

Variant Human Phosphoribosylpyrophosphate Synthetase Altered in Regulatory and Catalytic Functions

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ABSTRACT An inherited, structurally abnormal and superactive form of the enzyme 5-phosphoribosyl 1-pyrophosphate (PP-ribose-P) synthetase (EC 2.7.6.1) has been characterized in fibroblasts cultured from a 14-yr-old male (S.M.) with clinical manifestations of uric acid overproduction present since infancy. PP-ribose-P synthetase from the cells of this child showed four- to fivefold greater than normal resistance to purine nucleotide (ADP and GDP) feedback inhibition of enzyme activity and hyperbolic rather than sigmoidal inorganic phosphate (Pi) activation in incompletely dialyzed extracts. Excessive maximal velocity of the enzyme reaction catalyzed by the mutant enzyme was indicated by: enzyme activities twice those of normal at all concentrations of Pi in chromatographed fibroblast extracts; normal affinity constants for substrates and for the activator, Mg²⁺; and twofold greater than normal activity per immunoreactive enzyme molecule. The mutant enzyme thus possessed deficient regulatory and superactive catalytic properties, two mechanisms previously demonstrated individually to underlie the excessive PP-ribose-P and uric acid synthesis of affected members of families with superactive PP-ribose-P synthetases. Increased PP-ribose-P concentration (4-fold) and generation (2.7-fold) and enhanced rates of PP-ribose-P dependent purine synthetic reactions, including purine synthesis *de novo*, in S.M. fibroblasts confirmed the functional significance of this patient's mutant enzyme.

Diminished stability of the variant PP-ribose-P synthetase was manifested *in vitro* by increased thermal lability and *in vivo* by deficiency of enzyme activity at Pi concentrations >0.3 mM in hemolysates

and by an accelerated, age-related decrement in enzyme activity in lysates of erythrocytes separated by specific density. Despite the diminished amount of PP-ribose-P synthetase in the S.M. erythrocyte population, S.M. erythrocytes had increased PP-ribose-P concentration and increased rates of incorporation of [¹⁴C]adenine and hypoxanthine into acid-soluble nucleotides during incubation at 1 mM Pi. These findings provided further confirmation of the extent to which PP-ribose-P synthesis is modulated in the normal cell at physiological Pi concentration by purine nucleotide inhibition of PP-ribose-P synthetase.

The activity and kinetic characteristics of PP-ribose-P synthetase from fibroblasts of the mother of patient S.M. indicated that this woman was a heterozygous carrier of the enzyme defect expressed in hemizygous manner by her son.

INTRODUCTION

The high-energy sugar phosphate 5-phosphoribosyl 1-pyrophosphate (PP-ribose-P)¹ is an intermediate in the synthesis of purine, pyrimidine and pyridine nucleotides. The synthesis of PP-ribose-P from ATP and ribose-5-phosphate (Rib-5-P) is catalyzed by the enzyme PP-ribose-P synthetase (E.C. 2.7.6.1) in a reaction requiring Mg²⁺ and inorganic phosphate (Pi). Studies of PP-ribose-P production by intact cells (1, 2) and analyses of the kinetic characteristics of purified microbial (3-5) and mammalian (6-10) PP-ribose-P synthetases indicate that a substantial number of effector compounds including substrates, inhibitors, activators, and products influence enzyme activity.

¹Abbreviations used in this paper: FGAR, α -N-formylglycinamide ribotide; KRPG, Krebs-Ringer phosphate buffer (pH 7.4) with 5.5 mM glucose; Pi, inorganic phosphate; PP-ribose-P, 5-phosphoribosyl 1-pyrophosphate; Rib-5-P, ribose-5-phosphate.

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Correlation of the actions of effector compounds on the quaternary structure and activity of the enzyme (11–13) have led to a proposed molecular model (13) for the control of erythrocyte PP-ribose-P synthetase activity and thus for the regulation of PP-ribose-P synthesis in these cells.

Study of inherited abnormalities of human PP-ribose-P synthetase, in which structurally variant forms of the enzyme with excessive activity lead to increased PP-ribose-P concentration and generation in conjunction with purine nucleotide overproduction and clinical gout (10, 14–20), have provided one line of evidence supporting a role for PP-ribose-P in the regulation of the rate of purine synthesis *de novo* (21–22). Among these abnormal forms of the enzyme, superactive PP-ribose-P synthetases with either diminished sensitivity to feedback inhibition (16, 17) or excessive reaction velocity per molecule of enzyme (10) have been described and extensively characterized. To date, the clinical manifestations of all the males bearing superactive forms of this enzyme, the structural gene for which has been mapped on the X-chromosome (23–25), have been restricted to gouty arthritis, uric acid urolithiasis, and/or renal insufficiency first manifested in early adulthood (15, 18, 20).

In this study, we report the characterization of a superactive form of PP-ribose-P synthetase identified in fibroblasts cultured from a child (S.M.) whose clinical findings and uric acid overproduction, apparent since the first year of life, have been described (26). Our studies provide evidence for both feedback resistance and excessive maximal reaction velocity in the variant enzyme and indicate that the activity of the aberrant form of PP-ribose-P synthetase is abnormally labile in erythrocytes. In addition, correlation of the *in vitro* kinetic characteristics of the mutant enzyme with the capacity of intact cells from the affected individual to generate PP-ribose-P provides information about the intracellular regulation of PP-ribose-P synthesis.

METHODS

Materials. [8-¹⁴C]Hypoxanthine (52 mCi/mmol), [8-¹⁴C]guanine (46 mCi/mmol), [8-¹⁴C]adenosine (55 mCi/mmol), and sodium [¹⁴C]formate (59 mCi/nmol) were purchased from Amersham Corp. (Arlington Heights, Ill.). [8-¹⁴C]-Adenine (44 mCi/nmol) was a product of New England Nuclear (Boston, Mass.). All unlabeled purine compounds were obtained from Sigma Chemical Co. (St. Louis, Mo.). The dimagnesium salt of PP-ribose-P was purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.), and Rib-5-P, azaserine, and glucose-6-phosphate were obtained from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.). Dibutyl phthalate and dimethyl phthalate were products of Matheson, Coleman & Bell (East Rutherford, N. J.), and Sephadex chromatographic gels were purchased from Pharmacia Fine Chemicals (Piscataway, N. J.). Eagle's minimal essential medium and fetal calf serum were supplied by Grand Island Biologicals (Grand Island, N. Y.).

Tissue culture methods. Fibroblast cultures were initiated from skin biopsy specimens and were propagated as described (27). Strains were derived from: four normal male individuals; patient S.M.; Su.M., the mother of S.M.; and H.B., an adult male with gout whose purine nucleotide and uric acid overproduction is associated with excessive fibroblast and erythrocyte PP-ribose-P synthetase activity (18). Fibroblasts were grown in monolayer to confluence (corresponding to greater than $\sim 1.5 \times 10^6$ cells/75 cm² culture flask) under conditions described (20). For each fibroblast strain, biochemical determinations were made in duplicate on at least three separate occasions between the 6th and 14th passages in culture. Because no evidence was obtained for an influence of passage number on any of the values shown by an individual strain, the results of determinations have been combined for analysis. Frequent biochemical and microbiological assays (performed by Irvine Scientific, Santa Ana, Calif.) failed to demonstrate evidence for mycoplasma contamination of any of the fibroblast strains.

