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# Characterization of a Feedback-Resistant Phosphoribosylpyrophosphate Synthetase from Cultured, Mutagenized Hepatoma Cells That Overproduce Purines

**(HTC cells/somatic cell mutants/methylmercaptopurine ribonucleoside/purine biosynthesis/hyperuricemia)**

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**ABSTRACT** **A clone of cells in which the regulation of purine metabolism is genetically altered was selected and isolated from chemically mutagenized HTC cells (a line of rat hepatoma cells in continuous culture). The clone, designated MAU V, was selected for increased ability to salvage exogenous purines by isolating it in medium containing methylmercaptopurine ribonucleoside, adenine, and uridine, in which medium wild-type cells cannot divide. We have characterized these cells as having an increased rate of *de novo* purine biosynthesis, apparently as the result of an altered phosphoribosylpyrophosphate (PRPP) synthetase. The altered enzyme has normal catalytic properties but an altered sensitivity to feedback inhibition by purine and pyrimidine nucleotides. The types of inhibitions (competitive and uncompetitive) exerted by AMP, ADP, and TDP on the wild-type enzyme have been maintained in the altered enzyme, but values for $K_i$ have been increased by factors of 10, 17.5, and 5, respectively. The specific catalytic activities of AMP: pyrophosphate phosphoribosyltransferase and IMP:pyrophosphate phosphoribosyltransferase are normal. The mutant cell may serve as a model for a specific human disease, one type of dominantly inherited overproduction hyperuricemia.**

Purine biosynthesis in mammalian cells is regulated at numerous sites along the pathway (1). The relative importance of each of these sites in maintenance of the physiologic state is unclear. Aberrant regulation can result in human disease, most notably overproduction hyperuricemia leading to gout (2). We have been examining the regulation of purine biosynthesis in a line of rat hepatoma cells in continuous culture (HTC cells) (3), which we consider to be a model for the mammalian hepatocyte. Because of recent advances in the techniques for studying the genetics of somatic mammalian cells (4–6), and because genetic approaches have been so profitably employed in studies of procaryotic regulatory mechanisms (7), we have attempted to isolate an HTC cell in which regulation of the purine pathway has been genetically altered.

From chemically mutagenized HTC cells we have selected a clone of cells for increased purine salvage capabilities. We have characterized these cells as having an increased rate of *de novo* purine biosynthesis, apparently as the result of an altered phosphoribosylpyrophosphate (PRPP) synthetase. The altered enzyme has normal catalytic properties but an altered sensitivity to feedback inhibition by nucleotides of purine and pyrimidine. Comparative studies of this mutant provide information about the normal regulation of the purine pathway, and the mutant cell may serve as a model for a specific human disease, one type of dominantly inherited overproduction hyperuricemia.

## MATERIALS AND METHODS

Generally labeled [$^3$H]adenine (10 Ci/mmol), [$^3$H]hypoxanthine (10 Ci/mmol), the sodium salt of [$^{14}$C]formate (50 mCi/mmol), [*carboxyl*-$^{14}$C]orotic acid (10 mCi/mmol), [$^3$H]uridine (25 Ci/mmol), [$^3$H]uracil (25 Ci/mmol), [$^{14}$C]glycine (100 mCi/mmol), and Omnifluor scintillant were purchased from New England Nuclear Corp. Lyophilized orotidine monophosphate (OMP) pyrophosphorylase contaminated with OMP decarboxylase was purchased from PL Biochemicals, Inc. DE-81 ion-exchange paper discs were purchased from H. Reeve Angel & Co., Inc. All other reagents and chemicals were purchased from commercial sources and were of the purest grade available.

Scintillation counting fluid consisted of 4 g of Omnifluor, 700 ml of toluene, and 250 ml of Triton X-100, except that the fluid used when counting Hyamine papers contained 300 ml of 95% ethanol instead of the Triton.

