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INOSINE ANALOGS AS SUBSTRATES FOR ADENOSINE KINASE—INFLUENCE OF IONIZATION OF THE *N*-1 PROTON ON THE RATE OF PHOSPHORYLATION

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Abstract—This study was undertaken to attempt to rationalize previously obtained and apparently conflicting findings that although adenosine kinase (EC 2.7.1.20) from H.Ep. #2 cells did not accept inosine as a substrate, these cells became resistant to an inosine analog, 8-azainosine, only when activities of both hypoxanthine (guanine) phosphoribosyltransferase [H(G)PRTase] and adenosine kinase were lost. No evidence could be found for the presence of inosine kinase in H.Ep. #2 cells: crude supernatants from these cells converted ring-labeled inosine to IMP, but the conversion was prevented by the addition of hypoxanthine and therefore apparently was achieved by the alternative pathway involving the action of H(G)PRTase on hypoxanthine. An investigation of inosine and inosine analogs as substrates for adenosine kinase revealed that certain inosine analogs were substrates and that the substrate activity could be correlated with the degree of ionization of the N-1 proton. Of four 6-oxo and 6-thio nucleosides studied, 8-aza-6-thioinosine had the lowest pK_a (6.75) and was the only one that was a good substrate at pH 7.0; the K_m was 210 μM and the V_{max} was about 1.5 times that of adenosine. The rate of phosphorylation of 8-aza-6-thioinosine increased markedly as the pH of the reaction mixture was increased in the pH range 6.0 to 7.0. Phosphorylation of 8-azainosine (pK_a 7.45) and 6-thioinosine (pK_a 7.60) was much poorer and could be demonstrated at pH 7.0 only after overnight incubation with the kinase. The fact that 8-azainosine and 8-aza-6-thioinosine are substrates whereas ionsine is not, can be rationalized by the facts that (a) the substitution of an N-atom for the C-8 atom of the nucleoside lowers the pK_a so that the N-1 proton is more strongly ionized at physiological pH; and (b) the ionized form of a 6-oxo or 6-thio nucleoside resembles adenosine with respect to the bond structure at the 1- and 6-positions of the purine ring whereas the unionized form does not.

Adenosine kinase (ATP:adenosine-5′-phosphotransferase, EC 2.7.1.20), partially purified from H.Ep. #2 cells, catalyzes the phosphorylation of purine nucleosides of diverse structure but has no detectable activity for inosine or guanosine [1]. However, cultured H.Ep. #2 cells are sensitive to inhibition by an inosine analog, 8-azaHR*, and evidence has been obtained that in these cells 8-azaHR is phosphorylated directly [2]. The evidence is that a subline of H.Ep. #2 cells lacking activity of H(G)PRTase and resistant to 8-azaH is sensitive to 8-azaHR and that another mutant lacking activity of both H(G)PRTase and adenosine kinase is resistant to both 8-azaH and 8-azaHR. Two possible explanations are: (a) that H.Ep. #2 cells contain a kinase for inosine that is separate from adenosine kinase but is closely linked genetically so that mutation leading to loss of activity of adenosine kinase also results in loss of activity for inosine kinase; or (b) that adenosine kinase catalyzes the phosphorylation of 8-azaHR but not that of inosine. We report here investigation of these possibilities and the conclusion that certain inosine analogs are substrates for adenosine kinase and that the degree of ionization of the N-1 proton is critical for substrate activity.

*Abbreviations used in the text are as follows: 8-AzaHR, 8-azainsoine; 8-AzaH, 8-azahypoxanthine; 6-MP, 6-mercaptopurine; 6-MPR, 6-mercaptopurine ribonucleoside; 6-MeMPR, 6-methylthioinosine (6-methylmercaptopurine ribonucleoside); 8-aza-6-MPR, 8-aza-6-thioinosine (8-aza-6-MP-ribonucleoside); PRTase, phosphoribosyltransferase; and H(G)PRTase, hypoxanthine (guanine) phosphoribosyltransferase (EC 2.4.2.8).

