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Inhibition of Human 5-Phosphoribosyl-1-pyrophosphate Synthetase by 4-Amino-8-(β-D-ribofuranosylamino)-pyrimido[5,4-*d*]pyrimidine-5′-monophosphate: Evidence for Interaction at the ADP Allosteric Site

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SUMMARY

The kinetics of inhibition by the aminopyrimidopyrimidine nucleotide 4-amino-8-(β-D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine-5′-monophosphate (APP-MP) were assessed with two human isozymes of 5-phosphoribosyl-1-pyrophosphate synthetase (PRS) (PRS1 and PRS2) and a mutant enzyme, S.M. PRS1, derived from an individual with PRS hyperactivity. In the presence of 1 mM potassium phosphate, APP-MP inhibited PRS1 and PRS2 with half-maximal inhibition (IC_{50}) at 5.2 μM and 23.8 μM, respectively. The degree of inhibition for both enzymes was highly dependent on the phosphate concentration; IC_{50} values were 70 times higher in the presence of 50 mM potassium phosphate. APP-MP exhibited mixed noncompetitive-uncompetitive inhibition against PRS1, with a K_{i_i} value of 6.1 μM and a K_{i_s} value of 14.6 μM, and produced parabolic secondary plots of slope or intercept versus APP-MP concentration. In comparison, inhibition of PRS1 by ADP was of a mixed noncompetitive-competitive type, with a K_{i_i} value of 9.6 μM and a K_{i_s} value of 2.8 μM. A similar kinetic analysis was completed using S.M. PRS1, a mutant enzyme with a single amino acid substitution resulting in diminished sensitivity to feedback inhibition by nucleotides. The noncompetitive component of ADP inhibition of PRS1 was absent with S.M. PRS1 and ADP inhibition was purely competitive, with a K_i of 6.4 μM. APP-MP was a very poor inhibitor of S.M. PRS1, displaying uncompetitive characteristics and a K_i of 1.6 mM. These data indicate that APP-MP inhibits PRS1 with a strong element of noncompetitive inhibition and appears to interact specifically at the allosteric site used by ADP. These results contrast with those obtained with ADP, which has a strong component of ATP competitive inhibition and binds at the ATP site as well as at a second, allosteric, site.

PRPP is required by cells for the *de novo* biosynthesis of purine, pyrimidine, and pyridine nucleotides, as well as for the salvage of preformed purine and pyrimidine nucleobases. The enzyme PRS (EC 2.7.6.1) catalyzes the synthesis of PRPP by transfer of a pyrophosphoryl group from ATP to the 1-hydroxyl group of ribose-5′-phosphate, in a reaction requiring P_i and Mg^{2+}. The enzyme plays a crucial role in the maintenance and regulation of nucleotide pools and related intermediate metabolism. PRS has been isolated and characterized from several sources, including *Salmonella typhimurium* (1), *Escherichia coli* (2), *Bacillus subtilis* (3), rat liver (4, 5), and human erythrocytes (6). More recently, mammalian PRS has been found to exist in at least three independently active isoforms with differing characteristics and tissue distributions (7).

There is some indication that PRS activity is elevated in neoplastic tissue. Heinrich *et al.* (8) and Balo-Banga and Weber (9) have reported increased activity of PRS in transplanted rat tumors, and Natsumeda *et al.* (10) showed that rat hepatoma 3924A cells possessed an elevated pool of PRPP, relative to normal rat liver. The data suggest that there may be an enlarged pool of PRPP and an increased capacity for utilization of this metabolite in cancer cells and that elevated PRS activity in tumors may be linked to transformation and progression. These observations, and the critical role that this enzyme plays in the maintenance of metabolites essential for proliferation, make PRS an attractive target in cancer chemotherapy (11).

Attempts to identify chemical structures that inhibit PRS have been successful mainly through the discovery of an aminopyrimidopyrimidine nucleoside, APP (NSC 283867). This compound was first synthesized by Berman *et al.* (12) and was reported by Srivastava *et al.* (13) to have immunosuppressive and antileukemic activity in mice. In cells, APP

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ABBREVIATIONS: PRPP, 5-phosphoribosyl-1-pyrophosphate; APP-MP, 4-amino-8-(β-D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine-5′-monophosphate; PRS, 5-phosphoribosyl-1-pyrophosphate synthetase; APP, 4-amino-8-(β-D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine.

