ribonucleotide reductase from Ehrlich tumor cells.

Ehrlich ascites cells were taken from mice (Dub/ICR) 7 days after transplantation. The crude extract and pH 5.2 fraction were prepared from these cells by the method of Moore (2) except that all solutions contained 1 mM dithioerythritol. The protamine sulfate fraction was prepared as

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[3]Abbreviation used: Periodate-oxidized ATP: ATP-PI

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previously described (3). CDP and ADP reductase activities were determined by the methods of Steeper and Steuart (4) and Cory, et al. (5), respectively. The enzyme assays were carried out in triplicate. In those studies involving the measurement of inhibition as a function of pre-incubation time, the protamine sulfate enzyme fraction was incubated at 4° in the presence and absence of inhibitor. Aliquots were taken at the indicated time intervals and reductase activity determined.

Two identical columns of Sephadex G-25 (2 X 30 cm) were prepared and the void volume of each determined with Blue Dextran and shown to be equivalent (45 ml). Aliquots (2.0 ml) of the control and treated-enzyme fractions were applied to the G-25 Columns and eluted with Tris-HCl buffer (0.02 M, pH 7.0). Fractions (2.0 ml) were collected and the absorbance of the effluent was monitored at 280 and 260 nm. The protein eluting with the void volume was also assayed for CDP reductase activity. The absorbance at 260 nm was monitored to show the separation of the periodate-oxidized ATP from the protein peak in the void volume.

Periodate oxidation of ATP was carried out essentially by the method of Khym and Cohn (6). ATP-PI was separated by chromatography on a Sephadex G-10 column (1.9 X 105 cm). The presence of the aldehyde functional group of the ATP-PI was determined by the method of Zamicnik, et al. (7).

^{14}C-CDP (351 mCi/mmole) and ^{14}C-ADP (53.6 mCi/mmole) were purchased from New England Nuclear; ^{14}C-ATP (550 mCi/mmole) was purchased from Amersham-Searle.

RESULTS

Periodate-Oxidized ATP as Activator for CDP Reductase Periodate-oxidized ATP could not substitute for ATP in the standard assay mixture at 1 mM, 0.1 mM and 0.01 mM concentrations. However, when added in the presence of ATP caused marked inhibition of ribonucleotide reductase activity.

Inhibition of CDP Reductase Activity by Periodate-Oxidized ATP Periodate-oxidized ATP was a very effective inhibitor of CDP reductase activity.

Table I: Inhibition of CDP Reductase by ATP-PI.[a]

Concentration of Inhibition, mM	Preincubation Time, hr	nmoles deoxycytidine/ 30 min/mg protein	Inhibition
-	0	1.58	-
1.0	0	0.06	97
0.1	0	0.65	59
0.01	0	1.48	6
1.0	1	0.03	99
0.1	1	0.35	79
0.01	1	1.45	9
1.0	2	0.03	99
0.1	2	0.29	82
0.01	2	1.18	26
-	20	1.91	-
0.01	20	0.01	99
0.001	20	0.09	95
0.0001	20	1.17	40

[a]The enzyme was incubated with ATP-PI for the indicated time interval at 4° and then the enzyme assay was carried out at 37°. Different preparations of enzyme were used for the 0-2 and 20 hr experiments. A control containing no inhibitor was kept at 4° for 20 hr and assayed for enzyme activity along with the inhibitor-treated samples.

The inhibition by ATP-PI was found to be both concentration and time dependent. With no preincubation, essentially 100% inhibition was seen at 1 mM. The degree of inhibition observed at lower concentrations of inhibitor increased with time. Essentially 100% inhibition was observed with 1 μM ATP-PI and 40% inhibition with 0.1 μM ATP-PI after 20 hr of incubation. These data are shown in Table I. Controls were also run in which sodium iodate at 0.01 and 0.001 mM were incubated with the enzyme fraction for 2 and 20 hr, respectively. Absolutely no inhibition was observed in either case. These concentrations of sodium iodate are the highest that could possibly be present as a contaminant in the inhibitor preparation.

Irreversible Inhibition as shown by Ackermann-Potter Graph The method of Ackermann and Potter (8) was used to determine if the inhibition of ribonucleotide reductase by ATP-PI was reversible or irreversible. The experiment was set up in two ways. Varying concentrations of the enzyme fraction were mixed with ATP-PI (0.1 mM final concentration) and immediately assayed for reductase activity. In the second experiment varying concentrations of the enzyme fraction was incubated with ATP-PI at 0.1 μM for

