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PRODUCTION OF ADENOSINE AND NUCLEOSIDE ANALOGS BY THE EXCHANGE REACTION CATALYZED BY RAT LIVER ADENOSINE KINASE

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Abstract—We have previously shown [8] that rat liver adenosine kinase can produce [^{14}C]AMP from [^{14}C]adenosine (Ado) and unlabelled adenosine monophosphate (AMP), in the absence of ATP, by an exchange reaction. In this study, we investigated whether Ado or AMP could be replaced in this exchange reaction by other nucleosides or nucleoside monophosphates (NMP), respectively. In the presence of 1 mM of the unlabelled NMP analogs 7-deazaadenosine (tubercidin) 5′-monophosphate, 6-chloropurine riboside 5′-monophosphate, or N^6-methyl-AMP, [^{14}C]AMP was formed from 20 μM [^{14}C]Ado at up to 50% of the rate recorded with 1 mM unlabelled AMP. In the presence of 0.2 mM of the unlabelled analog nucleosides tubercidin, N^6-methyladenosine, or 6-methylmercaptopurine riboside, [^{14}C]Ado was generated from 1 mM [^{14}C]AMP at up to 60% of the rate recorded with 0.2 mM unlabelled Ado. Small amounts of [^{14}C]Ado were also formed from the natural nucleosides 5-amino-4-imidazolecarboxamide (AICA) riboside or 2′-deoxyadenosine. Administration of therapeutic anticancer and antiviral nucleosides that can serve as substrates for the exchange reaction catalyzed by adenosine kinase might, thus, result in a net production of Ado, a potent autacoid with physiological effects in numerous tissues.

Key words: exchange reaction; nucleosides; tubercidin; N^6-methyladenosine; 6-methylmercaptopurine riboside

Adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) catalyzes the conversion of Ado† and ATP into Ado monophosphate (AMP) and Ado diphosphate (ADP). It can also phosphorylate several Ado-related nucleosides and analogs into the corresponding monophosphates, preferentially using ATP or guanosine triphosphate (GTP) as the phosphoryl donor [1]. The kinetic characteristics of Ado kinase from different sources have been investigated in detail, and nearly all studies have concluded that the enzyme acts by way of an ordered Bi Bi mechanism. The order of binding of substrates and of release of products has, nevertheless, been reported to vary depending on cell type. For Ado kinase from Ehrlich ascites tumor cells, ATP was found to be the first substrate to bind and AMP the last product to be released [2]. For Ado kinase from human placenta [3], bovine adrenal medulla [4], human erythrocytes [5], and *Leishmania donovani* [6], Ado was shown to bind first, AMP remaining the last product to dissociate.

Recently, we have observed that in anoxic isolated rat hepatocytes and rat liver extracts, incubation with radiolabelled Ado resulted in the labelling of AMP in the absence of ATP by an exchange reaction between Ado and AMP, catalyzed by Ado kinase [7]. Subsequent kinetic studies have led us to the conclusion that Ado kinase from rat liver operates by way of a different ordered Bi Bi mechanism, in which ATP is the first substrate to bind and ADP the last substrate to be released [8]. In this mechanism (Fig. 1), the exchange reaction between Ado and AMP observed in the absence of ATP with the purified enzyme can only be explained if ADP is present. Accordingly, we found that analytical grade AMP was slightly contaminated by ADP (0.001%), and that the exchange reaction could be drastically decreased by purification of AMP.

In the present work we investigated whether Ado or AMP could be replaced in the ADP-dependent exchange reaction between Ado and AMP, catalyzed by rat liver Ado kinase, by nucleoside or NMP analogs, respectively. We also evaluated the ability of other nucleosides, in addition to ADP, to allow the exchange reaction between Ado and AMP to take place.

MATERIALS AND METHODS

Adenosine kinase

Ado kinase was purified to homogeneity from livers of fed male Wistar rats as described [8] to a specific activity of 3.2 μmol/min per mg protein when measured in the presence of 1 μM Ado.