TABLE 1

Specific activities of PRS1 and PRS2 at different phosphate concentrations

One unit of activity equals 1 μmol of product formed/min at 37°. Data are expressed as the mean ± standard error of three separate experiments. The enzyme assay procedure is described in Materials and Methods.

Phosphate concentration	Specific activity	
	Human PRS1	Human PRS2
mM	*units/mg of enzyme*	
1	11.2 ± 1.9	3.4 ± 0.2
5	19.5 ± 1.4	20.7 ± 0.7
20	25.1 ± 1.6	29.7 ± 1.2
50	29.6 ± 1.9	35.4 ± 1.0

is metabolized to APP-MP by adenosine kinase. The mononucleotide inhibits PRS, markedly reducing PRPP levels in cells treated with APP and causing severe depletion of purine and pyrimidine nucleotide pools (14–17). APP and the β-anomer of APP-MP have demonstrated antitumor activity against several *in vivo* mouse models, including L1210 leukemia, M16 mammary adenocarcinoma, and C26 colon carcinoma (14–17).

Previous results indicated that APP-MP was a noncompetitive inhibitor, with respect to ATP, of partially purified PRS from rat Novikoff hepatoma cells, with a K_i of 0.43 mM (14). In that study, an enzyme reaction buffer containing a high P_i concentration (50 mM) was used, and this may have affected the inhibition kinetics. Another study evaluated the 4-methoxy derivative against partially purified human erythrocyte PRS (16, 17), at a P_i concentration of 2 mM, and observed noncompetitive inhibition with respect to ATP, with an apparent K_i in the range of 190–260 μM. Competitive inhibition of PRS at the site utilized by P_i as a required cofactor (18–20) and an allosteric activator (21, 22) was proposed as the mechanism.

In this study, we have assessed the inhibitory properties of APP-MP against two human isozymes of PRS, PRS1 and PRS2. These enzymes were previously cloned, expressed in bacteria, and purified to homogeneity (20). We also present a detailed comparative kinetic analysis of the inhibition of PRS1 by APP-MP and ADP, the most potent natural feedback inhibitor of PRS. The results of these kinetic analyses with the normal human PRS1 and a mutant human PRS1 resistant to inhibition by purine nucleotides (23) support the view that APP-MP interacts specifically at the ADP allosteric site of PRS1.

Materials and Methods

Chemicals. APP-MP was synthesized as described previously (14). Ribose-5′-phosphate and all nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO). [^{32}P]ATP was obtained from Amersham Corp. (Arlington Heights, IL).

Enzymes. Pure recombinant human PRS1 and PRS2 were obtained from human cDNAs that were cloned into expression vectors and introduced into a mutant *Eschericha coli* strain that carries a deletion for the bacterial PRS. Complete details of the cloning, expression, and purification of these isozymes have been published previously (20). The cDNA for S.M. PRS1 was obtained by reverse transcription of total RNA, isolated from fibroblasts of a patient with PRS hyperactivity, and polymerase chain reaction amplification of PRS1 cDNA using appropriate human PRS1 primers (23). The cloning and expression of S.M. PRS1 have been described in a previous publication (23). These studies used recombinant S.M. PRS1 enriched to 8% of the total bacterial extract protein.

