Genotype-Phenotype Correlations in Lesch-Nyhan Disease

MOVING BEYOND THE GENE*

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Background: Mutations in the HPRT1 gene cause a spectrum of clinical phenotypes known as Lesch-Nyhan disease and its variants.

Results: The associated mutant enzymes demonstrate several different abnormalities in their kinetic properties.

Conclusion: Residual enzyme activity correlates with overall disease severity.

Significance: These studies provide a model for understanding general principles for genotype-phenotype correlations in human diseases.

Lesch-Nyhan disease and its attenuated variants are caused by mutations in the HPRT1 gene, which encodes the purine recycling enzyme hypoxanthine-guanine phosphoribosyltransferase. The mutations are heterogeneous, with more than 400 different mutations already documented. Prior efforts to correlate variations in the clinical phenotype with different mutations have suggested that milder phenotypes typically are associated with mutants that permit some residual enzyme function, whereas the most severe phenotype is associated with null mutants. However, multiple exceptions to this concept have been reported. In the current studies 44 HPRT1 mutations associated with a wide spectrum of clinical phenotypes were reconstructed by site-directed mutagenesis, the mutant enzymes were expressed in vitro and purified, and their kinetic properties were examined toward their substrates hypoxanthine, guanine, and phosphoribosylpyrophosphate. The results provide strong evidence for a correlation between disease severity and residual catalytic activity of the enzyme (k_cat) toward each of its substrates as well as several mechanisms that result in exceptions to this correlation. There was no correlation between disease severity and the affinity of the enzyme for its substrates (K_m). These studies provide a valuable model for understanding general principles of genotype-phenotype correlations in human disease, as the mechanisms involved are applicable to many other disorders.

In human genetics it is frequent that a specific gene defect is linked with a specific clinical phenotype, and later the same gene is linked with strikingly different phenotypes. The phenotypic heterogeneity leads to questions regarding the significance of the gene defects for the phenotypes and the mechanisms by which defects in a single gene might produce different clinical outcomes. Understanding genotype-phenotype correlations can provide important information about prognosis as well as clues to the pathogenesis of the clinical manifestations.

Elucidating these correlations, therefore, is a fundamental goal in human genetics.

Lesch-Nyhan disease (LND)2 historically has served as a model for understanding genotype-phenotype correlations. It is caused by mutations of the HPRT1 gene, which encodes the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGprt) (1, 2). LND was the first neurogenetic disorder to have its gene defect identified (3), and the HPRT1 gene is among the most intensively studied loci in human genetics (4). The classical clinical phenotype includes uric acid overproduction, motor dysfunction, cognitive disability, and self-injurious behavior (5). There also are attenuated variants where some features of the disease are missing or unusually mild (6). Three main phenotypic subgroups are recognized. The most severe subgroup is LND, where the complete classical syndrome occurs. The least affected subgroup is HGprt-related hyperuricemia (HRH), where patients exhibit overproduction of uric acid only. In HRH the neurobehavioral features of the phenotype are absent or sufficiently mild that they have no clinical significance. An intermediate subgroup is HGprt-related neurological dysfunction (HND), where patients exhibit overproduction of uric acid along with varying degrees of neurological impairments. However, HND patients do not have the self-injurious behaviors that are invariably seen in the classic LND syndrome.

The HPRT1 gene is ~45 kb in length on the X chromosome in the region Xq26–27, and it contains 9 exons and 8 introns (7–11). The mature mRNA is ~1.6 kb, in which a protein-encoding region containing 654 bp is translated into an enzyme of 218 amino acids with a predicted molecular mass of 24,579 Da. The active enzyme is thought to function as a dimer or tetramer (12). The mutations leading to clinical disease have a high degree of heterogeneity. More than 400 currently are known. Missense mutations, nonsense mutations, deletions, insertions, duplications, and translocations have been reported spanning the whole gene (1, 2). HGprt is an enzyme that plays a key role in

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2 The abbreviations used are: LND, Lesch-Nyhan disease; Hprt, hypoxanthine phosphoribosyltransferase; Gprt, guanine phosphoribosyltransferase; HGprt, hypoxanthine-guanine phosphoribosyltransferase; HND, HGprt-related neurological dysfunction; HRH, HGprt-related hyperuricemia; PRPP, phosphoribosylpyrophosphate.
the purine salvage pathway. The enzyme has two distinct functions; it catalyzes the conversion of hypoxanthine into IMP (Hp rt) and guanine into GMP (Gprt). Enzymatic studies with cultured fibroblasts have suggested that the degree of HGprt deficiency correlates with clinical severity (13, 14). Patients with LND generally have less than 1.5% of residual enzyme activity, those with HND have 1.5–8% residual activity, and those with HRH have more than 8% activity. However, methods for testing HGprt enzyme function are not standardized across laboratories, and several discrepancies between residual enzyme activities and clinical phenotypes have been reported using alternative assays (15–18).