HTC cells were grown as suspension cultures or as monolayers in Swim's 77 medium (3) containing 2 mM L-glutamine and 10% (vol/vol) calf serum (growth medium). Cells in monolayer culture were mutagenized for 2 hr with 30 $\mu$M *N*-methyl-*N*-nitroso-*N'*-nitroguanidine, conditions under which approximately 50% of the population survive, and were allowed to recover for 3 days before being selected for their ability to grow in the simultaneous presence of methylmercaptopurine ribonucleoside (MMPR) (0.2 mM), adenine (0.2 mM), and uridine (0.5 mM). Cells of the two surviving colonies from eight T-flasks were dispersed into single-cell suspensions in separate spinner flasks; an aliquot was then cloned in "cloning medium" (Swim's 77 medium containing 10% calf serum and 10% fetal-calf serum, supplemented with 2 mM glutamine and 1 mM pyruvate) that contained 0.3 g of agar/100 ml and the selective agents MMPR, adenine, and uridine at the above concentrations. Clones were picked from the semi-solid medium, grown as monolayers in nonselective cloning medium, placed into suspension culture, and routinely maintained there under nonselective conditions.

The rate of intracellular purine biosynthesis *de novo* was

Abbreviations: APRTase, AMP:pyrophosphate phosphoribosyltransferase (EC 2.4.2.7); HGPRTase, IMP:pyrophosphate phosphoribosyltransferase (EC 2.4.2.8); HTC cell, hepatoma tissue culture cell; MAU medium, growth medium containing MMPR, adenine, and uridine; MMPR, 6-methylmercaptopurine ribonucleoside; OMP decarboxylase, orotidine-5'-phosphate carboxy-lyase (EC 4.1.1.23); OMP pyrophosphorylase, orotidine-5'-phosphate:pyrophosphate phosphoribosyltransferase (EC 2.4.2.10); PRPP, phosphoribosylpyrophosphate; PRPP synthetase, ATP:D-ribose-5-phosphate pyrophosphotransferase (EC 2.7.6.1); R-5-P, D-ribose-5-phosphate.

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determined as previously described (8). The rate of purine release into the medium was determined by monitoring the incorporation of [$^{14}$C]formate into silver-precipitable purine (8). Approximately $5 \times 10^5$ cells growing as a monolayer in a 75-cm$^2$ Falcon culture flask were incubated for 6 hr with 20 ml of fresh growth medium containing 10 $\mu$Ci of [$^{14}$C]formate. At the end of the incubation 1 ml of the medium was assayed for radioactive purine (8). The cells were harvested, dissolved in 0.1 N NaOH, and assayed for protein by the method of Lowry *et al.* (9).

The relative rates of synthesis of PRPP were assayed by determining the rate of $^{14}CO_2$ evolution by intact cells incubated with [*carboxy*-$^{14}$C]orotic acid. $10^7$ Cells were incubated with gentle shaking at 37° in 5 ml of medium containing 2.5 $\mu$Ci of [$^{14}$C]orotic acid. The released $^{14}CO_2$ was absorbed by 0.5 ml of hydroxide of Hyamine 10-X on a 3 × 6-cm strip of filter paper.

All enzyme assays were performed on cell-free extracts prepared at 4° in the following manner. Cells grown in suspension culture were harvested by centrifugation at 200 × *g* and washed once with phosphate-buffered saline (pH 7.0) (10). The cell pellet was then suspended in the appropriate buffer at a cell density of $3.3 \times 10^7$ cells per ml. For assaying adenosine 5′-monophosphate pyrophosphate phosphoribosyltransferase (APRTase) and inosine 5′-monophosphate pyrophosphate phosphoribosyltransferase (HGPRTase), the buffer used was 50 mM Tris·HCl (pH 7.6), whereas, for PRPP synthetase we used 50 mM potassium phosphate (pH 7.4), 6 mM $MgCl_2$, and 0.1 mM dithiothreitol. The cells were disrupted by sonication for 2–5 sec using a Bronwill Biosonik III set at 30% output. The cell-free suspension was centrifuged at 20,000 × *g* for 20 min, and the supernatant was filtered through a column (10 × 1 cm) of fine Sephadex G-25 to remove all molecules of molecular weight less than 5000.