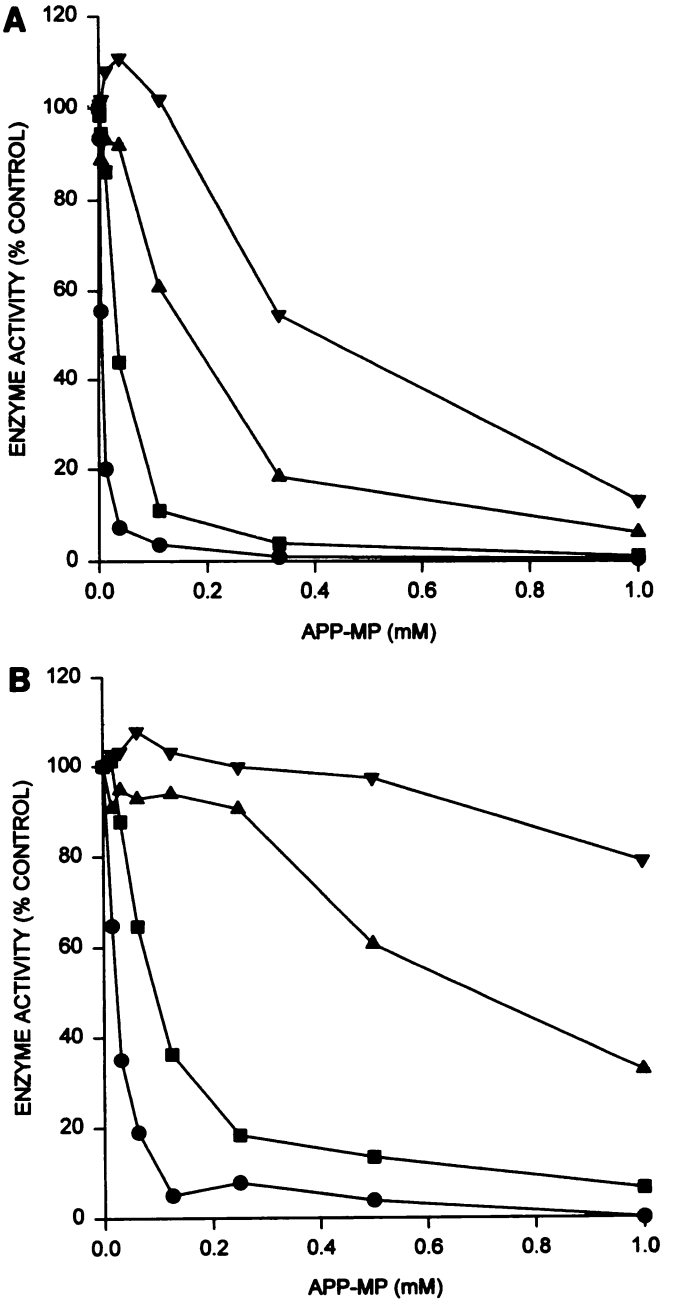

Fig. 1. Dose-response curves for the inhibition of PRS1 (A) or PRS2 (B) by APP-MP in the presence of various concentrations of potassium phosphate. Phosphate concentrations were 1 mM (●), 5 mM (■), 20 mM (▲), and 50 mM (▼).

Enzyme assays. PRS1 and PRS2 were assayed by a modification of the ^{32}P transfer method of Switzer and Gibson (24). The final volume of the reaction mixture was 250 μl, and the standard assay contained 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM ribose-5′-phosphate, 1 mM potassium phosphate, and 250 μM ATP. For certain experiments the potassium phosphate concentration was varied from 1 to 50 mM and for kinetic assays the ATP concentration was varied from 3 to 300 μM, as indicated in Results. PRS1 and PRS2 were diluted in 50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 6 mM $MgCl_2$, 1 mg/ml bovine serum albumin, with either 0.3 or 0.1 mM ATP, depending on the required final ATP concentration in the reaction. For enzyme kinetic studies corrections were made to ac-

TABLE 2

Inhibition of PRS1 and PRS2 by APP-MP at different phosphate concentrations

IC_{50} values represent the concentrations of APP-MP needed to inhibit enzyme activity by 50% and are the mean ± standard error of three experiments. The enzyme assay procedure is described in Materials and Methods.

Phosphate concentration	IC_{50}	
	Human PRS1	Human PRS2
mM	μM	
1	5.2 ± 0.3	23.8 ± 1.3
5	32.3 ± 1.1	87.0 ± 8.4
20	145 ± 9.3	653 ± 45.4
50	363 ± 16	1753 ± 109

count for the amount of ATP in the reaction mixture that was contributed by the enzyme solution. S.M. PRS1 was diluted in the same buffer with the addition of 0.1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. Reactions were performed for 10 min at 37°. The reaction rate was linear with the amount of enzyme and time under these conditions. Kinetic data were fitted by a nonlinear regression computer program (GraFit; Erithacus Software) to eq. 1, where I is the inhibitor concentration and v_0 is the initial reaction rate.