MATERIALS AND METHODS

Compounds. [2-^{14}C]8-AzaHR (Sp. act. 2.5 mCi/m-mole) and [^{35}S]-6-MeMPR (sp. act. 8.8 mCi/m-mole) were prepared as described elsewhere [3, 4]. [8-^{14}C]6-MPR (sp. act. 58 mCi/m-mole) was prepared by the reaction of [8-^{14}C]6-MP (New England Nuclear Corp., Boston, MA) with ribose-1-phosphate in the presence of purine nucleoside phosphorylase (Sigma Chemical Co., St. Louis, MO). The product was separated from unreacted [8-^{14}C]6-MP by high pressure liquid chromatography on a Partisil SAX column using a phosphate eluting system as previously described [5]. 8-Aza-6-MPR was also prepared in our laboratories [6]; the synthesis of this compound by another method has been reported by Hutzenlaub *et al.* [7]. [8-^{14}C]Inosine (sp. act. 35 mCi/m-mole) and [8-^{14}C]adenosine (sp. act. 1 mCi/m-mole) were obtained from New England Nuclear Corp.; [γ-^{32}P]ATP was obtained from Amersham/Searle, Arlington Heights, IL; and creatine phosphate and creatine phosphokinase were obtained from Sigma Chemical Co.

Cell cultures and enzyme assays. Human epidermoid carcinoma (H.Ep. #2) cells, established in culture by Moore *et al.* [8], were grown in suspension culture in SRI-14-medium [9]. For preparation of crude

supernatants, cells were harvested during the logarithmic phase of growth and homogenized in distilled water in a Potter–Elvehjem homogenizer; the homogenate was then centrifuged at 17,500 *g*. Adenosine kinase was purified as described earlier [1]; two preparations were used in the course of this study, one purified 430-fold and one 60-fold over the crude supernatant. The 430-fold-purified preparation was carried through all of the steps of the procedure, whereas the 60-fold-purified preparation was carried only through the first two steps, namely elution from columns of aluminum oxide and Sephadex-G100.

For assay of kinase activity for inosine, the conditions were essentially those used by Pierre and LePage [10] for determination of this enzyme in extracts of Ehrlich ascites cells. The reaction mixture contained, in a final volume of 0.5 ml: [8-^{14}C]inosine (0.4 mM); $MgCl_2$ (5 mM); ATP (1 mM); crude supernatant from H.Ep. #2 cells (1 mg protein); creatine phosphate (5 mM); creatine phosphokinase (39 μg); and Tris buffer (10 mM, pH 7.4). After incubation for 10 or 30 min at 37°, the reaction was stopped by immersion in a boiling water bath. The reaction mixture was subjected to chromatography on Whatman 3 M paper in a solvent consisting of equal volumes of 93.8% aqueous 1-butanol and 44% aqueous propionic acid. The paper strips were assayed for ^{14}C in a Packard chromatogram scanner, and the ^{14}C present at the R_f of IMP was determined.

Procedures for assays of nucleosides as substrates for partially purified adenosine kinase have been described elsewhere [1]. The composition of the reaction mixture used for non-labeled nucleosides is given in Fig. 1. For such substrates, the mixture contained [γ-^{32}P]ATP, and the reaction was measured by separation and assay of the ^{32}P-labeled nucleoside monophosphate that was formed. When a labeled nucleoside was used, the reaction mixture was the same except that unlabeled ATP replaced [^{32}P]ATP. The procedures for separation and assay of the products were the same as those described above for the assay of inosine kinase.

Determination of pK_a *values.* The pK_a values for nucleosides were determined by semimicrotitrations as described by Albert and Serjeant [11].

RESULTS

Absence of inosine kinase activity in H.Ep. #2 cells. Crude supernatants from H.Ep. #2 cells had some capacity to convert [^{14}C]inosine to [^{14}C]IMP (Table 1). The addition of non-radioactive hypoxanthine to the reaction mixture reduced the amount of [^{14}C]IMP formed; this was true for both incubation periods (10 and 30 min). In the 30-min assay, the addition of hypoxanthine in amounts equimolar to inosine reduced formation of IMP by about 80 per cent and higher concentrations reduced it by more than 95 per cent.