Preparation of fibroblast suspensions. Fibroblasts were removed from the surface of plastic vessels (Falcon Labware, Oxnard, Calif.) by treatment with trypsin and were washed twice as described (20). The supernatant layer from the final wash was then removed by aspiration, and the cells were resuspended at a density of $5\text{--}10 \times 10^6$ cells/ml in Krebs-Ringer phosphate buffer (pH 7.4) with 5.5 mM glucose (KRPG). The suspension was incubated at 37°C for 30 min with shaking in a water bath. During incubation, 100 μ l of cell suspension was diluted to 1 ml in KRPG containing 0.04% trypan blue, and this suspension was incubated 5–10 min before enumeration of the viable cells on a Spencer bright-line hemocytometer (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). Except for studies of enzyme activities, all values for determinations made in fibroblasts and described in Results are expressed per million viable cells. Suspensions in which <90% of cells were viable were discarded before analysis.

Preparation of fibroblast extracts for enzyme determinations. Trypsin treated, washed fibroblasts were resuspended at a density of $1\text{--}2 \times 10^7$ cells/ml in a buffer at pH 7.4, containing 2 mM sodium phosphate, 10 mM reduced glutathione, and 1 mM tetrasodium EDTA (extraction buffer). Cells were then disrupted by rapid freezing (in liquid nitrogen) and thawing of the suspension three times, after which the fibroblast extract was centrifuged at 48,000 *g* for 30 min at 4°C in a Sorvall RC2B refrigerated centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.). The resulting supernatant layer was then prepared by one of two alternative procedures for measurement of the enzyme activities described below. In the first procedure, the supernatant layer was dialyzed for 2 h at 4°C against 250 vol of extraction buffer before enzyme assay. In the second procedure, 0.4 ml of the supernatant layer was applied to a Sephadex G-25 column (15 cm \times 1.4 cm²) equilibrated with extraction buffer, and elution was carried out in this buffer at a flow rate of 0.5 ml/min. Material eluting in the void volume of the column was collected for enzyme assay. Protein determinations on cell extracts and on partially purified enzyme preparations (see below) were performed by the method of Lowry et al. (28) with bovine serum albumin as standard.

Partial purification of fibroblast PP-ribose-P synthetase. On several occasions, 1-ml samples of dialyzed fibroblast extracts, prepared from $\sim 5 \times 10^7$ normal and S.M. cells, were added to 1.5-ml suspensions of DEAE-cellulose (corresponding to 0.45 g of preswollen DE52; Whatman Chemicals, Div. W & R Balston, Maidstone, England) equilibrated with extraction buffer. After incubation at 4°C with stirring for 1 h, the suspensions were placed on Whatman No. 1 filter papers and filtered on Buchner funnels. The gels were

washed successively with 20 vol each of extraction buffer, 3 mM sodium phosphate buffer (pH 7.4), and 3 mM sodium phosphate buffer with 50 mM KCl (pH 7.4) (11). PP-ribose-P synthetase was then eluted from the gels by treatment with two 1.5-ml fractions of 50 mM sodium phosphate buffer containing 220 mM KCl, 0.3 mM ATP, 6.0 mM MgCl₂, and 1 mM dithiothreitol (pH 7.4). The fractions containing eluted enzyme were combined and, after centrifugation at 12,000 g for 10 min, the clear supernatant layers were dialyzed for 16 h at 4°C against 1,000 vol of extraction buffer. The purifications of PP-ribose-P synthetase achieved with this procedure ranged from 21- to 44-fold for the normal enzyme and from 19- to 46-fold for the S.M. enzyme, respectively. Recoveries of enzyme activity ranged from 66 to 79%.

Biochemical determinations in fibroblasts. The overall rate of the early steps of purine nucleotide synthesis *de novo* was estimated by measurement of the rate of incorporation of sodium [¹⁴C]formate into α -N-formylglycinamide ribotide (FGAR) in cells blocked in further conversion of this compound by the glutamine antagonist azaserine at a concentration of 0.3 mM (20, 29). In previous studies in fibroblasts, results of this method have correlated well with *in vivo* determinations of rates of purine synthesis *de novo* (20, 27, 30). The generation and extraction of labeled FGAR and separation of FGAR from other labeled compounds were carried out as described (27, 31).

Fibroblast PP-ribose-P concentration and generation (20) and activities of hypoxanthine-guanine phosphoribosyltransferase (E.C. 2.4.2.8) (32) and adenine phosphoribosyltransferase (E.C. 2.4.2.7) (33) were measured by previously described isotopic methods. Rates of incorporation of adenine, hypoxanthine, guanine, and adenosine into purine compounds including nucleotides, nucleosides, bases, and nucleic acids were measured using saturating concentrations of the respective ¹⁴C-labeled compounds as described by Rajivo and Seegmiller (34). Although the rate of adenine nucleotide synthesis at saturating concentrations of radioactive adenine has been used as an estimate of the rate of PP-ribose-P synthesis (35) (or PP-ribose-P availability [36]), we have found that PP-ribose-P generation, as defined (20), allows a more accurate estimate of production of this compound, particularly in cells with increased rates of PP-ribose-P synthesis. As shown in Fig. 1, where PP-ribose-P generation and availability are compared in normal fibroblasts and in cells from patient S.M., PP-ribose-P generation (Fig. 1A) and availability (Fig. 1B) were quite comparable in normal cells but showed a greater difference in S.M. fibroblasts. Incorporation of labeled adenine exceeded PP-ribose-P generation in S.M. fibroblasts because, in contrast to the case in normal cells, saturating concentrations of [¹⁴C]adenine do not deplete intracellular PP-ribose-P concentrations to nearly unmeasurable values during the course of the incubation period. As a result, PP-ribose-P availability slightly overestimates the apparent generation of the compound in these cells and underestimates the total of PP-ribose-P generation and PP-ribose-P concentration.