Although it is difficult to compare results for different patients across laboratories that use different assays, the discrepancies have questioned the validity of the correlation between enzyme activity and clinical phenotype and raised the possibility that other factors may play a role in the expression of the clinical phenotype (2). Another uncertainty in the relationship between the enzyme and clinical phenotype involves the dual functions of HGprt. This uncertainty exists because a few studies have suggested the possibility of significantly skewed loss of activity toward one or the other substrate (13, 14, 19), so it remains unclear whether the clinical phenotype results from reduced enzymatic activity toward hypoxanthine, guanine, or both. These observations suggest that clinical correlations may exist for one or both functions of the HGprt enzyme.

In the current study, a novel approach was developed to address the limitations of prior attempts to link residual enzyme function to clinical severity. Briefly, 44 HPRT1 mutations associated with a wide spectrum of clinical phenotypes were reconstructed by site-directed mutagenesis, the mutant enzymes were expressed in vitro and purified, and their kinetic properties were examined both toward hypoxanthine and guanine as well as the co-substrate PRPP. By testing the enzymes under standardized conditions, direct comparisons of their kinetic properties and relationships to known clinical phenotypes could be made without the need for fresh blood or fibroblast cultures from patients required for other assays. The results provide strong evidence for a correlation between residual enzyme activity and disease severity as well as some mechanistic explanations for why some exceptions may occur. Because these mechanisms are relevant to many genetic diseases, these studies provide a valuable model for understanding general principles for genotype-phenotype correlations in human disease.

**EXPERIMENTAL PROCEDURES**

**Expression Vector**—The construction of the cDNA clone encoding full-length human HGprt has been reported previously (20). PCR primers were designed to add the MGHHHH-HHQGGCCPGGCGG sequence to the N terminus, in which a tag containing six histidine residues facilitated protein purification. Sequences encoding the peptide CCPGCC also were added as a fluorescent signal. The cDNA was then subcloned into the pET24d(+) vector (Novagen, New Canaan CT) to create the parent vector (pET24d(+)/HGprt) for protein expression. Mutations were introduced into the parent vector by site-directed mutagenesis with the PCR-based QuikChange kit from Stratagene (La Jolla, CA), with primers designed via the QuikChange Primer Design Program. All coding sequences were confirmed before protein expression.

**Protein Expression and Purification**— Cultures of transformed *Escherichia coli* were started by streaking frozen glycerol stock cells into an agar plate containing 50 μg/ml kanamycin. The plate was incubated at 37 °C overnight. A single kanamycin-resistant colony was picked and inoculated in 20 ml of Luria-Bertani (LB) medium containing 50 μg/ml kanamycin at 37 °C until the absorbance at 600 nm reached 0.6. This starter culture was then inoculated into 500 ml of LB media containing 50 μg/ml kanamycin with an initial absorbance of 0.002 at 600 nm. The bacteria were allowed to grow until the absorbance reached 0.6. Next, isopropyl-β-d-thiogalactopyranoside was added to a final concentration of 0.5 mM after which the culture was allowed to proceed for an additional 5 h at 37 °C with agitation at 220 rpm. The bacteria were then harvested by centrifugation at 8000 × g for 15 min at 4 °C and stored at −80 °C.

Bacteria were frozen, then resuspended in 25 ml of 50 mM Tris buffer, pH 7.4, containing 5% glycerol, 300 mM NaCl, and 10 mM β-mercaptoethanol. One tablet of EDTA-free protease inhibitors was added (Complete Mini, EDTA-free, Roche Applied Science), and the bacteria were sonicated on ice. Insoluble debris were removed by centrifugation at 27,000 × g for 30 min at 4 °C. The supernatant was then applied to nickel-nitrotetraacetic acid affinity columns (Qiagen, Hilden, Germany) equilibrated with 50 mM Tris buffer, pH 7.4, 300 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, and 5% glycerol. The column was washed with 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5% glycerol, 20 mM imidazole, 10 mM β-mercaptoethanol, and stepwise increasing concentrations of 75 mM and 100 mM imidazole to eliminate nonspecific binding. Purified protein was eluted with 250 mM imidazole in buffer. The fractions containing HGprt were pooled and exchanged into 50 mM Tris, pH 7.4, containing 3 mM MgCl2, 5 mM DTT, and 5% glycerol using PD-10 desalting columns (GE Healthcare). The eluted protein was pooled and concentrated using Amicon Ultra centrifugal filter tubes with a 10,000-Da molecular mass cut-off (Millipore, Billerica MA). Concentrated protein was frozen rapidly in liquid nitrogen and stored at −80 °C. Protein purity was determined by SDS-PAGE followed by staining with Coo massie Blue. Protein quantification was carried out using the Bradford method with bovine serum albumin as a standard.

