Hypoxanthine Phosphoribosyltransferase Activity in Intact Fibroblasts from Patients with X-Linked Hyperuricemia

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ABSTRACT Discordance between clinical phenotype and the level of a mutant enzyme activity may reflect differences between enzyme function in vivo and that measured by the customary enzyme assays on cell extracts. In the present study, the conversion of hypoxanthine to phosphorylated products was measured in intact skin fibroblasts and in cell extracts from seven patients with mutant hypoxanthine-guanine phosphoribosyltransferase (HPRT) and six control subjects. The patient's phenotypes ranged from asymptomatic hyperuricemia to the Lesch-Nyhan syndrome. Although there was a general correlation between the HPRT activity in cell extracts assayed by the usual methods and the function of the purine salvage pathway in patients, as reflected by urinary oxypurine excretion, there were notable exceptions. A more accurate appraisal of the functioning of the pathway at the cellular level is achieved by measuring the conversion of substrate to product in the intact cell at physiological concentrations of substrates, activators, and product and metabolite inhibitors, and in a physiological ionic environment. In one of the seven patients, the standard enzyme assay indicated normal function, whereas measurements in the intact cell exposed severe dysfunction of the salvage system. In another, the standard assay suggested a severe deficiency not evident in the intact cell or in the patient.

INTRODUCTION

The enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) [1] (EC 2.4.2.8) catalyzes the transfer of the 5-phosphoribosyl moiety of 5-phosphoribosyl-1-pyrophosphate (PRPP) to guanine, hypoxanthine, or xanthine to form their respective ribonucleotide derivatives. Deficiencies in HPRT are associated with impaired function of the purine salvage pathway and with excess production and excretion of oxypurines. Severe or virtually complete deficiency results in the Lesch-Nyhan (LN) syndrome, characterized by massive uricosuria and signs of neurological dysfunction, e.g. spasticity and compulsive self-mutilation (1, 2). Patients with less severe HPRT deficiencies produce more moderate amounts of uric acid and may have gouty arthritis and uric acid calculi but do not have the neurologic or behavioral features characteristic of the classical LN syndrome (3–7).

Although there is a general correlation between clinical manifestations and enzyme activity in red cell lysates, there are some notable exceptions (8, 9). Dancis et al. (8) reported a pair of cousins with no detectable HPRT activity in their erythrocytes, a finding considered pathognomonic of the LN syndrome.

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[1] *Abbreviations used in this paper:* APRT, adenine phosphoribosyltransferase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; LN, Lesch-Nyhan; PRPP, 5-phosphoribosyl-1-pyrophosphate.

However, these patients have only modest uricemia and have none of the LN stigmata. Dancis et al. (8) suggested that this apparent discordance between clinical phenotype and erythrocyte enzyme activtiy may be a reflection of the difference between enzyme function in vivo and that measured by customary in vitro erythrocyte assays. A closer approximation of the function of the purine salvage pathway in vivo might be obtained by measuring the rate of conversion of hypoxanthine to phosphorylated products in intact nucleated cells.

To investigate this possibility, the conversion of hypoxanthine to phosphorylated products was measured in intact fibroblasts and fibroblast extracts from seven patients with mutant HPRT and from six subjects with normal HPRT. The clinical phenotype in the HPRT-deficient patients ranged from asymptomatic hyperuricemia to classical LN syndrome. For each mutant HPRT, the rates of conversion of hypoxanthine to phosphorylated products, relative to control, were compared in erythrocyte lysates, fibroblast extracts, and intact fibroblasts. For five of the patients there was good agreement among the three methods of assay. For two patients, however, the apparent HRPT activity in the intact fibroblast was found to differ from the catalytic activity determined in both erythrocyte lysates and fibroblast extracts. The rate of conversion of substrate to product in the intact cell provided the best correlation with the apparent function of the purine salvage pathway in these patients, as reflected by urinary oxypurine excretion.