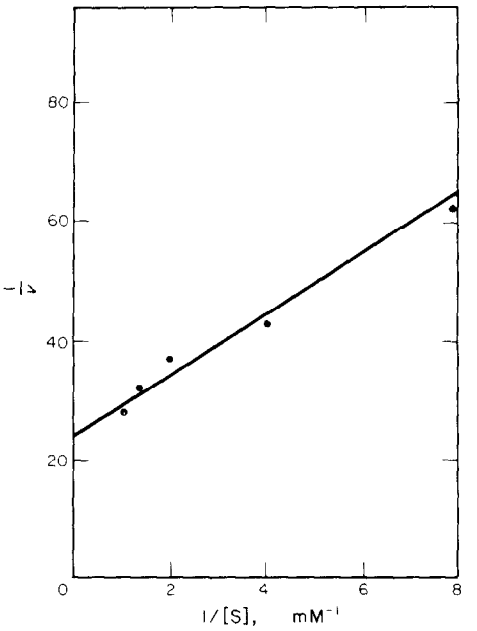


Fig. 1. Lineweaver Burk plots for the phosphorylation of 8-aza-6-MPR by adenosine kinase. The enzyme was 60-fold purified from H.Ep. #2 cells [1]. The incubation mixture contained, in a final volume of 0.2 ml: enzyme; 8-aza-6-MPR at the indicated concentrations; [γ-^{32}P]ATP, 2.5 mM; $MgCl_2$, 0.25 mM; and 50 mM potassium phosphate buffer, pH 7.0. After incubation at 25° for 30 min, the reaction was stopped by immersion in boiling water, and the reaction mixture was analyzed by paper chromatography as described in the text for assay of inosine kinase activity.

8-Aza-6-MPR as a substrate for adenosine kinase. As shown in Fig. 1, 8-aza-6-MPR was a substrate for adenosine kinase. The K_m was 210 μM and the V_{max} was 76 nmoles/min/mg; the corresponding values for adenosine were 1.8 μM and 50 nmoles/min/mg.

Table 1. Effects of non-radioactive hypoxanthine on the conversion of [8-^{14}C]inosine to [8-^{14}C]IMP in extracts of H.Ep. #2 cells*

Unlabeled hypoxanthine added (μmoles/μmole [^{14}C]inosine)	IMP formed (nmoles/μmole of substrate [^{14}C]inosine)	
	10 min	30 min
0	0.21	0.55
0.4	0.11	0.39
1.0	0.02	0.12
3.0	0.01	0.01
10.0	<0.01	<0.01

* Cell-free supernatants from H.Ep. #2 cells were incubated with [8-^{14}C]inosine, and the reaction mixture was described in the text in the presence or absence of unlabeled hypoxanthine. After 10 or 30 min the reaction was stopped and the amount of [^{14}C]IMP formed was determined. See text for details.

Table 2. Rates of phosphorylation by adenosine kinase and ionization constants of some nucleosides and nucleoside analogs*

Substrate	Formation of nucleotide: 45-min Assay (nmoles/min/mg)	Formation of nucleotide: Overnight assay	pK_a	Per cent ionization at pH 7.0
[8-^{14}C]adenosine	296	+		
[8-^{14}C]inosine	<0.8	–	8.74	2
[8-^{14}C]6-MPR	<15	+	7.60	20
[2-^{14}C]8-AzaHR	<15	+	7.45	26
[Non-radioactive]-8-aza-6-MPR	416	+	6.75	64
[^{35}S]-6-MeMPR	760	+		

* The adenosine kinase was 430-fold purified from H.Ep. #2 cells. The incubation mixtures for the 45-min assays and the overnight assays were the same as that given for Fig. 1. The phosphorylation of adenosine was linear during the 45-min assays. For the overnight assays a plus sign indicates that nucleotide was formed (no attempt was made at quantitation) and a minus sign means that no nucleotide was detectable. For other details see Fig. 1 and the text.