APRTase was assayed by measuring the production of radioactively labeled AMP from [$^3$H]adenine. The assay mix contained in 100 $\mu$l: 2.5 $\mu$mol of Tris·HCl (pH 7.6), 0.5 $\mu$mol of $MgCl_2$, 0.1 $\mu$mol of PRPP, 0.01 $\mu$mol of adenine (0.5 $\mu$Ci), and 50 $\mu$l of cell extract containing 10–50 $\mu$g of protein. The reaction was started by the addition of the extract to the assay mix at 37°, and stopped by placing 50 $\mu$l of the reaction solution onto a 25-mm DE-81 disc. All the discs from a single experiment were immersed together in 2 liters of distilled water and washed five times with 2 liters of water per wash. Each disc was placed in a scintillation vial with 0.4 ml of 1 N HCl and 16 ml of scintillation counting fluid. The assay was linear for 5 min, proportional to the mass of protein added, and showed maximal activity at pH 7.6.

HGPRTase was assayed by measuring the production of radioactively labeled IMP from [$^3$H]hypoxanthine. Again, the labeled product was separated from the substrate by absorption to DE-81 discs. The assay mix contained in 100 $\mu$l: 2.5 $\mu$mol of Tris·HCl (pH 7.6), 0.2 $\mu$mol of $MgCl_2$, 0.1 $\mu$mol of PRPP, 0.01 $\mu$mol of hypoxanthine (0.25 $\mu$Ci), and 50 $\mu$l of cell extract. The method was otherwise the same as that for APRTase.

PRPP synthetase was assayed by a modification of Method I of Kornberg, Lieberman and Simms (11). The reaction was coupled to OMP pyrophosphorylase and OMP decarboxylase, and the evolution of $^{14}CO_2$ from [*carboxyl*-$^{14}$C]orotic acid was measured. The reaction mix contained in 500 $\mu$l: 25 $\mu$mol of potassium phosphate (pH 7.4), 1.25 $\mu$mol of $MgCl_2$, 1.25 $\mu$mol of ATP, 1.25 $\mu$mol of D-ribose-5-phosphate (R-5-P), 0.06 $\mu$mol of [*carboxyl*-$^{14}$C]orotic acid, the appropriate amount of the indicated feedback inhibitor, 1 mg of OMP pyrophosphorylase–OMP decarboxylase, and 50 $\mu$l of cell extract. The reaction was started by adding the extract to the reaction mix, which had been warmed to 37°. The evolved $^{14}CO_2$ was absorbed by a filter paper strip in 0.5 ml of hydroxide of Hyamine:ethanol (1:1) contained in a 6 × 50-mm glass tube inserted into the 10 × 75-mm reaction tube, which was then corked. The 6 × 50-mm tube was removed at the end of the assay (15 min) and the paper strip was placed in a scintillation vial with 18 ml of scintillation fluid and counted. The reaction was dependent on R-5-P, ATP, and Mg, and was stimulated by inorganic phosphate. It was linear up to 15 min and proportional to protein added up to 400 $\mu$g of protein. When standard PRPP solutions were substituted for R-5-P in the reaction mix, $^{14}CO_2$ evolution was linear up to 25 nmol of PRPP and the reaction went to completion within 2 min. One nanomole of PRPP gave rise to 0.20 nmol of $^{14}CO_2$ detected on the paper strip.

Polyacrylamide disc-electrophoresis of radioactively labeled RNA extracted from wild-type and MAU V cells revealed no 16S ribosomal RNA, indicating that both types of cells were free of contamination by mycoplasma (12).