METHODS

Fibroblast strains

All fibroblast strains were initiated in our laboratory except cell strain C. M., obtained from Dr. Paul J. Benke. To avoid using cells in high passage, cultures were restarted from frozen stock at regular intervals. Fibroblast strains were obtained from three patients with classical LN syndrome. These patients, Bak., Sep., and Sch., were between 2 and 3 yr of age at biopsy. The origin of the other four HPRT-deficient cell strains used in this study and a brief description of each patient's clinical phenotype are given in Table I. Six fibroblast strains with normal HPRT served as controls for this study. Three of these strains were obtained from patients with other inborn errors (a 3-mo-old girl with hyperlipoproteinemia, Type I; a 48-yr-old man with hyperlipoproteinemia, Type II; and a yr-old boy with nonketotic hyperglycinemia), and three were started from foreskins obtained from apparently normal newborns.

For the intact cell studies, all cell strains were grown as monolayers in Eagle's minimal essential medium, made with Earle's balanced salt solution and supplemented with streptomycin (50 μg/ml), penicillin (50 U/ml), kanamycin (33 μg/ml), nonessential amino acids, glutamine (2 mM), vitamins, and fetal calf serum (15%). Cell extracts were prepared from monolayers cultured in Waymouth's medium (10) supplemented with streptomycin (50 μg/ml), penicillin

TABLE I

Indices of Purine Metabolism in Four Patients with X-Linked Hyperuricemia

Patient	Age*	Urinary uric acid		Urinary uric acid to creatinine ratio	Serum urate	Erythrocyte lysate HPRT activity	Symptoms and signs‡	Previous description
	yr	*mg/24 h*	*mg/kg body wt/24 h*		*mg/100 ml*	*% of control*		*ref. no*
HoRo.	14	832	13.2	0.81	10.0	<0.01	Asymptomatic hyperuricemia	8
C. M.	13	1,741§	31.1§	Not available	12.5§	104‖	Massive uricosuria, renal failure, mild neurological dysfunction	9
Bou.	16	1,173	14.3	1.00	17.8	0.84	Recurrent renal colic and hematuria	7: individual III-16 in the Da family
Ill.	54	976	14.0	0.56	15.0	0.15	Severe and protracted recurrent gouty arthritis	7: individual T.II. of the II. family
Lesch-Nyhan patients' range		660–920¶	25–143¶	2.07–4.68¶	4.9–25.1¶	<0.01¶		
Normal children								
Mean±SD		—	—	Age-dependent‡‡	3.6±1.2¶			
Range		—	<18¶	—	—			
Normal adults								
Mean±SD		—	—	0.49±0.15‡‡	4.9±1.4**			
Range		<590**	<7**	0.19–0.79‡‡	—			

* At time of serum and urinary urate determinations. At time of biopsy, HoRo., C. M., Bou., and Ill. were, respectively, 9, 18, 22, and 49 yr old.
‡ Before treatment.
§ Data from Sorensen and Benke (20).
‖ Data from Benke and Herrick (21).
¶ Data from Kelley and Wyngaarden (22).
** Data from Wyngaarden and Kelley (18). Mean serum urate value is for normal males (n = 2,987).
‡‡ Data from Kaufman et al. (23). The normal mean is 1.55 in the 1st wk of life and declines to 0.61 at age 10 and to 0.50 at age 14.

(50 U/ml), kanamycin (33 μg/ml), and fetal calf serum (10%). The HPRT activities of cells grown in Eagle's and Waymouth's media are similar.

Apparent enzyme activities in intact fibroblasts

The rate of conversion of substrate to product in the intact cell is not a determination of catalytic enzyme activity. Because the rate of conversion in the intact cell is dependent upon a number of factors in addition to true enzyme activity, this rate of conversion will be designated "apparent enzyme activity" as suggested by Snyder et al. (11).