$$v_0 = \frac{V_{max}[ATP]}{K_m\left(1 + \frac{I}{K_{i_s}}\right) + [ATP]\left(1 + \frac{I}{K_{i_i}}\right)} \quad (1)$$

Results

Inhibition of PRS1 and PRS2 by APP-MP. The inhibitory potency of APP-MP was assessed against PRS1 and PRS2 in the presence of different P_i concentrations. Both isozymes require P_i for activity, and Table 1 shows the specific activity for both proteins at each of the P_i concentrations used in this analysis. Fig. 1 shows inhibition curves for PRS1 and PRS2 with varying concentrations of APP-MP at different concentrations of P_i. The degree of inhibition was exquisitely dependent on the P_i concentration. IC_{50} values calculated from the titration curves showed that APP-MP was approximately 70 times more potent with 1 mM potassium phosphate than 50 mM (Table 2). Furthermore, APP-MP was clearly more potent (nearly 5-fold) against PRS1 than PRS2 (Table 2), similarly to previous observations for ADP (20). Because 1 mM P_i approximates intracellular concentrations, all subsequent experiments were performed with this concentration. The relative inhibition of PRS1 by APP-MP, in comparison with ADP and GDP, the most effective feedback inhibitors of the two isoforms (20) under these conditions, showed that APP-MP (IC_{50} = 4.9 μM) was approximately 2-fold more potent than ADP (IC_{50} = 10.5 μM) and 6-fold more potent than GDP (IC_{50} = 29 μM).

Inhibition kinetics. To gain insight into the mechanism of inhibition of PRS by APP-MP, comparative kinetic analyses of the inhibition of PRS1 by APP-MP and ADP were carried out with ATP as the variable substrate. Fig. 2A shows a double-reciprocal plot of enzyme activity versus ATP concentration at various concentrations of ADP. In agreement with previous findings with *Salmonella typhimurium* PRS (1), ADP inhibition was not simple; fitting the data to eq. 1 indicated both noncompetitive and competitive inhibition. The K_{i_s} and K_{i_i} values were 2.8 μM and 9.6 μM, respectively (Table 3). Secondary plots of the slopes and intercepts from