Mean chromosome numbers were determined by counting 50 mitotic figures, stained by the Giemsa banding technique (13) after isolation from cells cultured overnight in the presence of 0.1 $\mu$M desacetyl-methylcholchicine (Colcemid, Ciba).

## RESULTS

*Selection for a Mutant with Increased Purine Salvage Capability.* The rationale for the design of our selective medium was derived from three earlier observations: (*a*) HTC cells divide very slowly when 0.5 mM adenine is present in the culture medium (8); (*b*) the presence of MMPR in the medium inhibits *de novo* synthesis of purine ribonucleotides and kills HTC cells; and (*c*) MMPR or adenine in the medium impose on HTC cells a requirement for an exogenous pyrimidine ribonucleoside (Martin and Owen, unpublished observations).

A cell grown in the presence of MMPR would, therefore, be dependent upon exogenous purines and pyrimidine ribonucleosides for synthesis of nucleic acids. However, wild-type HTC cells apparently cannot salvage adenine sufficiently well to divide at a normal rate (8). Thus, a mutant HTC cell with an increased capability to salvage adenine would possess a definite survival advantage in the presence of MMPR, adenine, and uridine (MAU medium).

We selected from chemically mutagenized HTC cells several clones that were capable of dividing rapidly in MAU medium and forming colonies in 0.3% agar under the same selective conditions.

The data below are derived from studies of one of these independently derived clones, designated MAU V. The mean number of chromosomes from 50 MAU V cells is 57, whereas that of the wild-type cell is 58 per cell (3). The population of MAU V cells have been grown for as long as 3 months in nonselective medium without loss of ability to grow rapidly in MAU medium. HTC cells which had not been chemically mutagenized did not give rise ($<10^{-8}$) to any clones capable of growing in MAU medium.

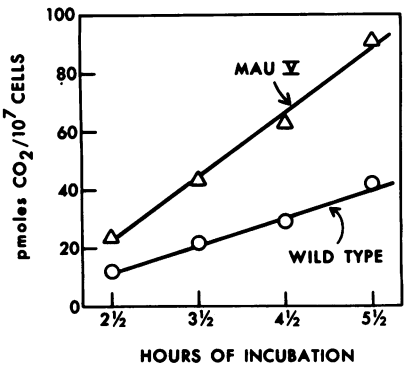


FIG. 1. Relative rates of PRPP synthesis *in vitro*. The evolution of $^{14}CO_2$ by cells incubated in the presence of [*carboxyl*-$^{14}$C]orotic acid was measured as described in *Materials and Methods*.

*De Novo Purine Biosynthesis and Purine Excretion in MAU V Cells.* An explanation for the ability of MAU V cells to grow in MAU medium could be that the cells were resistant to the inhibition of *de novo* purine biosynthesis by MMPR and/or adenine. We examined the rates of purine biosynthesis and found (Table 1) that with 0.2 mM adenine or MMPR in the medium, the *de novo* pathway in MAU V cells was inhibited to the same extent as in wild-type cells. In addition, MAU V cells synthesize purines *de novo* at nearly twice the rate of wild-type cells in the absence of exogenous adenine and MMPR. Since under nonselective conditions the MAU V cells do not appear to divide more rapidly than wild-type cells, we examined whether the purine-overproducing cells released more purines into their culture medium. We found that MAU V cells release purines labeled with [$^{14}$C]formate (see *Materials and Methods*) at a rate (9815 cpm/hr per mg of protein) nearly three times greater than wild-type cells (3690 cpm/hr per mg of protein).

*Formation of Intracellular PRPP in MAU V cells.* The observation that *de novo* purine biosynthesis is enhanced in MAU V cells and the fact that MAU V cells were selected for an increased purine salvage capability suggested that their intracellular concentrations of PRPP might be increased; our results suggest that the rate of formation of measurable PRPP in MAU V cells is approximately twice that in wild-type cells (Fig. 1). One possible explanation for this observation might be, by analogy to the Lesch–Nyhan syndrome (14), loss of a salvage enzyme, particularly HGPRTase, since MAU V cells are capable of salvaging adenine from the medium and, therefore, presumably possess APRTase. The specific catalytic activities of the HGPRTases in crude extracts from MAU V and wild-type cells are the same.