Measurements of exchange reactions

The exchange between [^{14}C]Ado and AMP or other NMPs was measured by following the formation of [^{14}C]AMP from [8-^{14}C]Ado and unlabelled NMPs in the absence of ATP, but in the presence of a maximally stimulatory concentration of ADP. Assays were performed at 37°C in a final reaction volume of 50 μL. The reaction mixture contained 50 mM Hepes, pH 7.2, 0.1

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† Abbreviations: Ado, adenosine; AICA, 5-amino-4-imidazolecarboxamide; AICAR or ZMP, 5-amino-4-imidazolecarboxamide riboside monophosphate; AMP-PNP, β,γ-imidoadenosine 5′-triphosphate; APS, adenosine 5′-phosphosulfate; NMP, nucleoside monophosphate; ZTP, 5-amino-4-imidazolecarboxamide riboside triphosphate.

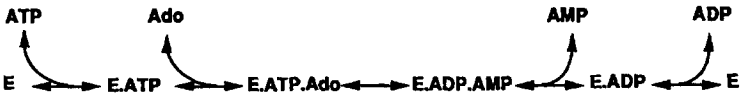

Fig. 1. Reaction mechanism of rat liver adenosine kinase. E, enzyme; Ado, adenosine.

mM ADP, 1 mM $MgCl_2$, labelled Ado and NMPs as indicated in the text. The reaction was initiated by addition of 5 μL of the enzyme preparation, diluted in 25 mM Hepes, pH 7, 0.1% bovine serum albumin, and 5 mM dithiothreitol. The reaction was stopped at suitable time intervals by spotting 10 μL of the incubation mixture on 2 cm × 2 cm pieces of Whatman DE-81 anion exchange paper, which were dipped into ice-cold 1 mM ammonium formate, pH 7 (at least 40 mL/paper) and washed for 15 min. After 2 additional successive 15-min washes at room temperature, the papers were rinsed with absolute ethanol, dried, and the radioactivity in AMP counted by liquid scintillation.

The exchange reaction between [^{14}C]AMP and Ado or other nucleosides was measured by following the formation of [^{14}C]Ado from [8-^{14}C]AMP and unlabelled nucleosides. The assay was performed in the same incubation medium as used for the [^{14}C]Ado-NMP exchange, but the reaction was stopped at suitable time intervals by spotting 10 μL of the incubate on polyethylenimine thin-layer cellulose chromatography plates (Merck, Darmstadt, Germany), on which carrier solutions (50 nmol/mL) of AMP and Ado had been applied. After development in water, the spot corresponding to Ado was cut out and its radioactivity counted.

Analytical methods

Protein was measured by the method of Bradford [9] with bovine γ-globulin as the standard. Analog NMPs formed by the exchange reaction and contaminants of ATP, GTP, and 2′-deoxy-ATP were identified by high pressure liquid chromatography (HPLC) on a 110 mm × 4.7 mm PartiSphere 5 SAX anion-exchange column (Whatman, Maidstone, Kent, U.K.) with a gradient from 100% buffer A (0.01 M $NH_4H_2PO_4$, pH 3.7) and 0% buffer B (0.48 M $NH_4H_2PO_4$, pH 3.7) to 100% buffer B over 40 min at a flow rate of 2 mL/min, adapted from [10]. Retention times were, AMP, 2.4 min; ADP, 17 min; ATP, 29.5 min; GMP, 9 min; GDP, 21 min; GTP, 34 min; 2′-deoxy-ADP, 18 min; 2′-deoxy-ATP, 30 min; tubercidin 5′-monophosphate, 1 min; N^6-methyl-AMP, 2.6 min; 6-methylmercaptopurine riboside 5′-monophosphate, 4.5 min.

Chemicals

[8-^{14}C]Ado (50 mCi/mmol) and [8-^{14}C]AMP (53 mCi/mmol) were purchased from Amersham International (Amersham, Bucks, U.K.). DEAE-Sepharose Fast Flow, Sephacryl S-200, 5′-AMP-Sepharose, Q-Sepharose Fast Flow, and Sephadex G-25 (fine grade) were from Pharmacia (Uppsala, Sweden). Ado, 2′-deoxyadenosine, 2′-deoxy-AMP, adenosine 5′-phosphosulfate (APS), 1,N^6-ethenoadenosine, 1,N^6-etheno-AMP, N^6-methyladenosine, N^6-methyl-AMP, adenine arabinoside, AICAriboside, AICAR or ZMP, 6-mercaptopurine riboside, 5-mercaptopurine riboside 5′-monophosphate, 6-methylmercaptopurine riboside, 6-chloropurine riboside 5′-monophosphate, 1,2,4-tri-azole-3-carboxamide riboside (ribavirin), 7-deazaadenosine (tubercidin), tubercidin 5′-monophosphate, AMP-PNP, ATP, 2′-deoxy-ATP, GDP, ZTP, and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Guanosine, inosine, AMP, ADP, GMP, GTP, IMP, UMP, UDP, UTP, CMP, CDP, and CTP were from Boehringer (Mann-heim, Germany). Bovine γ-globulin was from Bio-Rad (Richmond, CA, U.S.A.). Dithiothreitol was purchased from Janssen Chimica (Beerse, Belgium). All chemicals were of analytical grade.