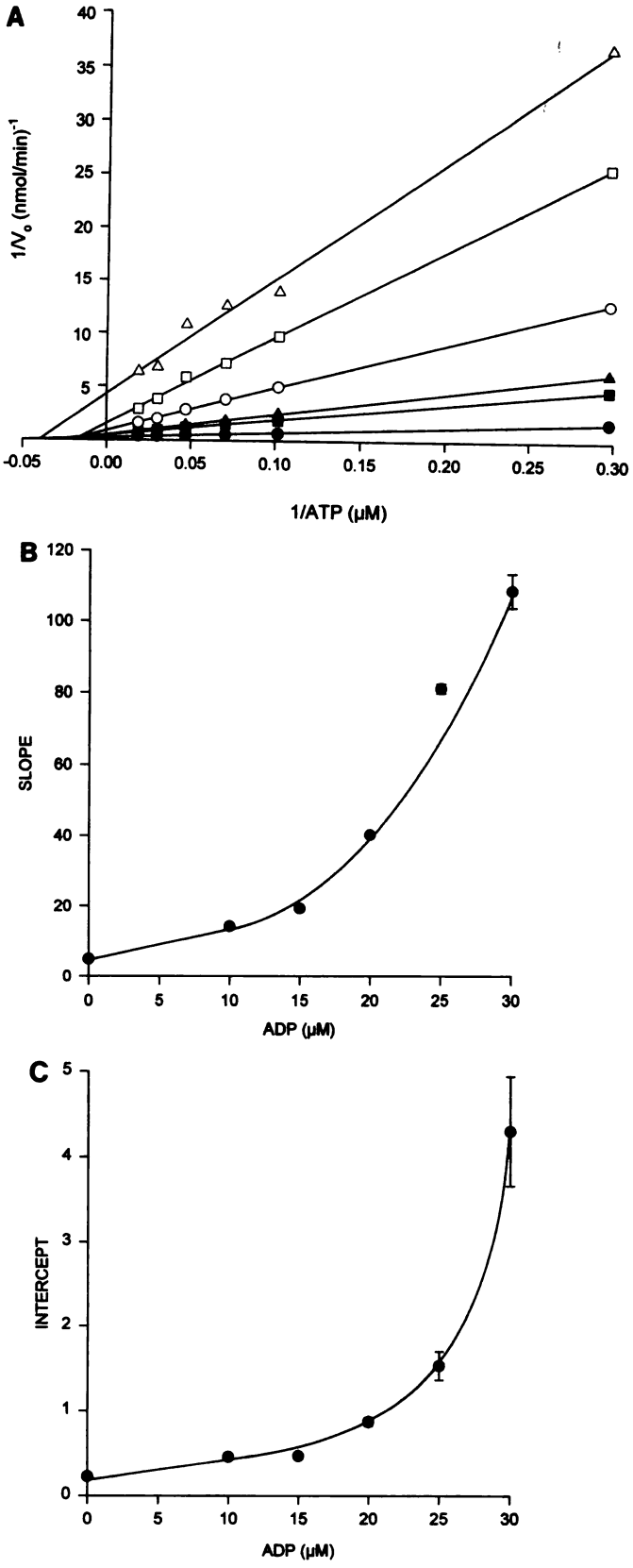

Fig. 2. A, Double-reciprocal plot for the inhibition of PRS1 by ADP at varying ATP concentrations. ADP concentrations were 0 (●), 10 μM (■), 15 μM (▲), 20 μM (○), 25 μM (□), and 30 μM (△). B, Secondary plot of slope versus ADP concentration. C, Secondary plot of intercept versus ADP concentration. Slopes, intercepts, and standard error bars were generated from the lines of plot A by linear regression.

TABLE 3

Kinetic parameters for the inhibition of wild-type PRS1 or mutant S.M. PRS1 by ADP or APP-MP

Details of the enzyme assays are described in Materials and Methods. Kinetic values are expressed as mean ± standard error of three separate experiments. All K_i values were obtained in the presence of 1 mM potassium phosphate, with ribose-5′-phosphate held constant at 500 μM and ATP as the variable substrate. The K_m values for ATP for wild-type PRS1 and S.M. PRS1 were 12.7 ± 1.7 and 11.6 ± 1.1 μM, respectively.

Enzyme	Inhibitor	Type of inhibition	K_{i_s}	K_{i_i}
			μM	μM
Wild-type PRS1	ADP	Mixed noncompetitive-competitive	2.8 ± 0.7	9.6 ± 2.3
Wild-type PRS1	APP-MP	Mixed noncompetitive-uncompetitive	14.6 ± 1.7	6.1 ± 0.2
S.M. PRS1	ADP	Simple competitive	6.4 ± 1.1	
S.M. PRS1	APP-MP	Simple uncompetitive		1605 ± 195

Fig. 2A versus the ADP concentration further emphasized the complexity of the inhibition, yielding parabolic functions (Fig. 2, B and C), again consistent with multiple modes of inhibition by ADP. Inhibition of human PRS1 by APP-MP showed both similarities and differences, compared with that noted with ADP. Fig. 3A shows a double-reciprocal plot of enzyme activity versus ATP concentration at various concentrations of APP-MP. Inhibition was once again not simple but differed from that shown by ADP in that the best fit to eq. 1 indicated both noncompetitive and uncompetitive inhibition, with K_{i_s} and K_{i_i} values of 14.6 μM and 6.1 μM, respectively (Table 3). A secondary plot of slope versus APP-MP concentration showed a parabolic function, similar to that produced by ADP (Fig. 3B). The secondary plot of intercept versus APP-MP concentration, however, produced what appeared to be a linear function, which illustrates a distinct difference between APP-MP and ADP (Fig. 3C).