Another explanation might be that the enzyme responsible for the salvage of adenine possesses altered substrate affinities that could account for the altered salvage capability and PRPP level. The catalytic parameters of the APRTases in crude extracts from MAU V and wild-type cells show no significant differences.

*Catalytic Properties of PRPP Synthetase from MAU V Cells.* In view of the above results we compared, in gel-filtered crude extracts, the PRPP synthetases from MAU V and wild-type cells. The specific catalytic activities (46 ± 2 nmol/hr per mg of protein) and the values for $K_m$ for R-5-P (200 μM), ATP (45 μM) and $P_i$ (8 mM) are essentially the same for the enzymes from MAU V and wild-type cells.

*Regulatory Properties of PRPP Synthetase from MAU V Cells.* PRPP synthetase has been reported to be sensitive to feedback inhibition both in bacteria (15, 16) and in mammalian cells (17–20). We compared the sensitivity to feedback inhibition of the enzyme from MAU V cells with the sensitivity of that from wild-type cells. We found that PRPP synthetase activity in gel-filtered, crude extracts from MAU V cells was significantly less inhibited by low concentrations of AMP, ADP, or TDP than was the enzyme in extracts of wild-type cells. The difference in response to ADP of the enzymes in the mutant and wild-type cells (Fig. 2) is similar to their different responses to the other two inhibitors we examined.

In view of the resistance to inhibition of PRPP synthetase from MAU V cells, we examined whether the type of inhibition remained the same as that for the enzyme in wild-type cells. Fig. 3 is double reciprocal plots of initial velocity versus substrate concentration at several concentrations of inhibitors (21). In all cases the type of inhibition appears to be the same for the PRPP synthetases from the two types of cells. Inhibition by AMP and TDP is competitive with respect to ATP, whereas in all other examined cases the type of inhibition appears to be uncompetitive. The altered sensitivity to inhibition of PRPP synthetase from MAU V cells can be seen most readily by comparing the positions of the lines representing its enzyme activity in the presence of 0.01 or 0.1 mM of inhibitor with those representing the enzyme activities from wild-type cells under identical conditions.

From the above data, values for the inhibitor constants ($K_i$) for the enzymes from the two cell lines were determined (21) (Table 2). The sensitivity of the enzyme from MAU V cells to inhibition by AMP, ADP, and TDP is decreased 10-fold, 17.5-fold, and 5-fold, respectively.

Since these experiments were performed using crude extracts, it was possible that the difference between MAU V cells and wild-type cells lay not in an altered PRPP synthetase but in an increased ability on the part of extracts from MAU V cells to inactivate the inhibitor molecules or in the presence of some inhibitor antagonist that might act directly on the enzyme. If either were the case, one would expect that, upon mixing an equal quantity of enzyme activity

TABLE 1. *Rate of de novo purine biosynthesis in MAU V and wild-type cells*

| Cell strain | Rate of purine synthesis (nmol purine per hr per $A_{260}$) | | |
|---|---|---|---|
| | Growth medium | Growth medium + 0.2 mM adenine | Growth medium + 0.2 mM MMPR |
| MAU V | 16.4 ± 0.4 | 2.8 | 3.0 |
| Wild-type | 9.9 ± 0.3 | 3.1 | 1.9 |

$5 \times 10^6$ Cells were incubated at 37° for 30 min in 10 ml of fresh growth medium containing 5 μCi of [$^{14}$C]glycine and the additions indicated above. At the end of the labeling period, the cells were harvested and the amount of [$^{14}$C]purine in the cells was determined (8).

from each cell type in the presence of 0.01 or 0.1 mM ADP (where the extents of inhibition of the two enzymes are different), the resultant enzyme activities would not be directly, additive. We found that the resultant enzyme activities and, therefore, the extents of inhibition, were directly additive, making the above explanations unlikely.