RESULTS

Exchange reaction between [^{14}C]Ado and NMPs

This exchange was investigated by measuring the formation of [^{14}C]AMP produced from [^{14}C]Ado by the following reaction:

[^{14}C]adenosine + analog NMP → [^{14}C]AMP + analog nucleoside

Reactions were performed with either 20 or 200 μM [^{14}C]Ado, whereas unlabelled NMP were at a concentration of 1 mM. All assays were performed in the presence of 1 mM $MgCl_2$ and 0.1 mM ADP, concentrations that we have shown previously to maximally stimulate the exchange reaction between adenosine and AMP [8]. As shown in Table 1, tubercidin 5′-monophosphate, 6-chloropurineriboside 5′-monophosphate, and N^6-methyl-AMP were found to generate [^{14}C]AMP from 20 μM [^{14}C]Ado at velocities reaching 50%, 28% and 20%, respectively, of that measured in the presence of unlabelled AMP and 20 μM [^{14}C]Ado. Among the natural NMPs, only ZMP and 2′-deoxy-AMP were found to phosphorylate [^{14}C]Ado, albeit at a low, though easily measurable rate. The other NMPs were ineffective as phosphate donors. Strikingly, when the exchange reaction was measured in the presence of 200 instead of 20 μM [^{14}C]Ado, a marked decrease in the generation of [^{14}C]AMP was recorded, which reached 60% in the presence of AMP, 75% in the presence of 6-chloropurineriboside 5′-monophosphate, and 80% in the presence of tubercidin 5′-monophosphate and N^6-methyl-AMP.

Exchange reaction between [^{14}C]AMP and nucleosides

This exchange was investigated by measuring the formation of [^{14}C]Ado, produced from [^{14}C]AMP by the following reaction:

analog nucleoside + [^{14}C]AMP → analog NMP + [^{14}C]Ado

The concentration of [^{14}C]AMP was 1 mM, and the unlabelled analog nucleosides were used at a concentration of 0.2 mM. Assays were also performed in the presence of 1 mM $MgCl_2$ and 0.1 mM ADP. As shown in Table 2, [^{14}C]Ado was generated in the presence of tubercidin or of N^6-methyladenosine at a velocity that reached 60% of that measured in the presence of unlabelled adenosine; in the presence of 6-methylmercaptopurine riboside, velocity reached 45% of that with

Table 1. Formation of [^{14}C]AMP from [^{14}C]adenosine and NMPs

NMP (1 mM)	[^{14}C]AMP formed (nmol/min per mg protein) with [^{14}C]adenosine (20 μM)	with [^{14}C]adenosine (200 μM)
AMP	1500 (1480–1620)	672 (654–690)
Tubercidin 5′-monophosphate	750 (732–768)	138 (150–126)
6-Chloropurineriboside 5′-monophosphate	425 (420–430)	110 (115–105)
N^6-Methyl-AMP	300 (291–309)	51 (50–52)
ZMP	2 (2–2)	<1
2′-Deoxy-AMP	2 (2–2)	<1
GMP	<1	<1
IMP	<1	<1
CMP	<1	<1
UMP	<1	<1
6-Mercaptopurineriboside 5′-monophosphate	<1	<1
1,N^6-Etheno-AMP	<1	<1

The exchange reaction was measured in the presence of 0.1 mM ADP, 1 mM $MgCl_2$, and [8-^{14}C]Ado and NMPs as indicated. Values are means of two determinations given between parentheses.

Ado. It was verified by HPLC that generation of [^{14}C]Ado from [^{14}C]AMP in the presence of these analog nucleosides was accompanied by production of the corresponding analog NMP. Among the naturally occurring nucleosides, only AICAriboside and 2′-deoxyadenosine could be slightly phosphorylated in the presence of [^{14}C]AMP, as evidenced by the minimal formation of [^{14}C]Ado.