Inhibition kinetics with mutant S.M. PRS1. PRS forms from certain individuals with enzyme superactivity are insensitive to feedback inhibition by purine nucleotides, including ADP (25, 26). PRS1 cDNAs from two of these individuals have been cloned and sequenced, and they were found to possess point mutations, i.e., an adenine to guanine transition at nucleotide 341, predicting an asparagine to serine change at amino acid residue 113, in N.B. PRS1 and a guanine to cytosine transversion at nucleotide 547, yielding an aspartic acid to histidine change at amino acid 182, in S.M. PRS1 (23). S.M. PRS1, which has reduced sensitivity to ADP through apparent alteration of the allosteric binding site, was used in this study to assess the role of this site in the inhibitory actions of APP-MP. The concentrations of ADP necessary to inhibit the wild-type and mutant enzymes by 50% were 10.5 and 120 μM, respectively. Similarly, the mutant enzyme was 130-fold less sensitive to APP-MP, with 50% inhibition at 688 μM, compared with 4.9 μM for wild-type PRS1. Fig. 4 shows a double-reciprocal plot of S.M. mutant PRS1 activity versus ATP concentration at various concentrations of ADP. The type of inhibition was simple competitive, with a K_i of 6.4 ± 1.1 μM (Table 3), a mechanism distinctly different from the mixed noncompetitive-competitive inhibition shown by wild-type PRS1 (Fig. 2A). A secondary plot of slope versus ADP concentration showed a linear function (data not shown), in contrast to the parabolic function obtained with wild-type enzyme (Fig. 2B). These data are consistent with the view that amino acid substitution in S.M. PRS1 inactivates the allosteric feedback inhibition site for nucleotides, thus eliminating the noncompetitive component of the inhibition by ADP. A similar analysis with APP-MP revealed a much different pattern of inhibition. First, S.M. PRS1 was remarkably insensitive to APP-MP; millimolar concentrations were required to produce significant inhibition. Second, the inhibition appeared to be more uncompetitive in nature, with a K_i of 1.6 mM (Fig. 5). These results indicate that, at physiologically relevant concentrations, virtually all of the inhibitory properties of APP-MP are mediated through the allosteric feedback inhibitory site.

Discussion

The current study provides evidence that APP-MP inhibits human PRS1 quite specifically at the allosteric site for ADP. This conclusion is based in part on similarities in the behavior of ADP and APP-MP in several aspects of PRS inhibition kinetics. First, APP-MP, like ADP (20), is severalfold more potent in inhibiting PRS1 than PRS2. Second, both APP-MP and ADP show very strong components of noncompetitive inhibition against PRS1. Third, and perhaps most significantly, the noncompetitive component of inhibition of PRS1 in response to both APP-MP and ADP is abolished with S.M. PRS1, a variant enzyme with diminished sensitivity to feedback inhibition by nucleotides (23). There are, however, several distinct differences between APP-MP and ADP with regard to the inhibitory effects of these compounds. Although a noncompetitive mechanism of inhibition is prominent with both, ADP inhibition displays a competitive component as well, consistent with previous findings implying that ADP binds at both the ATP site and an allosteric site (27). APP-MP inhibition of PRS1 shows no competitive component and, in fact, displays weak uncompetitive features. This difference was made clear when inhibition of S.M. PRS1 was studied. ADP was a pure competitive inhibitor of the mutant enzyme, with a K_i of 6.4 μM. In contrast, APP-MP was virtually without inhibitory potency for S.M. PRS1, showing uncompetitive effects only at very high concentrations (1–2 mM). The basis of the latter mode of inhibition is unclear. Because it is demonstrable only at very high concentrations of APP-MP, weak residual binding at the allosteric site or weak specific or nonspecific binding at alternative sites on the enzyme, with the formation of a dead-end complex between enzyme, ATP, and APP-MP, is a possibility. Nevertheless, at low concentrations APP-MP displays one dominant mechanism of inhibition of wild-type PRS1 and that is noncompetitive. When that mechanism is abolished, as with the mutant enzyme, APP-MP is essentially inactive at physiologically relevant inhibitor concentrations. In contrast, ADP retains a reasonably potent competitive mechanism of inhibition of S.M. PRS1, with respect to ATP, even in the absence of the noncompetitive mechanism. Despite retention of the competitive mechanism of ADP inhibition, S.M. PRS1 displays considerably reduced sensitivity to ADP inhibition,