Apparent HPRT activity. Replicate flasks (25 cm^2 Falcon plastic [BioQuest, Oxnard, Calif.]) of each fibroblast strain were fed every 4 days and on the day before assay. The growth medium employed was Eagle's minimal essential medium, supplemented as described above. Cell cultures were selected for assay when the monolayers were almost confluent (late log phase). The growth medium was removed from each flask and replaced by 1 ml of prewarmed (37°C) growth medium containing [8-^{14}C]hypoxanthine (15 mCi/mmol; 0.4 mM). After a 30-min incubation on a rocking table at 37°C, the flasks were placed on a layer of crushed ice. The labeled medium was removed from each flask by suction, and the cell layer was covered immediately with 5 ml of ice-cold Hanks' balanced salt solution. The flask was rocked gently for approximately 30 s, and then the rinse was removed by suction. The rinsing procedure was repeated five times. (The effectiveness of this rinsing regimen was tested by counting aliquots from each successive rinse on several occasions). After the final rinse, the neck was broken off each flask to facilitate the removal of the cells with a rubber scraper. The cells from each flask were harvested into a total of 2 ml of distilled water containing 1 mM adenine 5'-monophosphate (AMP) and 1 mM inosine 5'-monophosphate (IMP), to provide excess unlabeled substrate for the endogenous phosphatases released when the cells are lysed. The cells were transferred to a chilled tube (16 × 100-mm, Pyrex) and disrupted by freezing and thawing (five times) in dry ice-acetone and room-temperature water baths, respectively. Tubes containing the disrupted cell suspensions were kept on ice. A uniform supension was maintained by frequent vortexing. Aliquots of each suspension were removed for subsequent protein determination (12). Aliquots were spotted onto each of several 2-inch squares of DEAE cellulose paper (Whatman DE-81, Whatman Chemicals, Div. W. & R. Balston, Maidstone, Kent, England). These papers were thoroughly dried at room temperature. One set of squares was washed in several changes of 2 mM sodium formate, once in distilled water, and twice in 95% ethanol to remove nonphosphorylated compounds (13). The second set of squares was not washed. All squares were dried and counted in a toluene-based liquid scintillation fluid. The unwashed DEAE squares provided a measurement of total isotope accumulation. Correction for the small amount of extracellular isotope not removed from the monolayer during the rinsing with Hanks' solution was determined for each batch of labeled medium by incubating each of two flasks for 30 s at 4°C and then assaying as usual. The effectiveness of the product isolation was checked at frequent intervals by including in the wash DEAE squares spotted with a reference solution containing either [8-^{14}C]hypoxanthine or [8-^{14}C]AMP.

Apparent adenine phosphoribosyltransferase (APRT) activity. The assay conditions were as described for the HPRT intact cell assay except that [8-^{14}C]adenine (15 mCi/mmol; 0.5 mM) was substituted for hypoxanthine.

Enzyme assays in fibroblast extracts

Fibroblasts growing in monolayer in 1-liter rectangular Blake bottles were fed every 5 days and on the day before harvesting with Waymouth medium supplemented with antibiotics and fetal calf serum as described above. Cells, harvested from intact, confluent monolayers by trypsinization, were washed three times with ice-cold phosphate-buffered saline (pH 7.4), resuspended in 0.3 ml of 0.01 M potassium phosphate (pH 7.0), and disrupted by rapid freezing and thawing. Debris was removed by centrifugation at 1,000 *g* for 15 min at 4°C. The supernate was removed to a chilled tube. Both HPRT and APRT enzyme assays were performed simultaneously on this freshly prepared cell extract.

The method used was modified from Atkinson and Murray (14) and Rubin et al. (15). For the HPRT assays, incubation mixtures of 185 μl contained 54 mM Tris buffer, pH 8, 5.4 mM $MgCl_2$, 0.27 mM PRPP, 55 μM [8-^{14}C]hypoxanthine (15 mCi/mmol), 1.22 mM AMP, and cell extract (50–75 μg protein). Reactions were initiated by adding the hypoxanthine and AMP to a solution containing the other components. After 20 min of incubation at 37°C, the reactions were terminated by rapid freezing in a dry ice-acetone bath. The samples were allowed to thaw in an ice-water bath. Aliquots (50 μl) of each reaction mixture were spotted on 1-inch squares of DEAE paper. The papers were air-dried at room temperature and then washed in several changes of 2 mM sodium formate, once in distilled water, and twice in 95% ethanol to remove nonphosphorylated compounds. The papers were then dried and counted in a toluene-based liquid scintillation fluid.

The assay conditions for APRT were as described for HPRT except that 110 μM [8-^{14}C]adenine (15 mCi/mmol) replaced hypoxanthine and 1.22 mM IMP replaced AMP in the incubation mixtures.

Chemicals

[8-^{14}C]Adenine (60 mCi/mmol) was obtained from New England Nuclear, Boston, Mass. [8-^{14}C]Hypoxanthine was prepared from [8-^{14}C]adenine by the method of Rubin et al. (15) and purified by ion-exchange chromatography on Dowex 50 (Dow Chemical Co., Midland, Mich.) AMP, IMP, and PRPP were purchased from Sigma Chemical Co., St. Louis, Mo. Cell culture media and fetal calf serum were obtained from Grand Island Biological Co., Grand Island, N. Y. All other reagents were of the highest purity obtainable from commercial sources.