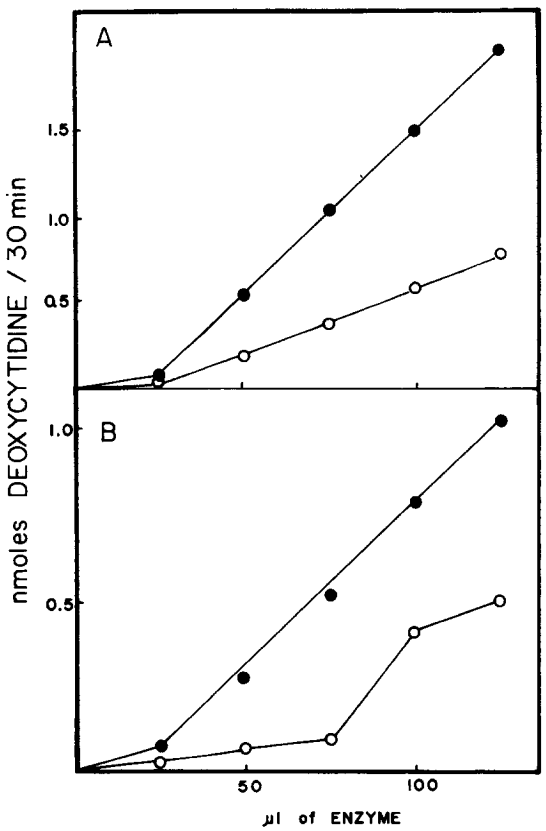

Figure 1: Ackermann-Potter plot. In panel A, periodate-treated ATP (0.1 mM) was added to the enzyme fraction, and the substrate mixture was added and the ribonucleotide reductase assay carried out. There was no preincubation period. In panel B, periodate-treated ATP (0.1 µM) was incubated with the enzyme fraction 20 hrs at 4° prior to carrying out the reductase assay. The volume of the enzyme fraction added was varied as indicated. The final volume in all cases was 150 µl. The reductase activity of the controls is shown by the closed circles (●), while the activity of the samples with periodate-treated ATP is shown by the open circles (o).

20 hr at 4°, and then assayed for reductase activity. Untreated controls were run in both cases. The Ackermann-Potter graphs of these data are shown in Figure 1. Panel A shows the results obtained with no preincubation. These data suggest that the inhibition is reversible. However, with preincubation of the enzyme with inhibitor at much lower concentrations (0.1 µM), the inhibition was shown to be irreversible since the enzyme was effectively titrated out with a fixed concentration of inhibitor. In both cases, the activity of the control at the lowest concentration of enzyme

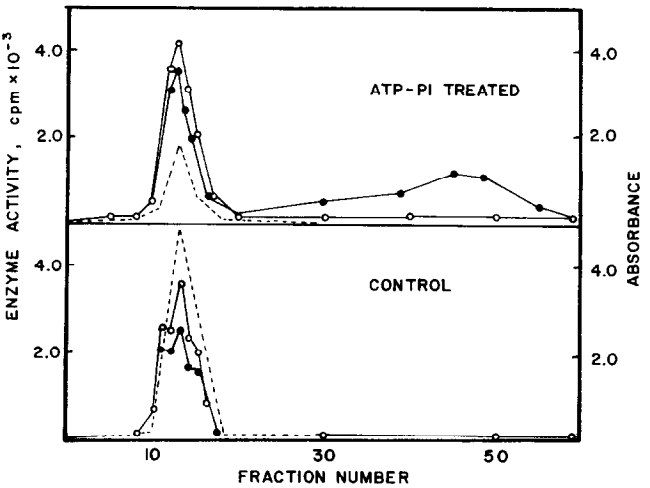

Figure 2: Chromatography of the enzyme fraction treated with ATP-PI on Sephadex G-25. The absorbance at 280 nm is shown by the open circles (○), the absorbance at 260 nm by the closed circles (●) and the CDP reductase activity by the dashed lines (----). The ATP-PI (1 mM) was incubated with the enzyme fraction 3.0 hr prior to passage over Sephadex G-25.

(25 µl of the fraction) was lower than would be expected from the assays run at higher concentrations (100 µl of enzyme fraction/reaction mixture). However, this was probably due to a dilution effect and has been previously reported for ribonucleotide reductase (9,10).

Sephadex G-25 Chromatography of Inhibitor-Treated Enzyme The enzyme fraction was incubated with ATP-PI (1 mM) for 0.75, 1.5 and 3.0 hr at 4°. The inhibitor-treated and control enzyme fractions were put over identical Sephadex G-25 columns and the enzyme eluted with buffer. Under these conditions, it was observed that there was 36, 58 and 64% inhibition in the enzyme samples incubated for 0.75, 1.5 and 3.0 hr, respectively with ATP-PI prior to chromatography on G-25 columns (the % inhibitions were determined on the basis of enzyme activity/mg protein of the ATP-PI-treated samples compared to the untreated controls after passage over the G-25 columns). The data for the samples incubated for 3 hr are shown in Figure 2. The data obtained at the other time intervals were similar except for the degree of inhibition observed. Complete activity was not restored even though the ATP-PI was removed from the protein fraction by this method.