Activity of PP-ribose-P synthetase in fibroblast extracts and partially purified fibroblast preparations was determined at several concentrations of Pi using a minor modification of the two-step method described (10). In the first step, in which PP-ribose-P is produced from MgATP and Rib-5-P, concentrations of the substrates were 100 and 200 μ M, respectively. This reduction in substrate concentrations was prompted by the observation that at Pi concentrations below 2.0 mM, considerable substrate inhibition of enzyme activity occurred in the presence of the usual substrate concentrations, 500 μ M MgATP and 350 μ M Rib-5-P. At the reduced substrate concentrations, substrate inhibition was not apparent, and the maximal velocities of the enzyme reaction at

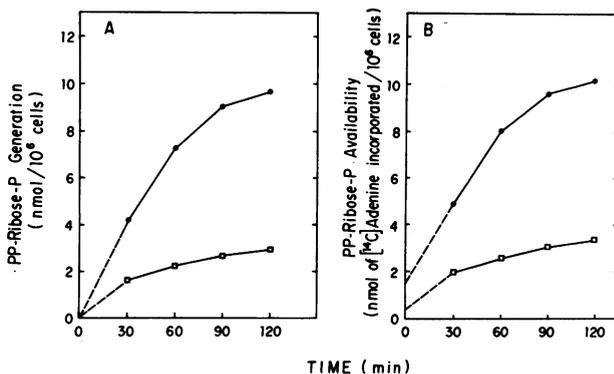


FIGURE 1 PP-ribose-P generation (A) and availability (B) in normal fibroblasts (□) and fibroblasts from patient S.M. (●). Duplicate fibroblast suspensions containing 1.0×10^6 normal or S.M. cells in 400 μ l KRPG were incubated for the appropriate times at 37°C with either unlabeled or labeled adenine (80 μ M). At the points, PP-ribose-P concentrations were determined in cells incubated with unlabeled adenine, and incorporation of ¹⁴C into purine compounds was determined in cells incubated with [8-¹⁴C]adenine. (A) Values indicated were calculated by the formula as described (20), in which PP-ribose-P concentration at zero min and at the appropriate incubation time are considered as well as the incorporation of label; in contrast, (B) values shown indicate only incorporation of label (except at zero min, where base-line PP-ribose-P concentrations are plotted).

Pi concentrations from 2.0 to 32 mM were within 2% of those measured at the higher substrate concentrations.

For studies of the kinetics of the PP-ribose-P synthetase reaction, the two-step assay was employed, but the reaction mixture for the first step was modified to contain identical concentrations of each of the reactants in 0.2 ml, 0.1 of the volume of the standard assay. Studies were carried out at final Pi concentrations of 1.0 and 32 mM. Conditions used for the determination of affinity constants (K_m) and inhibitory constants (K_i or $I_{0.5}$) were identical to those described elsewhere (10, 18) except for appropriate modifications in substrate or inhibitor concentrations.

Thermal inactivation of partially purified fibroblast PP-ribose-P synthetase was studied at 54°C (10). Immunochemical inactivation of PP-ribose-P synthetase in fibroblast extracts prepared by Sephadex G-25 chromatography was investigated by titration of the extracts with the immunoglobulin (Ig)G fraction of monospecific rabbit antiserum to purified normal erythrocyte PP-ribose-P synthetase (19). Measurements of the protein contents of the immunoprecipitates resulting from addition of antiserum to fibroblast extracts were performed as described (10). Mobilities of PP-ribose-P synthetase on cellulose acetate gel were examined with an activity stain (37) after electrophoresis of fibroblast extracts in several buffer systems (10, 24, 37).

Biochemical determinations in erythrocytes and erythrocyte lysates. Activities of PP-ribose-P synthetase (measured as described above), hypoxanthine-guanine phosphoribosyltransferase (38), adenine phosphoribosyltransferase (30), and glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) (39) were determined in dialyzed erythrocyte lysates prepared from freshly drawn heparinized venous blood. Erythrocyte PP-ribose-P (18) and Pi concentrations (40) were measured after extraction from freshly prepared cells by heating in 50 mM Tris-HCl, 1 mM EDTA (pH 7.4) and by addition of 10% trichloroacetic acid, respectively. rates of incorporation

of [¹⁴C]adenine and [¹⁴C]hypoxanthine into purine nucleotides (34) were determined after incubation of intact erythrocytes in isotonic saline solutions at pH 7.4 containing 1 and 32 mM Pi.

On two occasions, erythrocytes of four specific density classes were prepared from heparinized blood by means of a two-stage centrifugation procedure described in detail elsewhere (41). Correspondence of the least dense cells with the youngest erythrocytes and the most dense cells with the oldest has previously been established (41, 42). Erythrocytes in each density class were washed three times with 20 mM Tris-HCl, 150 mM NaCl (pH 7.4) and then prepared for measurements of PP-ribose-P concentration and activities of the enzymes listed above.

On a single occasion, normal and S.M. erythrocyte PP-ribose-P synthetases were partially purified (60- and 44-fold, respectively) by means of the DEAE-cellulose batch elution step described by Fox and Kelley (11). These preparations were used to study kinetic properties of the respective enzymes.

RESULTS

PP-ribose-P metabolism and purine nucleotide synthesis in S.M. fibroblasts. Comparative values for PP-ribose-P metabolism and purine nucleotide syn-

thesis in cultured fibroblasts derived from patient S.M. and from normal individuals are shown in Table I. An increased rate of PP-ribose-P synthesis in the cells of patient S.M. was indicated by the increased intracellular PP-ribose-P concentration and rate of generation (Fig. 1A) shown by these cells. The resulting increased availability of PP-ribose-P (Fig. 1B) for the pathways of purine base salvage and purine nucleotide synthesis *de novo* was reflected by increased rates of incorporation of labeled hypoxanthine and guanine as well as adenine into purine compounds and by an increased rate of incorporation of [¹⁴C]formate into FGAR in the presence of azaserine. In contrast with the accelerated rates of incorporation of labeled precursors into purine compounds through PP-ribose-P-dependent biosynthetic pathways shown by S.M. fibroblasts, rates of [¹⁴C]adenosine incorporation were nearly identical in S.M. and normal fibroblasts. These results suggested that the increased rate of intracellular PP-ribose-P synthesis underlay the excessive rate of purine synthesis *de novo* in patient S.M. fibroblasts.

TABLE I
PP-ribose-P Metabolism and Purine Nucleotide Synthesis in Fibroblasts Cultured from Four Normal Individuals and from Patient S.M.

Cell strain	PP-ribose-P		FGAR accumulation cpm/h/10 ⁶ cells	Rate of incorporation of ¹⁴ C				Fibroblast extract enzyme activity		
	Concentration nmol/10 ⁶ cells	Generation nmol/h/10 ⁶ cells		Ade	Hyp	Gua	Ado	PP-ribose-P synthetase nmol/h/mg protein	Hyp-Gua	Ade
									Phosphoribosyltransferase	
Normal				nmol/h/10 ⁶ cells				nmol/h/mg protein		
W.O.	0.37	2.76	2,600	2.97	1.41	1.92	2.74	448	126	210
L.E.O.	0.35	2.21	2,220	2.42	1.23	1.71	2.81	412	138	196
M.G.	0.42	3.14	3,140	3.47	1.44	1.64	3.59	464	147	188
O.N.	0.29	2.48	2,550	2.71	1.36	1.68	2.27	380	131	218
Patient										
S.M.	1.69	7.34	7,240	8.15	2.76	4.73	2.66	922	133	211

Fibroblasts were resuspended at cell densities of 5–10 × 10⁶/ml in KRPG and incubated at 37°C for 30 min before determination of PP-ribose-P concentration and generation, rate of purine synthesis *de novo* (FGAR accumulation) and rates of incorporation of labeled purine bases and adenosine. For measurement of PP-ribose-P concentration, 0.2–0.4 ml of cell suspension was centrifuged for 5 min at 1,400 g, and the cell pellet was resuspended in 0.3 ml 50 mM Tris-HCl, 1 mM EDTA (pH 7.4) before extraction of PP-ribose-P by heating the 6 × 50-mm test tube in boiling water for 45 s. For measurement of rates of incorporation of labeled compounds, 0.2–0.4 ml of cell suspension was incubated in KRPG with the appropriate labeled base (each at a final concentration of 80 μM) or adenosine (50 μM) for 60 min at 37°C before addition of perchloric acid to a final concentration 0.4 N. After neutralization of the extract, purine compounds were separated as described (34) and counted. FGAR accumulation was measured after extraction and separation of this compound (27, 31) from 1 to 2 × 10⁶ cells incubated for 60 min at 37°C in KRPG with 4 mM L-glycine, 20 mM L-glutamine, 1.27 mM sodium [¹⁴C]formate, and 0.3 mM azaserine as described (20).