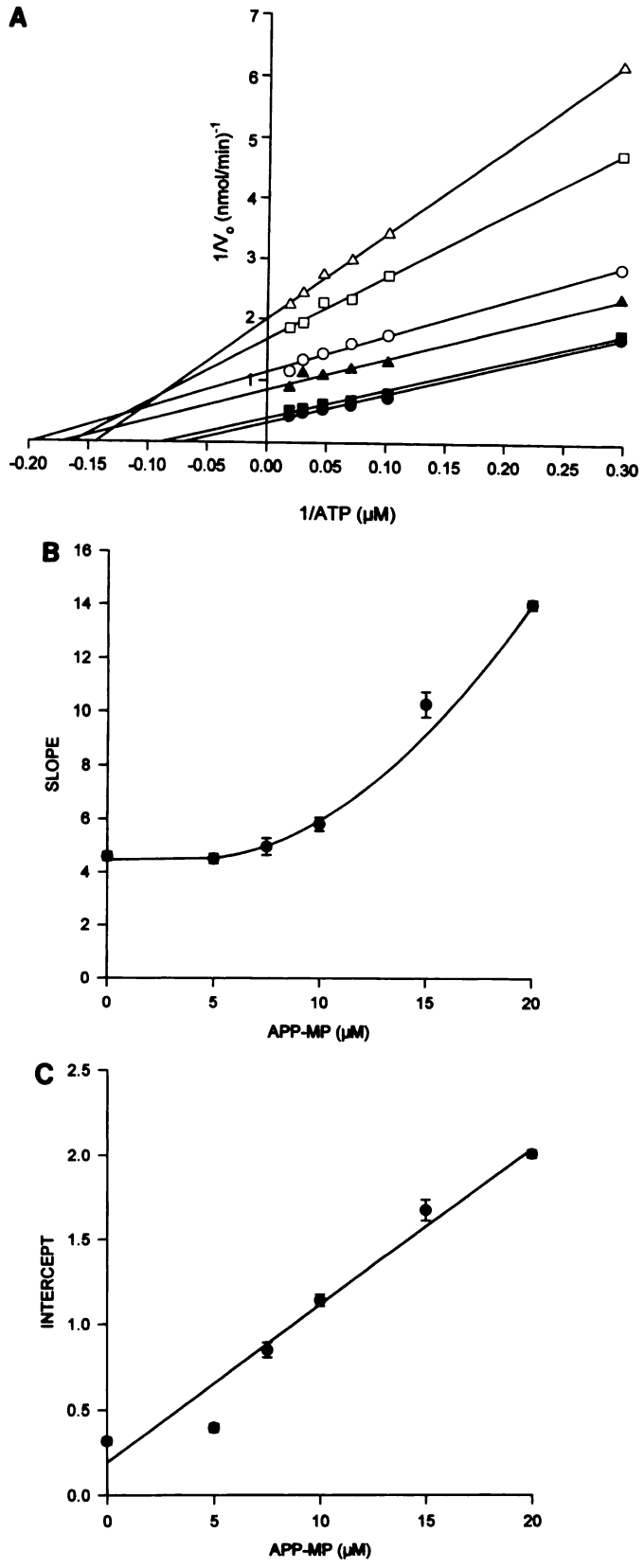

Fig. 3. A, Double-reciprocal plot for the inhibition of PRS1 by APP-MP at varying ATP concentrations. APP-MP concentrations were 0 (●), 5 μM (■), 7.5 μM (▲), 10 μM (○), 15 μM (□), and 20 μM (△). B, Secondary plot of slope versus ADP concentration. C, Secondary plot of intercept versus ADP concentration. Slopes, intercepts, and standard error bars were generated from the lines of plot A by linear regression.