Influence of nucleotides on the exchange reaction between [^{14}C]AMP and Ado

In the kinetic mechanism depicted in Fig. 1, ADP allows the exchange reaction between Ado and AMP to take place because it induces the formation of the E.ADP complex, which binds AMP. To assess the specificity of ADP for the exchange reaction, the influence of several nucleoside diphosphates on the exchange reaction between [^{14}C]AMP and Ado was investigated. The use of this exchange reaction rather than that between [^{14}C]Ado and AMP also allowed us to test the permissive effect of nucleoside triphosphates on the exchange reaction without interfering with the Ado kinase reaction. The nucleoside di- and triphosphates were used at a concentration of 10 μM to minimize their contamination. ATP and 2′-deoxy-ATP were contaminated by 0.8% ADP and 2′-deoxy-ADP, respectively, and GTP by 14% GDP. As shown in Fig. 2, the exchange reaction was stimulated most potently by ADP and ATP, GDP, GTP, and 2′-deoxy-ATP, in that order. Natural pyrimidine nucleotides were devoid of stimulatory effect. APS, a po-

Table 2. Production of [^{14}C]adenosine from [^{14}C]AMP and nucleosides

Nucleoside (0.2 mM)	[^{14}C]adenosine formed (nmol/min per mg protein)
Adenosine	730 (740–720)
Tubercidin	442 (464–420)
N^6-Methyladenosine	440 (468–412)
6-Methylmercaptopurine riboside	325 (348–302)
AICAriboside	3 (3–3)
2′-Deoxyadenosine	2 (2–2)
6-Mercaptopurine riboside	<1
Inosine	<1
Adenine arabinoside	<1
Ribavirin	<1
1,N^6-Ethenoadenosine	<1
Guanosine	<1

The exchange reaction was measured in the presence of 0.1 mM ADP, 1 mM $MgCl_2$, and 1 mM [8-^{14}C]AMP. Values are means of 2 determinations given between parentheses.

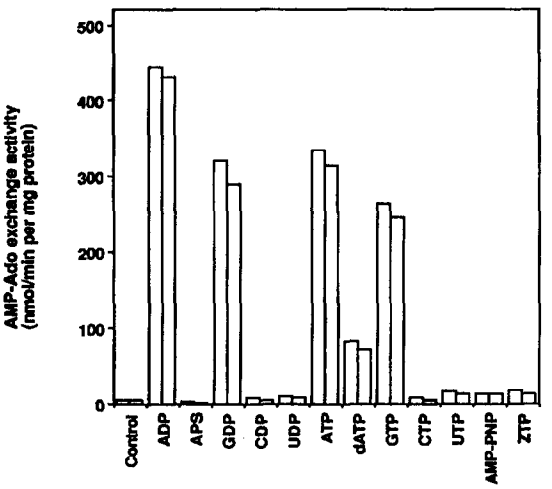

Fig. 2. Effect of nucleoside di- and triphosphates on the exchange reaction between [8-^{14}C]AMP and Ado. The exchange reaction was measured in the presence of 70 μM [8-^{14}C]AMP, 20 μM Ado, and 1 mM $MgCl_2$. Nucleotides were added at a concentration of 10 μM. Ado, adenosine; APS, adenosine 5′-phosphosulfate; AMP-PNP, β,γ-imidoadenosine triphosphate; ZTP, AICAriboside triphosphate. Values shown are 2 individual determinations.

tent stimulator of Ado phosphotransferase [11], ZTP, and AMP-PNP had little or no effect.

DISCUSSION

This study demonstrates that rat liver Ado kinase, in addition to catalyzing an exchange reaction between Ado and AMP, can also accomplish an exchange between [^{14}C]Ado and a number of synthetic analog NMPs, as well as between similar analog nucleosides and [^{14}C]AMP. Moreover, it shows that other nucleoside di- and triphosphates in addition to ADP can allow the AMP-Ado exchange reaction to occur.