For determination of enzyme activities, fibroblasts were resuspended at cell densities of 1–2 × 10⁷/ml in 2 mM sodium phosphate, 10 mM reduced glutathione, and 1 mM EDTA (pH 7.4) before repeated (three times) freezing in liquid nitrogen and thawing. After centrifugation of the cell extract at 48,000 g for 30 min, the resulting supernatant layer was dialyzed for 2 h against the extraction buffer. Activities of hypoxanthine-guanine and adenine phosphoribosyltransferases were measured at saturating substrate concentrations using the appropriate [¹⁴C]purine base as described (32, 33). PP-ribose-P synthetase activity was determined with a modified two-step assay (10) in which Pi, MgATP and Rib-5-P concentrations were 32 mM, 100 μM, and 200 μM, respectively.

PP-ribose-P synthetase in S.M. fibroblasts. An enzymatic basis for the increased rate of PP-ribose-P production in S.M. fibroblasts was provided by the results of determinations of PP-ribose-P synthetase activity in extracts of fibroblasts cultured from this child. S.M. PP-ribose-P synthetase activity, measured at saturating substrate and optimal cofactor concentrations, was twice that found in extracts of normal fibroblasts (Table I). In studies in which activities of PP-ribose-P synthetase were determined (at 32 mM Pi) in fibroblast extracts prepared from cultures harvested at cell densities ranging from 0.7 to 3.6×10^6 /flask, the magnitude of the excessive PP-ribose-P synthetase activity in fibroblasts from patient S.M. was less than that observed in cells from patient H.B. whose superactive enzyme shows increased maximal reaction velocity per enzyme molecule (10). Nevertheless, enzyme activities in S.M.'s cells exceeded those in normal fibroblasts by at least twofold at each cell density. The greater PP-ribose-P synthetase activity in S.M. extracts is not, therefore, a result of variation in enzyme activity with culture density (43).

Activation by Pi of S.M. and normal PP-ribose-P synthetases in partially dialyzed fibroblast extracts is shown in Fig. 2A. In addition to greater enzyme specific activity in S.M. fibroblast extracts, there were distinct differences in the shapes of the Pi activation curves (Fig. 2A, inset). In contrast with the sigmoidal Pi activation curve of the normal enzyme in incompletely dialyzed (or crude undialyzed) extracts, hyperbolic activation of S.M. PP-ribose-P synthetase was apparent. At 32 mM Pi, S.M. PP-ribose-P synthetase specific activity was 2-fold greater than normal, whereas in the more physiologic range of Pi concentrations (~0.5–2 mM), S.M. PP-ribose-P synthetase activities were nearly 10-fold greater than normal. The sigmoidal shape of the normal Pi activation curve of PP-ribose-P synthetase in crude or incompletely dialyzed extracts results from greater efficiency of feedback inhibitors of enzyme activity at lower Pi concentrations (1, 16). Thus, these studies suggested diminished responsiveness of S.M.'s enzyme to feedback inhibitors. In contrast with a described (14, 17) feedback-resistant human PP-ribose-P synthetase, however, S.M. PP-ribose-P synthetase showed significantly greater activity than normal at high as well as low Pi concentrations. This finding thus raised the additional possibilities of increased maximal reaction velocity and/or increased quantity of enzyme per cell to explain the increased activity of S.M. PP-ribose-P synthetase. To assess each of these potential mechanisms for excessive enzyme activity, procedures more effective than dialysis in removing small molecule inhibitors from the milieu of the enzyme were undertaken. Chromatography of crude fibroblast extracts on Sephadex G-25 resulted in hyperbolic Pi activation curves for

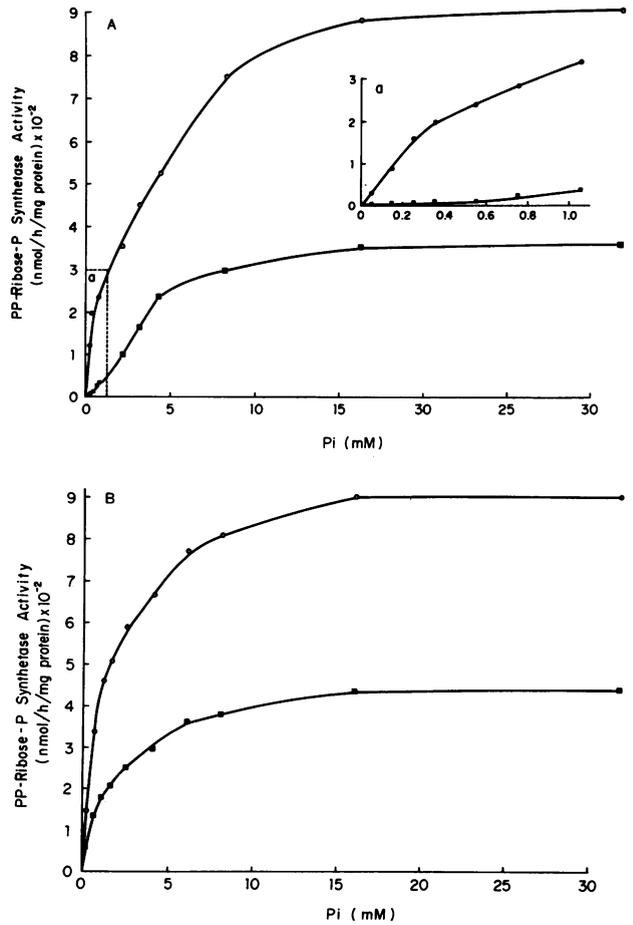


FIGURE 2 Activation of normal (■) and SM (○) fibroblast PP-ribose-P synthetases by Pi in (A) incompletely dialyzed extracts and (B) extracts chromatographed on Sephadex G-25. In A, inset shows enzymes activities over the range from 0.05 to 1.05 mM Pi in greater detail.

both normal and S.M. PP-ribose-P synthetases, and the variant form of the enzyme exhibited twofold greater activities at all Pi concentrations (Fig. 2B). Partially purified preparations of normal and S.M. PP-ribose-P synthetases also showed hyperbolic Pi activation patterns with a constant relative difference between normal and S.M. enzyme activities when the preparations were studied in the absence of inhibitor compounds.