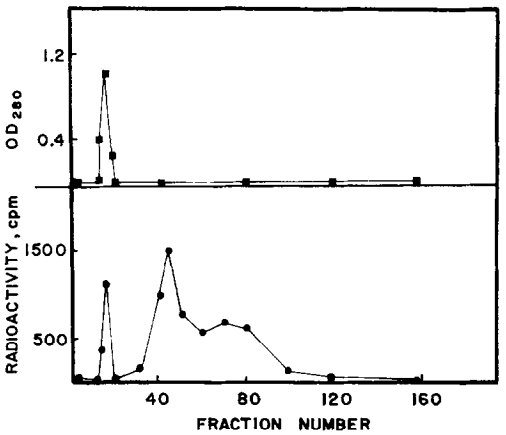

Figure 3: Binding of ^{14}C-ATP-PI to protein eluting with the void volume of a Sephadex G-25 column. The enzyme fraction was incubated with ^{14}C-ATP-PI (156,000 cpm) for 24 hr at 4° prior to chromatography. Aliquots of the fractions were taken for absorbance measurements at 280 nm (■) and for measurement of radioactivity (●).

^{14}C-ATP-PI Binding Studies The enzyme fraction was incubated with ^{14}C-ATP-PI for 24 hr at 4° and the mixture applied to a Sephadex G-25 column. Fractions were collected and the absorbance measured at 280 and 260 nm and aliquots were taken for measurements of radioactivity. The results of such an experiment are shown in Figure 3. There was a discreet peak of radioactivity eluting with the protein in the void volume indicating binding of the periodate-oxidized ATP to protein. In a similar type experiment, the ^{14}C-ATP-PI and enzyme fraction mixture was applied to a Sephadex G-200 column. A discreet peak of radioactivity was found associated with the main protein peak eluting near the void volume (ribonucleotide reductase activity is eluted in this region).

Effect of ATP-PI on CDP and ADP Reductase Activities The effect of ATP-PI on the reduction of ADP and CDP was determined. The results are shown in Table II. The ATP-PI was not preincubated with the enzyme prior to assay. It is seen that ATP-PI inhibits both CDP and ADP reductase activities and even appeared to be more inhibitory to the ADP reductase activity at 0.1 mM.

DISCUSSION

The activity of ribonucleotide reductase from both bacterial and

Table II: Effect of ATP-PI on CDP and ADP Reductase Activities[a]

ATP-PI (mM)	CDP Reductase[b]	% Inhibition	ADP Reductase[c]	% Inhibition
0	1.42	-	0.61	-
1.0	0.10	93	0.01	99
0.1	0.58	59	0.08	85

[a]The CDP and ADP reductase activities were determined in an ammonium sulfate fraction (20-40%) prepared from the protamine sulfate fraction. [b]CDP reductase activity is expressed in terms of nmoles of deoxycytidine/30 min/mg protein. [c]ADP reductase activity is expressed in terms of nmoles of deoxyadenosine/30 min/mg protein.

mammalian cells is subject to strict allosteric control. ATP is an activator of CDP and UDP reduction, while dGTP and dTTP are the prime activators of ADP and GDP reduction, respectively. On the other hand, dATP very effectively inhibits the reduction of all four substrates. The importance of the sugar moiety in the nucleotide effector has been shown not only by these very divergent effects of ATP and dATP on CDP reduction, but also by other studies which showed that adenine arabinoside 5'-triphosphate inhibited ribonucleotide reductase (11) and that 3'-deoxyadenosine 5'-triphosphate (cordycepin triphosphate) did not effectively replace ATP as an activator (12).

The data presented in this report show that periodate-oxidized ATP cannot replace ATP as a positive effector of CDP reduction, but is a very effective, irreversible inhibitor of ribonucleotide reductase activity (Table I).

The time dependence of the inhibitor suggested that ATP-PI was acting as an irreversible-type inhibitor. This was confirmed by the graphic method of Ackermann and Potter (8). That the inhibitor was bound tightly to the enzyme was shown by the studies using Sephadex G-25 chromatography. Even though the ATP-PI was well separated from the enzyme activity eluting in the void volume on Sephadex G-25, and though the protein eluting in the void volume was diluted approximately 10-fold, the ribonucleotide reductase activity in the protein peak was still inhibited. With the use ^{14}C-ATP-PI, it was shown that the periodate-oxidized ATP bound to protein and was eluted in a discreet peak with the protein. It should be pointed out that the enzyme

preparation is not homogeneous enough for us to conclude that the ^{14}C-ATP-PI was bound only to ribonucleotide reductase, but the experiments with the G-25 and G-200 columns do show that this compound does bind to protein. In both cases, the peak of ^{14}C-ATP-PI coincides with the peak of protein having ribonucleotide reductase activity. It is not possible at this time to determine where the ATP-PI is bound. It is known that dialdehydes of the type generated from nucleotides do form Schiff bases with amino groups (13,14). Conclusive data on whether this is specific binding to reductase must await final purification of the mammalian ribonucleotide reductase.

It has been shown for the mammalian reductase system (15) that dATP is "competitive" with ATP. That is, ATP can partially reverse the effects of dATP. The fact that ATP-PI inhibits not only CDP reductase but also ADP reductase activity suggests that ATP-PI is acting as an analog of dATP.

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