**Steady-state Kinetic Analysis**—The steady-state kinetics of normal and mutant HGprt were measured by monitoring the rate of the production of IMP or GMP in triplicate in 96-well UV compatible microplates with a SpectraMax M5e spectrophotometer (Molecular Devices, Sunnyvale CA). Apparent *Km* and *kcat* values for the purine bases were determined by measuring initial velocities with the concentration of PRPP fixed at 1 mM and varying concentrations of hypoxanthine or guanine from 2 to 200 μM. Apparent *Km* and *kcat* values for PRPP were determined with the concentration of either hypoxanthine or guanine fixed at 200 μM and varying concentration of PRPP from 5 to 1000 μM. The reactions were initiated by adding HGprt protein at 37 °C in a 200-μl reaction volume containing 100 mM Tris-HCl and 12 mM MgCl2 at pH 7.4. The amount of protein typically was 100–200 ng for each reaction, although
amounts up to 4000 ng were required for some proteins with very low activity. The production of IMP from hypoxanthine or GMP from guanine was measured at 245 and 257 nm, respectively. The change in extinction coefficient used for hypoxanthine to IMP was 1770 M⁻¹ cm⁻¹ and for guanine to GMP was 5146 M⁻¹ cm⁻¹. The Michaelis-Menten \( K_m \) and \( k_{cat} \) were calculated with SigmaPlot (Systat Software Inc., San Jose CA) by nonlinear regression of initial velocities at each substrate concentration.

**Enzyme Stability Assay**—The stabilities of normal HGprt and its mutants were compared by measuring residual activity after incubation at 37 °C over 84 h. An aliquot of the protein at a concentration of 20 μg/ml was heated to the target temperature in 50 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, and 100 mM NaCl. The maximal enzyme activities then were tested at specific time points in triplicate with 1 mM PRPP and 200 μM purine base in 100 mM Tris-HCl, pH 7.4, with 12 mM MgCl₂.

**Data Analysis**—To determine kinetic parameters, each enzyme was studied in triplicate with each substrate concentration. The enzyme activity assay was verified by three independent assays on normal HGprt. Steady-state apparent kinetic parameters \( k_{cat} \) and \( K_m \) were evaluated by applying initial velocity data using a substrate concentration that maximized the enzyme activities then were tested at specific time points in triplicate with 1 mM PRPP and 200 μM purine base in 100 mM Tris-HCl, pH 7.4, with 12 mM MgCl₂.

The correlation coefficient value \( R \) was always greater than 0.95 for each assay (Fig. 1).

To compare kinetic parameters of the enzyme with the clinical phenotype, patients were subgrouped as LND \( (n = 15) \), HND \( (n = 15) \), or HRH \( (n = 8) \). When the same mutation was reported for multiple phenotypes, the mutation was assigned to the most frequently reported phenotype. Because data were not normally distributed, they are presented as box-whisker plots. The Kruskal-Wallis test for non-parametric measures was applied to compare the kinetic parameters of the subgroups, with \( p < 0.05 \) as the criterion for statistical significance. The correlation between Hprt and Gprt activities was performed by linear regression using SigmaPlot (Systat Software, Inc. San Jose, CA).

**RESULTS**

**Selection of Mutants for Evaluation**—Among more than 400 mutations reported, 44 representative mutations were selected for evaluation. The selection criteria involved three main factors. Because the goal of these studies was to correlate residual enzyme function with clinical phenotype, the first criterion involved selecting genetic mutations that theoretically might yield mutant enzymes with measurable residual activity. We excluded mutations predicted to cause a null enzyme for which informative data could not be obtained such as nonsense mutations, deletions, insertions, and other frame-shifting mutations. Splice site mutations also were excluded because the amount of residual HGprt function depends more on variations in the fidelity of splicing than on the mutant enzyme itself. Most mutations, therefore, were single base substitutions leading to single amino acid changes, except for one that involved a double point mutation leading to two amino acid changes.