To determine whether the increased enzyme activity apparent in S.M. fibroblast extracts resulted from an increased number of enzyme molecules or from an enzyme with increased catalytic activity per molecule of enzyme, normal and S.M. fibroblast extracts, prepared by Sephadex G-25 chromatography and equated for protein concentration, were titrated with the IgG fraction of rabbit antiserum to normal erythrocyte PP-ribose-P synthetase (Fig. 3). At each point on the titration curve, inactivation of S.M. PP-ribose-P

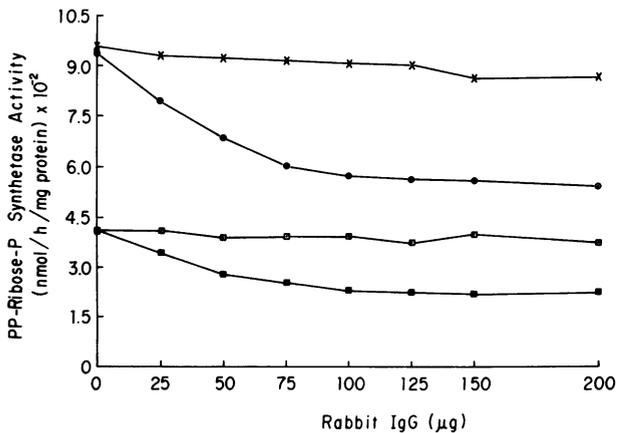


FIGURE 3 Inactivation of normal and SM fibroblast PP-ribose-P synthetases by rabbit IgG. Normal and SM fibroblast extracts were prepared by Sephadex G-25 chromatography and were equated for protein concentration. Each extract was then titrated with increasing quantities of IgG purified from the sera of an unimmunized rabbit and a rabbit immunized with highly purified normal erythrocyte PP-ribose-P synthetase (19). Incubation mixtures of 100 μ l contained the following: chromatographed fibroblast extract (25 μ g of protein); sodium phosphate buffer (pH 7.4), 2 μ mol; NaCl 10 μ mol; reduced glutathione, 0.33 μ mol; EDTA, 0.03 μ mol; and an amount of rabbit IgG as indicated. After incubation for 30 min at 37°C and 30 min at 0°C, 75 μ l of the incubation mixture was assayed for PP-ribose-P synthetase activity at 32 mM Pi. Normal extract incubated with: unimmunized IgG, (□); immunized IgG, (×). S.M. extract incubated with: unimmunized IgG, (■); immunized IgG (○).

synthetase was twofold greater than that of the normal enzyme even though, in parallel incubations, protein content of the immune precipitates resulting from addition of antibody to either of the extracts was identical for each ratio of antibody to enzyme. (Residual enzyme activities in the titration curves reflect activity of PP-ribose-P synthetase in the enzyme-antibody complex as previously demonstrated [25].) Thus, similar quantities of PP-ribose-P synthetase were present in normal and S.M. fibroblast extracts, but S.M. fibroblast PP-ribose-P synthetase had twofold greater catalytic activity per immunoreactive enzyme molecule.

Experiments were next undertaken to delineate the substrate binding and inhibitor responsiveness of partially purified normal and S.M. fibroblast PP-ribose-P synthetases (Table II). Apparent binding constants (K_m) for MgATP, Rib-5-P, and Mg^{2+} were similar for normal and S.M. enzymes studied at either 1 mM or 32 mM Pi. In contrast, the concentrations ($I_{0.5}$) of ADP and GDP at which 50% inactivation of PP-ribose-P synthetase was observed were distinctly different for S.M. and normal enzymes. S.M. enzyme was more resistant than normal enzyme to inactivation by ADP and GDP at 1 mM and 32 mM Pi. Fig. 4A and 4B show that with increasing ADP and GDP concentration, normal PP-ribose-P synthetase was four-

fivefold more sensitive to these inhibitors at 1 mM Pi than was the variant enzyme. At 32 mM Pi the K_i slope for inhibition of the normal enzyme by ADP was 22 μ M compared to 80 μ M for the enzyme from patient S.M. These findings indicated that despite normal affinities for substrates, S.M. PP-ribose-P synthetase was relatively more resistant than normal enzyme to the inhibitory effects of nucleotide compounds. Thus, the variant enzyme showed altered regulatory properties with respect to inhibitors as well as increased maximal reaction velocity when studied in the absence of negative effectors.

Thermal inactivation of S.M. PP-ribose-P synthetase at 54°C was substantially more rapid than that of normal fibroblast enzyme (Fig. 5) suggesting decreased stability of the variant enzyme. Electrophoretic mobilities of normal and S.M. PP-ribose-P synthetases were identical on cellulose acetate gel in several buffer systems over a range of pH from 7.5 to 9.0.

PP-ribose-P synthetase in S.M. erythrocytes. In contrast with the increased specific activity of S.M. PP-ribose-P synthetase in fibroblast extracts (Fig. 2A and B), PP-ribose-P synthetase activities in dialyzed or chromatographed erythrocyte lysates from this patient were considerably lower than those in similarly prepared hemolysates from normal individuals, measuring <10% of the mean activities in normal hemolysates at Pi concentrations >1 mM (Fig. 6). Despite the apparent deficiency in S.M. erythrocyte enzyme activity, PP-ribose-P synthetase in hemolysates from this patient showed hyperbolic Pi activation similar to that observed in his fibroblast preparations and, at the lowest Pi concentrations tested, enzyme activity in partially dialyzed S.M. hemolysates slightly exceeded those in comparably prepared hemolysates from normal individuals (Fig. 6, inset).

Several possible explanations for these findings were investigated. First, mixtures of normal and S.M. hemolysates failed to provide evidence for differences between these extracts in the content of either inhibitors or activators of PP-ribose-P synthetase. Second, under conditions of incubation with Pi, Mg^{2+} , and MgATP previously established to promote aggregation of normal PP-ribose-P synthetase subunits to active polymers (13), stimulation of S.M. and normal PP-ribose-P synthetase activities were proportionally similar. In addition, the elution profiles of normal and S.M. PP-ribose-P synthetase activities from Sephadex G-200 after chromatography of erythrocyte lysates were identical. Thus, the lower specific activity of the S.M. erythrocyte enzyme did not appear to result from an altered state of subunit aggregation.