pK_a values and activity of nucleosides as substrates for adenosine kinase. Three analogs of inosine were available for this study: 6-MPR, 8-azaHR, and 8-aza-6-MPR. Table 2 presents data on the activity of these nucleosides and inosine as substrates for adenosine kinase and shows also pK_a values and per cent of ionization at pH 7.0. For comparison, data on rates of phosphorylation are also given for 6-MeMPR and adenosine. Two types of assays for substrate activity for adenosine kinase were performed. One was our standard 45-min assay [1]; the other consisted of an overnight incubation with enzyme and was designed for study of nucleosides that are phosphorylated too slowly to have detectable substrate activity in the shorter assay. This overnight assay does not allow calculation of the rate of reaction but gives a qualitative indication as to whether a nucleoside is phosphorylated. No phosphorylation of inosine could be detected even in the overnight assay with [^{14}C]inosine of high specific activity. Phosphorylation of 6-MPR and 8-azaHR could not be detected in the 45-min assay but was detected in overnight assays. As already noted, 8-aza-6-MPR was a good substrate; it was the only one of the three inosine analogs that was phosphorylated at a rate detectable in the 45-min assay. It was also the only 6-oxo or 6-thio nucleoside that was more than 50 per cent ionized at pH 7.0.

Phosphorylation as a function of pH. The results of Table 2 indicate that the ionized form of a 6-oxo- or 6-thiopurine nucleoside is phosphorylated; if so, the rate of phosphorylation of these substrates should be increased by increasing the pH of the incubation mixture. Figure 2 shows the phosphorylation of 8-aza-6-MPR, 6-MeMPR, and adenosine over the pH range 6.0 to 8.0. Increase of pH from 6.0 to 6.5 almost doubled the amount of phosphorylation of 8-aza-6-MPR but had only slight effects on the phosphorylation of adenosine or 6-MeMPR. Further increase of pH to 7.0 produced an additional 25 per cent increase in the amount of 8-aza-6-MPR phosphorylated, and again there was little change in the phosphorylation of the control substrates. Raising the pH to 7.5 produced no further increase in the phosphorylation of 8-aza-6-MPR and caused a decrease in the phosphorylation of both control substrates. Above pH 7.5, phosphorylation of all three substrates declined, possibly due to inactivation of the enzyme. 6-MeMPR and adenosine were included as control substrates because it would be expected that pH changes in this range would produce no significant change in their structures, whereas the ionization of 8-aza-6-MPR, which has a pK_a of 6.75, would be markedly increased as the pH was raised from 6 to 8.

We have also accomplished studies with [^{14}C]-6-MPR at pH 7.0 to 8.0. The rate of phosphorylation was higher at higher pH values but still too low for proper quantitation; therefore these results are not shown.

DISCUSSION

Although inosine kinase has been reported in other mammalian cells [10, 12–14], our attempts to

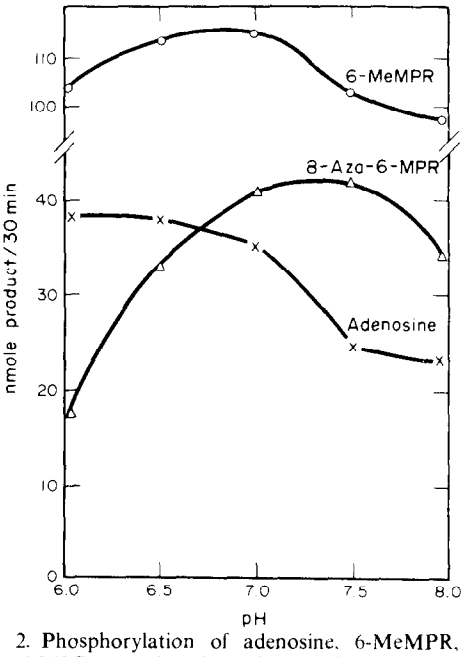
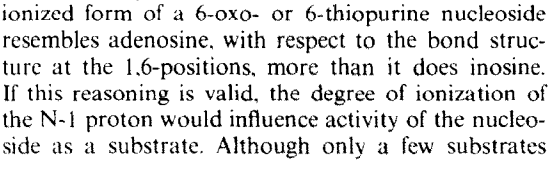