## DISCUSSION

It is important to know whether the altered regulatory characteristics of PRPP synthetase in the MAU V cell are the result of a mutational event and, if they are, whether the mutation occurred in the structural gene(s) for PRPP synthetase. The phenotype of MAU V cells is stable under nonselective conditions, and similar cells have not appeared in populations of wild-type cells which were not chemically mutagenized. In addition, the stable phenotypic difference between MAU V and wild-type cells can be explained by the identified alteration in a single enzyme. Thus, it is likely that a mutational event did occur and that the mutation is located in a structural gene for PRPP synthetase. It is unlikely that the altered phenotype is the result of severe changes of chromosomal constitution, since no gross alterations were detected.

We presume that MAU V cells contain a mutation which has altered the regulation of PRPP synthetase by nucleotide inhibitors. HTC cells are polyploid, and it is probable that the MAU V cells are heterozygous for the gene(s) for PRPP synthetase. One would predict that a regulatory mutation of this type would be dominantly expressed in a heterozygote. However, it is not evident whether MAU V cells contain two populations of PRPP synthetase molecules, one wild-type and one mutant form; or whether there is one form of hybrid molecule containing both mutant and wild-type subunits (22).

Some useful information which can be derived from our present studies concerns the mechanism by which AMP inhibits activity of the enzyme. Inhibition by AMP, an immediate product of the catalysis, is competitive with ATP, a substrate for the reaction; thus, one might suppose that AMP inhibits at the catalytic site. However, the $K_i$ for AMP has been altered 10-fold in the mutant PRPP synthetase while the $K_m$ for ATP, as well as for R-5-P, has remained unchanged indicating that the mutation has not affected the catalytic site. Thus, it appears that AMP inhibits at a site other than the catalytic site, i.e., an allosteric site.

The overproduction of purines in cells containing a feedback-resistant PRPP synthetase demonstrates the importance

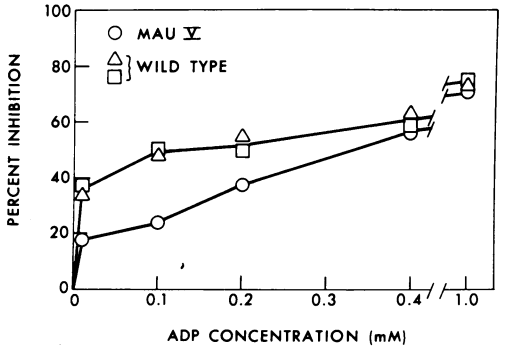


FIG. 2. Inhibition by ADP of PRPP synthetase from MAU V and wild-type cells. Cell-free extracts were made from one suspension culture of MAU V cells (○) and two of wild-type cells (△ and □). They were assayed for enzyme activity in the presence of ADP as described in *Materials and Methods*.