RESULTS

In late log phase cultures of fibroblasts with normal HPRT, the rate of conversion of hypoxanthine to phosphorylated products remained constant for at least 2 h (Fig. 1). Apparent HPRT activities determined in intact fibroblasts with normal and mutant HRPT are shown in Table II. Intact fibroblasts from three patients with LN syndrome had no detectable activity. The apparent HPRT activities in the intact fibroblasts from the other four patients ranged from severe deficiency (Ill.) to virtually normal (HoRo.).

The rate of conversion of adenine to phosphorylated products by intact fibroblasts with normal HPRT ranged

TABLE II

The Conversion of Hypoxanthine to Phosphorylated Products by Fibroblast Extracts and by Intact Fibroblasts from Patients with X-Linked Hyperuricemia

Fibroblast strain	HPRT activity* fibroblast extracts	Apparent HPRT activity‡ in intact fibroblasts	
		Range	Mean±SD
	nmol product/mg protein/h		
Controls	99±22	3.8–6.6	5.0±0.72
Partial HPRT deficiency			
HoRo.	19±2	3.6–5.2	4.42±0.54
C. M.	119±7	1.8–2.8	2.16±0.38
Bou.	18±1	1.2–1.6	1.40±0.10
Ill.	7±3	0.2–0.4	0.28±0.12
LN syndrome			
Bak.	<1	<0.01	<0.01
Sep.	<1	<0.01	<0.01
Sch.	<1	<0.01	<0.01

Data are given as mean±SD. Control values were determined by duplicate assays performed on extracts prepared from four different normal fibroblast strains on each of two occasions.

‡ Apparent HPRT activity in intact fibroblasts in log phase monolayer cultures (20–30 μg total cell protein/cm² of growing surface). Range for at least six determinations for each HPRT-deficient fibroblast strain and for four determinations on each of six fibroblast strains with normal HPRT.

from 6.8 to 10.4 nmol/mg cell protein per h with a mean of 8.6, and was constant for at least 2 h. Apparent APRT activities in intact fibroblasts from the seven patients with mutant HPRT were normal (data not shown).

HPRT activity in extracts from control fibroblast cultures ranged from 81 to 127 nmol IMP/mg protein per h with a mean of 99. APRT activity in these same extracts ranged from 116 to 176 nmol AMP/mg protein per h with a mean of 158. HPRT activities in fibroblast extracts from the patients with X-linked hyperuricemia are presented in Table II. APRT activities in these same extracts were normal (data not shown).

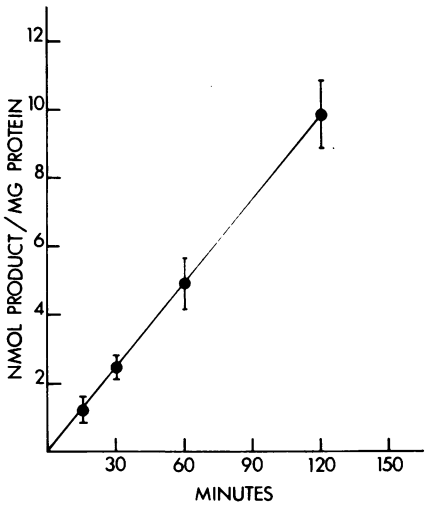

FIGURE 1 The synthesis of IMP from hypoxanthine in late log phase cultures of human diploid fibroblasts with normal HPRT. Each point represents the mean±SD for duplicate determinations on each of four normal fibroblast strains.

DISCUSSION

HPRT activity is usually determined in lysates of erythrocytes. This has served as an effective screening procedure for LN disease. However, as shown in the present study, the results of such assays may be misleading. For example, erythrocytes from HoRo. had no detectable HPRT activity, a finding believed to be diagnostic for LN disease, but this patient showed only moderate uricosuria and hyperuricemia (Table I) and none of the neurological symptoms associated with LN disease (8). C. M. had normal HPRT activity in red cell lysates but had marked uricosuria and some neurological dysfunction (9). Thus, a normal HPRT assay does not exclude the possibility of deficient HRPT activity in vivo, nor is the residual activity predictive of the degree of impairment of purine salvage in patients with partial deficiences.