Fig. 2. Phosphorylation of adenosine, 6-MeMPR, and 8-aza-6-MPR as a function of pH. The reactions were accomplished by incubations for 30 min under the conditions described in Fig. 1 except for the differences in pH. The enzyme preparation was also the same as that used for Fig. 1.

demonstrate its presence in H.Ep. #2 cells failed (see Table 1). Crude supernatants from these cells did convert inosine to IMP, but addition of hypoxanthine to the reaction mixture prevented the formation of IMP, indicating that inosine was converted to IMP via the alternative pathway involving the action of H(G)PRTase. Although no PRPP was added to the reaction, it is possible, as pointed out by Schrecker [15], that such crude systems would be likely to contain the necessary enzymes for the generation of PRPP from inosine.

The absence of any detectable activity of inosine kinase in H.Ep. #2 cells would indicate that adenosine kinase from these cells would not have activity for inosine. This has been consistently the case. Inosine kinase activity was absent in our first purified adenosine kinase [1], and all later preparations have been devoid of detectable activity for inosine. In the present study we made another effort to detect activity for inosine by using [^{14}C]inosine of high specific activity and incubating overnight. Even under these conditions no phosphorylation of inosine could be detected.

Previous studies with adenosine kinase have indicated that the bond structure at the 1–6 positions of the purine ring is a critical factor for substrate activity [1, 4]. Thus, 6-methoxypurine ribonucleoside and 6-MeMPR were good substrates, whereas inosine and 6-MPR were essentially unacceptable as substrates [1] (see Table 1). Since adenosine exists almost entirely in the amino form, whereas 6-oxo- and 6-thiopurines exist almost entirely in the oxo or thione forms [16], it is likely that this dissimilarity is responsible for inactivity of the nucleosides of 6-oxo- and 6-thiopurines as substrates. The nucleosides of 6-methoxypurine and 6-methylthiopurine, however, are more similar to adenosine than to inosine in that they have a double bond between the 1- and 6-positions and no proton on N-1. The fact that 8-aza-6-MPR is a good substrate whereas 6-MPR is not indicates that the introduction of the 8-N atom may cause a change in the bond structure at the 6-position such that the molecule resembles 6-MeMPR rather than 6-MPR. Introduction of an N atom in place of the 8-carbon atom of a 6-oxo-or 6-thiopurine atom is known to increase the ionization of the N-1 proton [17, 18]. As shown below, the ionized form of a 6-oxo- or 6-thiopurine nucleoside resembles adenosine, with respect to the bond structure at the 1,6-positions, more than it does inosine. If this reasoning is valid, the degree of ionization of the N-1 proton would influence activity of the nucleoside as a substrate. Although only a few substrates



Inosine (O) 6-MPR(S) | Adenosine | 6-MeMPR | 8-Aza-6-MPR (ionized)

were available for a test of this hypothesis, the results support the concept. The four 6-oxo- or 6-thio nucleosides that were studied range in pK_a values from 8.74 to 6.75. Under the assay conditions at pH 7.0, inosine is essentially un-ionized and had no substrate activity. 6-MPR is about 20 per cent ionized and had minimally detectable activity in the overnight assay. 8-Azainosine is slightly more ionized and had activity consistently detectable in the overnight assay. Of the four nucleosides, only 8-aza-6-MPR is more than 50 per cent ionized at pH 7.0; it was the only good substrate, with a V_{max} of the same order of magnitude as that for 6-MeMPR [1]. Further evidence of the influence of ionization on substrate activity is shown by the effect of increased pH of the reaction mixture on the phosphorylation of 8-aza-6-MPR (see Fig. 2). The increased rate of phosphorylation of 8-aza-6-MPR and 6-MPR at the higher pH values probably is due to effects on the substrate rather than on the enzyme, since the rates of phosphorylation of other substrates (adenosine and 6-MeMPR) were not similarly affected. Additional evidence on this point would be provided by a full kinetic study of the activities of the subject substrates as a function of pH.