The best substrates for the exchange reaction between [^{14}C]Ado and NMPs were the synthetic AMP analogs, tubercidin 5′-monophosphate, 6-chloropurineriboside 5′-monophosphate, and N^6-methyl-AMP. Among the natural NMPs, only 2′-deoxy-AMP and ZMP could serve as phosphoryl donors for [^{14}C]Ado, although their efficiency was minimal when compared with that of the synthetic analogs. Other AMP analogs, namely 6-mercaptopurineriboside 5′-monophosphate and 1,N^6-etheno-AMP, were ineffective. Similarly, the best substrates for the exchange reaction between [^{14}C]AMP and nucleosides were the synthetic Ado analogs tubercidin, N^6-methyladenosine, and 6-methylmercaptopurine riboside. Other analogs and natural nucleosides were poor or ineffective substrates. The capacity of nucleosides to function as substrates for the exchange reaction with AMP, catalyzed by Ado kinase, clearly correlates with their ability to be phosphorylated by this enzyme. Indeed, N^6-methyl-adenosine and 6-methylmercaptopurine riboside are readily phosphorylated by rabbit liver Ado kinase, with Km values of 6 and 2 μM, respectively [12]. In contrast, 2′-deoxyadenosine [13] and AICAriboside (unpublished experiments from this laboratory) are poor substrates of rat liver Ado kinase when compared to Ado. Guanosine and inosine are not or only minimally phosphorylated by ATP [14, 15].

The inhibition of the exchange reaction between [^{14}C]Ado and NMPs observed when the concentration of [^{14}C]Ado was increased from 20 to 200 μM accords with the known inhibitory effect of excess Ado on the activity of Ado kinase. This inhibition has been explained by binding of Ado to a regulatory site distinct from the Ado and ATP substrate sites [5, 16]. It is noteworthy that the inhibitory effect of excess Ado, already recorded at substrate concentrations slightly above 1 μM on the Ado kinase reaction, was not seen at up to 20 μM Ado concentration on the exchange reaction (unpublished observations from this laboratory). Hawkins and Bagnara [5] have observed that the inhibitory effect of excess Ado was diminished by addition of low concentrations of AMP, suggesting binding of the latter to the regulatory site. The high, 1 mM concentration of AMP and NMP analogs present in the assay of the exchange reaction between [^{14}C]Ado and these compounds might, thus, explain the less potent inhibitory effect of excess Ado on the exchange reaction. The observation that the elevation of the concentration of Ado from 20 to 200 μM did not decrease the formation of [^{14}C]AMP to the same extent in the presence of the different NMP analogs, might be explained by differences in their affinity for the regulatory site.

In addition to ADP, a number of triphosphates and GDP were found to enable the exchange reaction between [^{14}C]AMP and Ado to take place. That 2′-deoxy-ATP and GTP were efficient substrates accords with the knowledge that they are also good substrates of Ado kinase [12]. The contamination of ATP by 0.8% ADP and, particularly, of GTP by 14% GDP might intervene owing to the stimulatory property that both diphosphates display on their own, but is not sufficient to explain the effect.

A recently described placental Ado phosphotransferase [11], which is different from Ado kinase, has several features in common with the exchange activity of Ado kinase from rat liver. Both activities have strong preference for the Ado moiety in both acceptor and donor and are strongly stimulated by ADP. APS, a potent stimulator of Ado phosphotransferase [11], had, however, no effect on the exchange reaction between [^{14}C]AMP and Ado. In addition, hydrolysis of AMP can be accomplished in the absence of an acceptor nucleoside with placental Ado phosphotransferase, but not with Ado kinase (unpublished observations from this laboratory). The cytosolic IMP-GMP 5′-nucleotidase has also been shown to catalyze a nucleoside/NMP exchange, but it proceeds most preferentially between inosine and IMP [17, 18]. It also occurs with other nucleosides, however, including acyclovir [19] and 2′,3′-dideoxyinosine [20]. The exchange reaction between Ado and AMP, discovered in rat liver, is not restricted to this tissue but may be a property of the enzyme from various sources. Its existence has been demonstrated in renal brush border membranes from rat and pig [21]. Preliminary results obtained with filtered cytoslic fractions indicate that it can take place in rat heart, brain, and erythrocytes, and in human red blood cells (unpublished observations from this laboratory).

An important conclusion from our observations is that the exchange reaction between Ado and analog NMPs results in net formation of AMP, whereas the exchange reaction between AMP and analog nucleosides results in net production of Ado. Administration of a number of therapeutic anticancer and antiviral nucleosides, as well as of Ado receptor ligands which are substrates of Ado kinase [16], might, thus, result in the release of Ado, a potent autacoid with numerous physiological effects in a variety of tissues (reviewed in [22–25]).

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