One of the factors leading to the discrepancy between red cell HPRT assays and phenotype is readily apparent. Erythrocytes are anucleate cells, in which enzyme replacement or synthesis is reduced or absent. Hence, enzyme levels determined in erythrocytes are particularly susceptible to alterations in the rate of enzyme degradation. The absence of HPRT activity in HoRo.'s erythrocytes probably reflects extreme instability of his mutant enzyme, since in his leukocytes, nucleated cells in which enzyme synthesis continues, enzyme activity was readily demonstrable (8). Conversely, higher than normal enzyme activity in red cell assays may reflect a decreased rate of degradation for the enzyme under study. Elevated APRT activity, about twice the normal level, has been a consistent finding in the red cells of LN patients (2). The rate of synthesis of the enzyme is normal, but the half-life is prolonged due to stabilization, presumably by increased PRPP concentration (15).

Enzyme levels determined in nucleated cells are probably more representative of enzyme function in the total organism. However, enzyme assays carried out on disrupted nucleated cells also failed in two of the patients to correlate satisfactorily with the deficiency of purine salvage. Extracts of fibroblasts from HoRo., a patient with minor clinical evidence of HPRT deficiency, had only 20% of normal activity. In contrast, C. M., a patient with massive uricosuria and neurologic abnormality, had normal HPRT activity in fibroblast extracts assayed in the usual way. Measurements of purine salvage in intact fibroblasts provided a more satisfactory correlation. Under these conditions, HoRo.

had 87% of normal activity and C. M. had only 42% of normal.

Explanations can be offered based upon classical enzyme kinetics for the different results obtained with intact cells and with cell lysates. C. M.'s mutant enzyme has a low affinity for PRPP (9). Hence, this mutant enzyme has less than normal activity at the low intracellular PRPP concentration in the intact fibroblast. This decreased affinity for PRPP is not evident in the assay of fibroblast extracts, performed at high PRPP concentration. The explanation for the performance of HoRo.'s mutant HPRT may lie in the limitations placed on the activity of the normal HPRT by the low intracellular concentration of PRPP, with an effect opposite to that observed in C. M. The intracellular concentration of PRPP is about 10 μM (16). The PRPP K_m for normal HPRT is approximately 200 μM (17). Thus, the intracellular concentration of PRPP is substantially below the K_m for the enzyme and is rate-limiting. Under these conditions, the mutant enzyme of HoRo. almost achieves the limited performance of the normal enzyme. The assay of fibroblast extracts with excess PRPP demonstrates the potential superiority of the normal enzyme.

Enzyme assays are usually performed in the presence of saturating amounts of substrates and an excess of cofactors at optimal pH. These methods were developed by enzymologists to characterize the enzyme and do not necessarily reflect in vivo function where the multiplicity of coincident reactions may modify the effectiveness of the enzyme under study. A more accurate appraisal of enzyme function at the cellular level is achieved by measuring the conversion of substrate to product in the intact cell at physiological concentrations of substrates, activators, and product and metabolite inhibitors, and in a physiological ionic environment. A better correlation with purine salvage in the patient can be reasonably expected.

It should not be expected, however, that all manifestations in the individual attributable to the enzyme defect will be consistently and accurately predicted by results obtained with skin fibroblasts in tissue culture. Although the amount of uric acid excreted in the urine is clearly influenced by the degree of impairment of purine salvage, it is also affected by a number of other factors (18). For example, the relatively small amount of uric acid appearing in the urine of Ill. (Table I), despite the enzyme defect, may be related to his severe renal disease. Gout appears to be dependent on elevated serum urate, but there are many other contributory factors, some less well-defined (18). Neurological symptoms are regularly seen in LN disease, indicating a close relation to the virtual absence of enzyme activity (19). Neurological symptoms have been only sporadically reported in variants of HPRT deficiency, making their relation to the partial deficiency less certain and less predictable (18, 19). In spite of these limitations, measurement of function at the cellular level may provide interesting clues to in vivo function, as exemplified by the present series, and it may serve as a useful approach to the study of inherited metabolic disease.

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