To investigate the possibility that instability of S.M. PP-ribose-P synthetase might account for the observed deficit in erythrocyte enzyme activity, fractionation of normal and S.M. erythrocytes by specific density was carried out using the dimethyl phthalate-dibutyl phthal-

TABLE II
Comparison of Kinetic Constants of Purified Normal and S.M. Fibroblast PP-ribose-P Synthetases

Effector	Variable substrate	Pi concentration	Normal enzyme			S.M. enzyme		
			K_m	$I_{0.5}$	K_i slope	K_m	$I_{0.5}$	K_i slope
		mM	μM					
Substrates								
MgATP	MgATP	1	16	—	—	15	—	—
		32	24	—	—	22	—	—
Rib-5-P	Rib-5-P	1	12	—	—	15	—	—
		32	27	—	—	33	—	—
Activator								
Mg ²⁺	MgATP	1	36	—	—	32	—	—
		32	44	—	—	49	—	—
Inhibitors								
ADP	MgATP	1	—	22	—	—	85	—
		32	—	135	22	1	280	80
GDP	MgATP	1	—	34	—	—	153	—
		32	—	320	—	—	>750	—

Normal and S.M. PP-ribose-P synthetases, purified ~30-fold, were studied at 1 mM and 32 mM Pi. For studies of affinity constants (K_m), incubation mixtures of 0.2 ml contained the components of the standard reaction mixture at the usual concentrations except that when MgATP was the variable substrate, concentrations of MgATP ranged from 0 to 100 μM , and when Rib-5-P was the variable substrate, concentrations of this compound ranged from 0 to 350 μM . When binding of magnesium was studied, the concentration of MgCl₂ was varied from 0.1 to 5.0 mM in excess of the variable MgATP concentration. Affinity constants and the inhibitory constant (K_i) for ADP were determined as described (10). For the latter determination MgATP concentrations were varied from 0 to 100 μM in the presence of fixed ADP concentrations ranging from 0 to 100 μM . Additional inhibitory constants ($I_{0.5}$) were calculated from studies in which increasing concentrations of ADP and GDP were added to the first step of standard incubation mixtures (MgATP and Rib-5-P concentrations, 100 and 200 μM , respectively) for measurement of PP-ribose-P synthetase activity at either 1 mM or 32 mM Pi. Inhibitor concentrations ranged from 0 to 250 μM at 1 mM Pi (Fig. 5) and 0–750 μM at 32 mM Pi.

ate density centrifugation procedure. Hemolysates were prepared from each fraction, and activities of PP-ribose-P synthetase, hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, and glucose-6-phosphate dehydrogenase were determined (Fig. 7). S.M. and normal erythrocytes were comparable in their patterns of hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, and glucose-6-P dehydrogenase activities relative to increasing erythrocyte density, the latter two activities diminishing with increasing mean erythrocyte specific density as described (44, 45). (Increased adenine phosphoribosyltransferase activity in hemolysates from patient S.M. [26] was confirmed in these studies.) PP-ribose-P synthetase in the erythrocytes of normal individuals showed a slight decline in activity with increasing mean cell age, whereas activity of the enzyme in the erythrocytes of patient S.M. was virtually undetectable in all but the least dense erythrocyte population. This study suggested marked instability of PP-ribose-P synthetase in erythrocytes from patient S.M.

Despite the evidence for diminished quantities of active PP-ribose-P synthetase in S.M. erythrocytes,

intact, unfractionated erythrocytes from this patient showed increased capacity to synthesize PP-ribose-P at physiological Pi concentration. First, the intracellular PP-ribose-P concentration of freshly isolated S.M. erythrocytes (5.26 nmol/ml packed cells) exceeded that of normal erythrocytes (2.91 + 0.50 SD). The overall increase in PP-ribose-P concentration of S.M. erythrocytes resulted from a much greater PP-ribose-P concentration in the youngest cells from S.M. than in cells of comparable density from normal individuals (12.70 vs. 3.21 nmol/ml packed cells); in fact, in the most dense erythrocyte fractions, PP-ribose-P concentration in normal cells was 1.92 nmol/ml packed cells while PP-ribose-P was undetectable in S.M. cells. The higher PP-ribose-P concentration in unfractionated S.M. erythrocytes thus appeared to reflect the distinctly different distributions of S.M. and normal PP-ribose-P synthetase activities demonstrated by the density-fractionation procedure.

Second, intact S.M. erythrocytes showed greater than normal rates of incorporation of labeled adenine (Fig. 8) and hypoxanthine into acid-soluble nucleotides when incubated at 1 mM Pi but lower than normal rates of incorporation of these labeled bases when in-

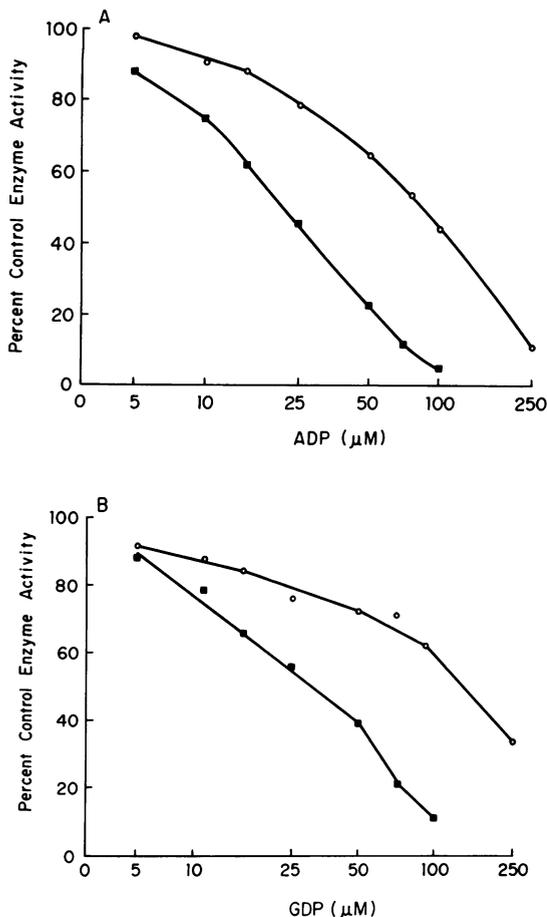


FIGURE 4 Inhibition of partially purified normal (■) and SM (○) fibroblast PP-ribose-P synthetases by (A) ADP and (B) GDP during incubation at 1 mM Pi. Enzyme activities in partially purified preparations of normal and mutant PP-ribose-P synthetases (containing equal protein concentrations) were determined during 30 min incubation at 37°C in the presence of the concentrations of inhibitory nucleotides indicated.

cubated at 32 mM Pi. These findings appear to be interpretable in light of the differences between the Pi activation curves of S.M. and normal erythrocyte PP-ribose-P synthetases (Fig. 6) and the diminished sensitivity of partially purified S.M. erythrocyte PP-ribose-P synthetase to nucleotide inhibition ($I_{0.5}$ ADP at 1 mM Pi for normal and S.M. enzymes were 19 and 103 μ M, respectively; $I_{0.5}$ GDP at 1 mM Pi were 36 and 300 μ M, respectively, for normal and S.M. enzymes). That is, at low Pi concentrations, S.M. enzyme resists the normally profound attenuation of PP-ribose-P synthetase activity by physiological concentrations of intracellular inhibitors; thus, despite the diminished quantity of active enzyme in the S.M. erythrocyte population, the synthesis of PP-ribose-P proceeds at an increased rate leading to a greater than