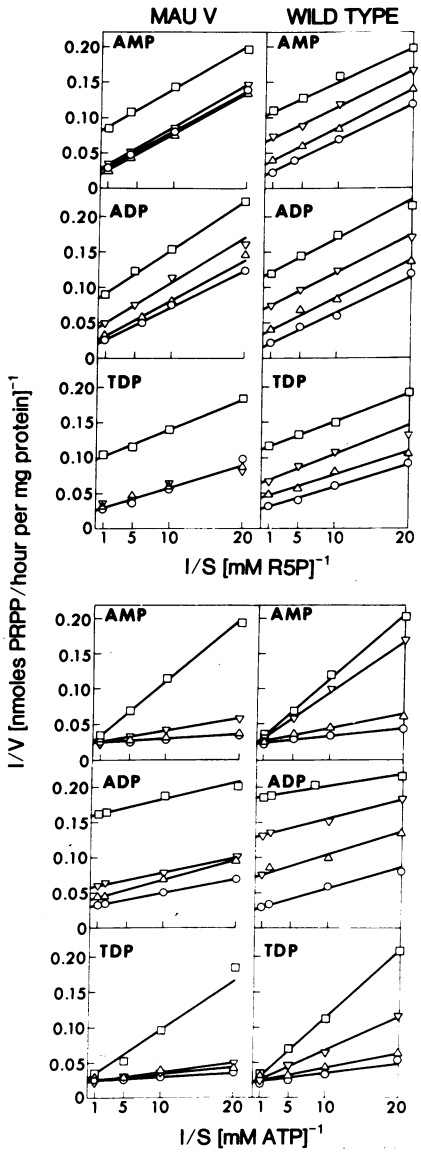


FIG. 3. Comparison of types of inhibition by nucleotide inhibitors of PRPP synthetase from MAU V and wild-type cells. Enzyme activities were assayed in crude extracts as described in *Materials and Methods*. Shown are double reciprocal plots of initial velocity vs. R-5-P concentration (*top*) and ATP concentration (*bottom*). ○, no inhibitor; △, 0.01 mM; ▽, 0.1 mM; and □, 1 mM inhibitor. The particular inhibitor is indicated in the *upper left* corner of each plot.

of feedback inhibition of this enzyme in the normal regulation of the *de novo* purine pathway. In MAU V cells the enzyme continues to produce PRPP in the presence of concentrations of nucleotides at which it would normally be inhibited. The intracellular concentration of PRPP has been implicated as an important regulator of *de novo* purine synthesis (23–26), and our observations support this. PRPP apparently affects the purine pathway by influencing the equilibrium between active and inactive forms of PRPP glutamylamidotransferase (27), the first enzyme unique to the purine pathway (1).

In lower organisms, mutations have been described that affect the feedback sensitivities of enzymes. These mutations range from complete loss of sensitivity (28, 29) to enhanced sensitivity (30, 31) to feedback inhibition, and have been

TABLE 2. *Inhibition constants for PRPP synthetase from MAU V and wild-type cells*

| Cell strain | $K_i$ (μM) | | |
|---|---|---|---|
| | AMP | ADP | TDP |
| MAU V | 100 | 175 | 750 |
| Wild-type | 10 | 10 | 150 |

Each $K_i$ was determined from a plot of the reciprocal of the initial velocity versus the inhibitor concentration (21).

associated with normal (30, 32, 33) or altered catalytic functions (32). PRPP synthetase from MAU V cells is apparently the only reported feedback-resistant mammalian enzyme (33, 34).

The overproduction of purines in man is associated with disease states (2, 35–37). The biochemically delineated causes of overproduction hyperuricemia in man are all associated with altered PRPP metabolism (35, 36, 38, 39). Two of these reported biochemical diseases directly involve the enzyme PRPP synthetase (34, 37). In both instances the catalytic activities of the enzyme are increased in the absence of inhibitors. Although the maximal specific catalytic activity of PRPP synthetase from MAU V cells is not increased, it has abnormally high activity under physiologic conditions in the cell. Thus, if one were attempting to identify a mutant PRPP synthetase, examining the maximal specific catalytic activity of the enzyme *in vitro* would not detect the altered enzyme, nor would the defect be discovered by examining the activity of PRPP synthetase in the presence of maximally effective concentrations of inhibitors. To recognize the aberrancy, one would at least have to determine the $K_i$ of the enzyme for the nucleotide inhibitor.

We suggest that our approach, that of constructing specific mutants of somatic mammalian cells in culture, can serve as a source of analogs of human biochemical diseases. These analogs might then provide experimental models for study of the molecular mechanisms underlying human pathologic conditions and provide suitable systems in which to explore new approaches to diagnosis and therapy.

## NOTE ADDED IN PROOF

Sperling *et al.* (40) have recently described a PRPP synthetase with decreased sensitivity to feedback inhibition from the erythrocytes of one of two male siblings with overproduction hyperuricemia and gout.

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