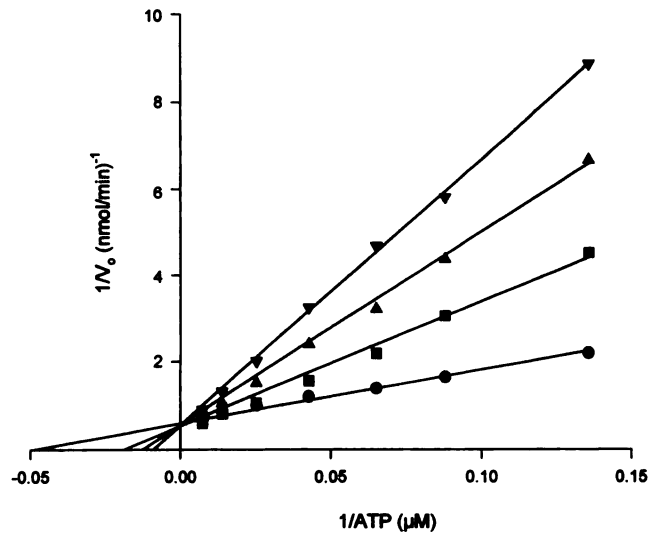

Fig. 4. Double-reciprocal plot for the inhibition of S.M. PRS1 by ADP at varying ATP concentrations. ADP concentrations were 0 (●), 10 μM (■), 35 μM (▲), and 50 μM (▼).

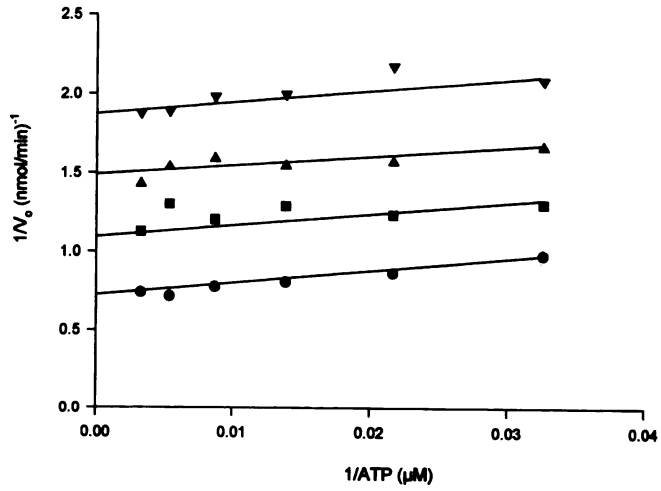

Fig. 5. Double-reciprocal plot for the inhibition of S.M. PRS1 by APP-MP at varying ATP concentrations. APP-MP concentrations were 0 (●), 0.5 mM (■), 2 mM (▲), and 3 mM (▼).

compared with wild-type PRS1. This likely reflects both the loss of the noncompetitive inhibition mechanism with the mutant enzyme and the relatively less prominent contribution of the competitive mechanism of ADP inhibition when enzyme activity is measured at nearly saturating concentrations of ATP.

The degree of inhibition of PRS by APP-MP is dependent on the concentration of P_i, a finding that has led to the proposal that APP-MP binds at the P_i site and that inhibition by this compound results from reduced activation by P_i (16, 17). The current study, however, provides some evidence against this possibility, because APP-MP was inactive against S.M. PRS1, which retains more modest but still substantial dependence on P_i for its activity (23). Although the P_i effector site on PRS1 is unknown and may be at least in part contiguous with the allosteric nucleotide inhibitor site, the virtual inactivity of APP-MP against S.M. PRS1 is clearly a consequence of the loss of the ADP allosteric inhibitory site,

providing strong evidence that the latter is the major site of interaction with APP-MP.

The basis for antitumor selectivity of APP-MP remains unclear at this time. A tissue distribution study of PRS1 and PRS2 mRNA in rats, mice, and humans, using Northern blot analysis, showed that PRS2 was present mainly in rapidly growing tissues such as thymus, lung, stomach, small intestine, spleen, and testis. PRS1 was prevalent in nonproliferating or relatively less proliferating tissues, such as brain and adrenal gland (7). Although these data might suggest a rationale for the selective inhibition of rapidly growing tumors by APP-MP, PRS2 was less sensitive to inhibition by this compound than was PRS1. Isozyme distribution, however, cannot be completely ruled out as a contributing factor to tumor selectivity, because a detailed study assessing the expression of PRS isozymes has not yet been completed in tumor tissues or even in normal tissues. It is of interest, however, that PRS1 and PRS2 transcripts were equally expressed in regenerating liver and Yoshida sarcoma cells (7). Among other factors that may govern the antitumor effects of APP-MP are intracellular P_i concentrations and greater utilization of nucleic acid precursors by tumors, with the consequent increased requirement for PRPP.

Acknowledgments

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