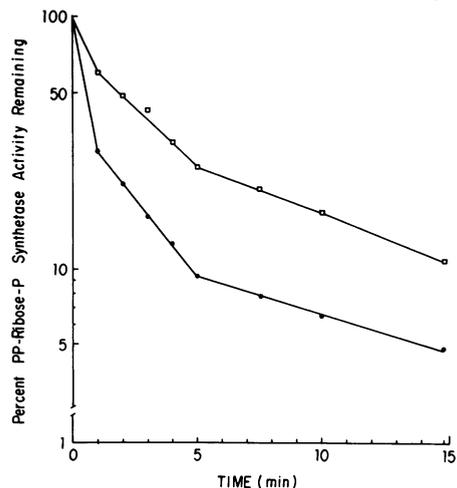


FIGURE 5 Thermal inactivation at 54°C of partially purified normal (□) and SM (●) PP-ribose-P synthetases. Each assay tube contained 4 μ g of enzyme preparation purified 30-fold.

normal rate of incorporation of labeled bases into nucleotides. Since, however, at the intracellular Pi concentration resulting from incubation of cells at 32 mM Pi (2.6 mM Pi for both S.M. and normal erythrocytes), the maximal reaction velocity of S.M. PP-ribose-P synthetase is not significantly greater than that achieved during incubation at 1 mM Pi (1.1 and 1.2 mM Pi for S.M. and normal erythrocytes, respectively), the rates of synthesis of PP-ribose-P by S.M. erythrocytes are similar at both high and low Pi concentrations. This contrasts with the situation in normal erythrocytes which have a greater quantity of potentially active PP-ribose-P synthetase that is expressed by sigmoidal activation over the range of increasing intracellular Pi concentrations. As a result,

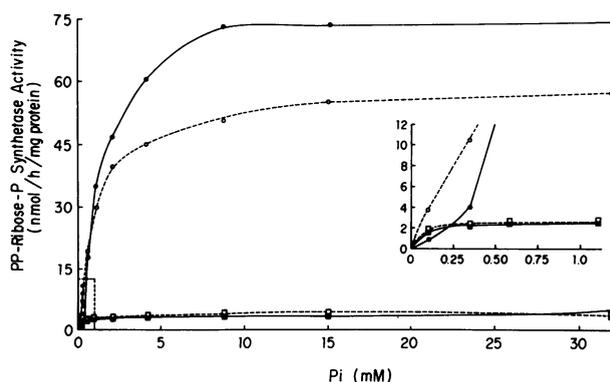


FIGURE 6 Activation of normal and S.M. erythrocyte PP-ribose-P synthetases by Pi in partially dialyzed hemolysates and in hemolysates chromatographed on Sephadex G-25. Inset shows enzyme activation over the range from 0.1 to 1.1 mM Pi in greater detail. Dialyzed normal hemolysate, (●); dialyzed S.M. hemolysate, (■). Chromatographed normal hemolysate, (○); chromatographed S.M. hemolysate, (□).

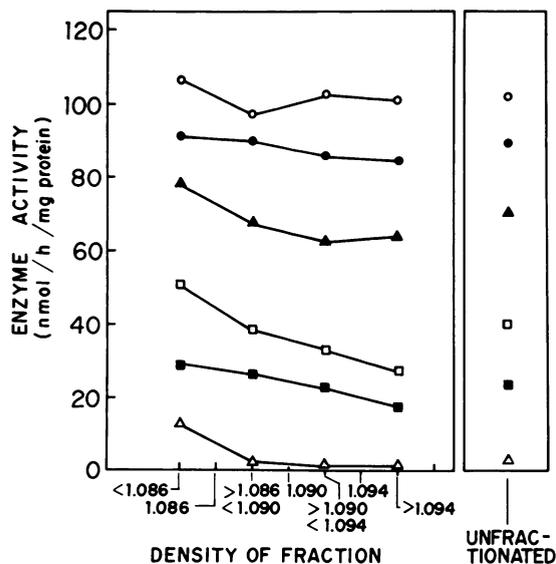


FIGURE 7 Activities of normal (closed symbols) and S.M. (open symbols) PP-ribose-P synthetase (Δ), adenine phosphoribosyltransferase (\square), and hypoxanthine-guanine phosphoribosyltransferase (\circ) in hemolysates from unfractionated erythrocytes and hemolysates prepared from erythrocytes fractionated by specific density. Activities of glucose-6-phosphate dehydrogenase (not shown) were also determined and were (in order of increasing density): 9.1, 7.3, 5.8 and 4.6 B \ddot{u} cher units per 10^9 normal cells and 8.8, 7.0, 5.8 and 4.4 B \ddot{u} cher units per 10^9 S.M. cells.

rates of PP-ribose-P synthesis and purine base incorporation by normal erythrocytes are much greater during incubation at 32 mM than at 1 mM Pi and at the higher concentration, exceed the rates of these processes in S.M. cells.

PP-ribose-P synthetase in erythrocytes and fibroblasts from Su.M., mother of patient S.M. Activities of PP-ribose-P synthetase in dialyzed erythrocyte lysates from the mother of S.M. were in the normal range of values for this enzyme at all Pi concentrations studied, and the Pi activation curve was sigmoidal. In contrast, evidence for the presence of the abnormal PP-ribose-P synthetase in cells of Su.M. was obtained by the study of the enzyme in extracts of fibroblasts cultured from this woman. Activities of PP-ribose-P synthetase in dialyzed fibroblast extracts were intermediate between those in normal and S.M. extracts, and hyperbolic Pi activation of the enzyme was found. In cells harvested from confluent cultures, Su.M. PP-ribose-P synthetase activity at 32 mM Pi was 740 nmol/h per mg protein, a value greater than those of normal cells (range, 285–486 nmol/h per mg protein) but less than that of S.M. fibroblasts (904 nmol/h per mg protein). In addition, nucleotide inhibition of PP-ribose-P synthetase activities in Su.M., S.M., and normal fibroblast extracts prepared by Sephadex G-25 chromatography were compared. At 1 mM Pi, $I_{0.5}$ of ADP and

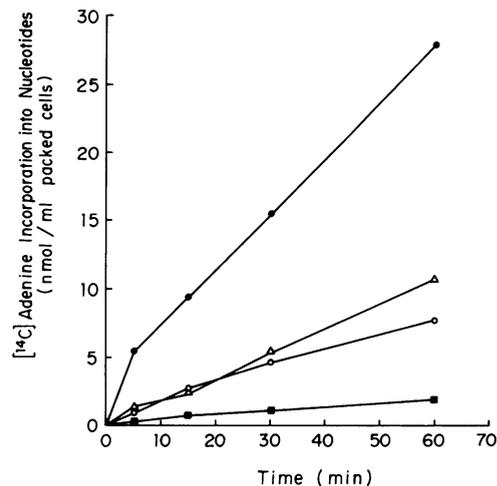


FIGURE 8 Incorporation of [14 C]adenine into acid-soluble nucleotides of normal and S.M. erythrocytes during incubation at 1 mM and 32 mM Pi. Erythrocytes were suspended in isotonic saline containing the appropriate Pi concentration. After addition of [8- 14 C]adenine (final concentration, 80 μ M), incubations were carried out at 37°C for the times indicated before perchloric acid extraction and subsequent isolation and counting of labeled nucleotides. Normal cells: at 1 mM Pi, (\blacksquare); at 32 mM Pi, (\bullet). S.M. cells: at 1 mM Pi, (\circ); at 32 mM Pi, (\blacktriangle).