From the results with these four substrates, it would appear that ionization may be required for a 6-oxo- or 6-thiopurine nucleoside to have good substrate activity, and it would also follow that any such nucleoside should be a substrate if the reactions were carried out at a pH in the neighborhood of, or above, the pK_a. Inosine (pK_a 8.74) itself should have detectable substrate activity at pH values above 8; it was not possible to make this determination because of the loss of activity of the enzyme at the higher pH values. The rate of phosphorylation of [^{14}C]-6-MPR was higher at pH values above 7, but was still too low for proper quantitation.

Ionization of the N-1 proton is not the sole feature critical for the activity of a 6-oxo- or 6-thiopurine nucleoside as a substrate for the kinase. For example, the degree of ionization of 8-aza-6-MPR at pH 6 (15 per cent) and that of 6-MPR at pH 7 (20 per cent) are not greatly different; yet the phosphorylation of 8-aza-6-MPR was readily detectable at pH 6 (see Fig. 2), whereas that of 6-MPR at pH 7 was detectable only in the overnight assay. Thus, the 8-aza substitution must have some effect other than on ionization that increases activity of a substrate for the kinase. Evidence for this is that 8-azaadenosine is phosphorylated more rapidly than adenosine [1]; ionization obviously is not responsible for this difference.

Nucleotides of purine analogs may be formed either by the action of a PRTase on the base or by the action of a kinase on the nucleoside, and, for analogs with biological activity, the relative rates of these two reactions would determine whether the base or its nucleoside is the more effective inhibitor. Kong and Parks [19], in a study of the relationship between pK_a and the activity of purine bases as substrates for H(G)PRTase, found that this enzyme preferred the un-ionized form. Thus, for 6-oxo- and 6-thiopurines, structural alterations that change the pK_a value substantially would have opposite effects on the activity of the base as a substrate for the PRTase and on the activity of the nucleoside as a substrate for adenosine kinase.

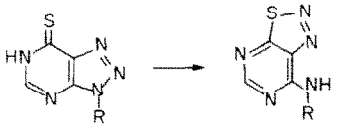
It should be emphasized that the conclusions drawn from the present study apply only to adenosine kinase from H.Ep. #2 cells. Kinases from other sources might have different substrate specificities. However, insofar as they have been examined, adeno-

sine kinases from several different mammalian sources have had similar substrate preferences [1, 20–23].

Kinase activity for inosine has been reported in crude preparations of several types of mammalian cells [10, 12–14]. The question is still unsettled as to whether these other cells have separate kinases for inosine and adenosine or a single kinase with broader substrate preference than the kinase from H.Ep. #2 cells. This problem is under study in our laboratories.

The results obtained in the present study, in addition to adding to knowledge of structural requirements for activity of nucleosides as substrates for adenosine kinase, are of potential interest in the design of new nucleoside analogs as growth inhibitors, since they indicate that any change in a 6-thio or 6-oxo nucleoside that increases the ionization of the N-1 proton should enhance its phosphorylation.

With regard to the observed kinetic constants for the phosphorylation of 8-aza-6-MPR, mention should be made of the presence in the reaction mixture of a small amount of a product resulting from rearrangement of the substrate. 8-Aza-6-thiopurines are known to rearrange to 1,2,3-thiadiazolo-(5,4-*d*)pyrimidines [17, 24, 25]. This rearrangement, observed originally with the bases, also occurs at the nucleoside level.* It proceeds slowly in solution at room temperature,



and within the 30–45 min period of these assays less than 1 per cent of the initial nucleoside undergoes rearrangement. However, the rearrangement product is also phosphorylated by enzyme(s) present in H.Ep. #2 cells. Hence, even though at the concentrations used in Figs. 1 and 2 as much as 50 per cent of the substrate is phosphorylated, the phosphorylation of the rearrangement product makes some small contribution to the calculated kinetic constants. The metabolism and metabolic effects of the rearrangement product are under study.*

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