GDP for Su.M. PP-ribose-P synthetase were 50 μ M and 72 μ M, respectively, values intermediate to those for normal and S.M. enzymes (Table II). These studies provide evidence for the presence of the superactive, feedback-resistant form of PP-ribose-P synthetase in the fibroblasts of Su.M., consistent with the hypothesis that this woman is a heterozygous carrier of the aberrant enzyme expressed in hemizygous form by her son, S.M.

DISCUSSION

The mutant form of PP-ribose-P synthetase characterized in this investigation combines two of the molecular defects previously demonstrated individually to underlie superactivity of this enzyme and result in excessive PP-ribose-P, purine nucleotide and uric acid production in man. PP-ribose-P synthetase from patient S.M. is abnormally resistant to purine nucleotide feedback inhibition of enzyme activity and, in crude or dialyzed enzyme preparations, shows a hyperbolic rather than the normal sigmoidal curve of Pi activation. In these respects, S.M. PP-ribose-P synthetase resembles the feedback-resistant (regulatory-deficient) enzyme studied in detail by Sperling et al. (14, 16) and Zoref et al. (17). In contrast with the findings of these investigators, however, S.M. fibroblast PP-ribose-P synthetase in crude and dialyzed extracts shows at least twofold greater than normal enzyme activity throughout the entire range of Pi concentra-

tions studied, and after Sephadex G-25 chromatography, activity of PP-ribose-P synthetase from S.M. fibroblasts is twice that of normal enzyme at all Pi concentrations. These findings, together with the demonstration of normal affinity constants of S.M. PP-ribose-P synthetase for MgATP, ribose-5-P and Mg^{2+} , and immunochemical inactivation data showing that S.M. PP-ribose-P synthetase has twofold greater activity per immunoreactive enzyme molecule, are consistent with a catalytic defect in the enzyme resulting in excessive maximal velocity of the enzyme reaction. Thus, S.M. PP-ribose-P synthetase resembles, in this respect, the superactive mutant enzyme with increased maximal velocity described by Becker et al., (10, 18, 19).

In addition to possessing abnormal regulatory and catalytic properties, S.M. PP-ribose-P synthetase is evidently less stable than the normal enzyme. This instability is manifested *in vitro* by increased thermal lability of S.M. fibroblast enzyme activity and *in vivo* both by marked deficiency of enzyme activity at high Pi concentrations in lysates of unfractionated S.M. erythrocytes and by an accelerated, age-related decrement in enzyme activity in lysates of density-fractionated erythrocytes. Together, the data indicate that the enzyme studied in S.M. is a new variant, distinct from those previously described.

Comparison of PP-ribose-P production in S.M. and normal erythrocytes provides some insight into the process by which PP-ribose-P synthesis in intact cells is ordinarily modulated under physiological conditions of Pi concentration. Although the *in vitro* activity of normal erythrocyte PP-ribose-P synthetase was much greater than that of S.M. enzyme at 1 mM Pi, intracellular PP-ribose-P concentration and the rates of synthesis of purine nucleotides from purine bases at 1 mM Pi were greater for S.M. than for normal erythrocytes. Thus, despite evidence for a markedly reduced quantity of mutant PP-ribose-P synthetase in the S.M. erythrocyte population, generation of PP-ribose-P by these cells exceeded generation of this compound by normal cells. This finding is best explained as reflecting the considerable inhibition of intracellular PP-ribose-P synthesis exerted by nucleotide feedback inhibitors in normal cells at physiological Pi concentrations (1, 2), a regulatory process to which the feedback-resistant S.M. cells are relatively refractory. Mutant cells thus generate PP-ribose-P at an excessive rate despite a reduced amount of enzyme. In a prior study (1), a discrepancy of several hundred-fold between erythrocyte PP-ribose-P synthetase catalytic capacity and PP-ribose-P synthesis was found; our studies confirm at least this much modulation of PP-ribose-P synthetase activity in the normal cell and provide substantiation of the role of nucleotide inhibitors in accounting, at least in part, for this regulation.

It is tempting to speculate about the relationship between the biochemical consequences of the abnormalities identified in PP-ribose-P synthetase from this family and the clinical findings in the hemizygous male patient, S.M., and his heterozygous mother, Su.M. In contrast with previously described males with superactive PP-ribose-P synthetases (15, 18, 20) whose uric acid overproduction has resulted in gouty arthritis and/or uric acid-related renal disease in adulthood, clinical manifestation of hyperuricemia and excessive uric acid excretion occurred in S.M. during the first year of life (26). In addition, a variety of developmental and behavioral abnormalities, including mental retardation and autistic behavior were reported in this patient (26). A recent clinical and psychological re-evaluation of S.M. at 14 yr of age has shown a severe sensorineural hearing loss, making an accurate assessment of the reported behavioral abnormalities difficult. Nevertheless, it is of interest that this patient's mother, in whom gout, uric acid urolithiasis and purine overproduction have been documented, shares with her son a significant hearing loss. Whether the combined regulatory and catalytic defects in PP-ribose-P synthetase in this family account for the early onset of clinical findings in S.M. and the symptomatic expression of the carrier state in Su.M. on the basis of a more severe functional abnormality in PP-ribose-P metabolism than is the case in previously described families or whether additional modifying factors account for these clinical features is uncertain. Similarly, the role of the aberrant enzyme and its metabolic sequelae in the hearing loss of S.M. and Su.M. and the additional developmental and behavioral abnormalities in S.M. require study of additional family members or of families with comparable enzymatic defects.

The results of measurements of PP-ribose-P synthetase activity in erythrocyte lysates from patient S.M. and from his mother have implications pertinent to screening for abnormalities of this enzyme. In the case of patient S.M., initial screening studies at high Pi concentrations suggested a deficiency of the enzyme which was clearly unexpected in the particular clinical setting. This points out the difficulty in devising a convenient but accurate screening procedure for PP-ribose-P synthetase. In fact, the range of molecular abnormalities associated to date with superactivity of this enzyme is sufficiently broad (10, 16, 17, 20) that the majority of these variant forms would not have been identified by routine assay procedures.

Although the evidence for X-linkage of the gene for PP-ribose-P synthetase (23-25) is compelling, the use of erythrocyte lysates to identify heterozygous female carriers of superactive variants of the enzyme is unreliable. As previously described (15, 18), enzyme

activities intermediate to those of normal individuals and affected males, as predicted by the Lyon (46) hypothesis, seem to be the exception in erythrocyte lysates from heterozygous carriers of PP-ribose-P synthetase variants. The normal enzyme activity in hemolysates from Su.M. emphasizes this point. Measurement of enzyme activity in fibroblasts cultured from women suspected to carry variant forms of PP-ribose-P synthetase appears to provide an accurate basis for heterozygote detection (17, 20, 24), and the availability of cultured fibroblasts provides material for a thorough evaluation of the biochemical consequences of aberrations in this enzyme (17, 18, 20).

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