The second criterion involved the certainty of the associated clinical phenotype. We focused on mutations from patients who were clinically evaluated directly by our group \( (n = 30) \) or by other members of the International Lesch-Nyhan Disease Study Group with standardized protocols \( (n = 14) \). We also aimed to include mutations where multiple patients were reported with a consistent phenotype independently from different groups whether or not they had been evaluated clinically by us. There were eight mutations with multiple independent reports including R48H \( (n = 9) \), G70E \( (n = 7) \), F74L \( (n = 6) \), R45K \( (n = 4) \), Y72C \( (n = 4) \), L68P \( (n = 3) \), G71V \( (n = 3) \), and H204D \( (n = 3) \).

The last criterion involved predictions from x-ray crystallography and studies of protein structure. Although early studies suggested clinically relevant mutations clustered around the active site of the enzyme, subsequent studies revealed many mutations far from the active site, such as the dimer interface (12). Several mutations at the active site and others far from the active site were, therefore, selected. Mutants affecting the active site included 12 predicted to involve substrate binding including L68P, G70E, G71V, G71D, S104R, D135V, T139C, G140D, V188A, D194E, D194N, and R200T. Residues LKGG from 68–71 are conserved across the phosphoribosyltransferase family of proteins and involved in PRPP binding (21, 22). Other substrate binding sites were determined from the human HGprt crystal structure in complex with transition-state analog inhibitor (23). From the total of 44 selected mutants, 6 could not be expressed for technical reasons as described below, leaving a total of 38 mutants for full biochemical evaluation. Table 1 summarizes the 38 mutations evaluated, the associated clinical phenotype, and the location of the mutation with respect to the structure of HGprt.

**Expression of Recombinant HGprt**—Recombinant HGprt proteins were purified to near homogeneity with an apparent molecular mass of ~26 kDa. For 36 of the 44 mutants selected, the standard methods provided large quantities of purified proteins sufficient for evaluation. Eight mutants could not be produced even after multiple attempts: I42F, I42T, R45K, A50P, L65P, F74C, R45K, and A161E. Sufficient material for evaluation could be produced for two of these mutants (I42T and R45K) by scaling up the production volumes 10-fold. The reasons for poor production of the remaining six could not be determined but may reflect poor expression levels, poor protein stability or solubility, or incompatibility with the purification protocol.

**Enzyme Kinetics for Normal HGprt**—The kinetic properties of the normal human HGprt enzyme were determined on three independent runs with separate assays for each substrate. With hypoxanthine as substrate, the apparent \( k_{cat} \) of Hpirt was 10.1 s⁻¹ with \( K_m \) of 7.7 μM for hypoxanthine and 26 μM for PRPP. With guanine as the substrate, the \( k_{cat} \) of Gprt was 15.2 s⁻¹ with \( K_m \) of 5.6 μM for guanine and 56 μM for PRPP (Fig. 1). These values are similar to those previously published for the native enzyme purified from human tissues or as a recombinant protein (Table 2). These results indicate that the addition of the small N-terminal peptide to the enzyme for purification did not significantly alter enzyme kinetics.
Enzyme Kinetics of Mutant HGprt toward Hypoxanthine—The apparent steady-state kinetic parameters for the mutant enzymes are shown in Table 3, and the distributions of residual activities are shown as box-whisker plots according to clinical subgroup in Fig. 2. Five active site mutations (G70E, G71D, T139C, G140D, and D194N) exhibited no activity, so the corresponding kinetic parameters could not be determined. For the remaining mutants, the apparent $k_{cat}$ for the Hprt reaction with hypoxanthine as the varied substrate ranged from null to 13.2 s$^{-1}$. Kruskal-Wallis one-way ANOVA revealed significant differences among the clinical subgroups ($p < 0.005$). The median residual Hprt activities were 13% for LND, 54% for HND, and 87% for HRH. When PRPP was the varied substrate, the $k_{cat}$ ranged from null to 15.7 s$^{-1}$. There again were significant differences among the clinical groups ($p < 0.003$). The median residual Hprt activities were 6% for LND, 55% for HND, and 67% for HRH. These results suggest a correlation between clinical severity and residual $k_{cat}$ for Hprt when using either hypoxanthine or PRPP as the varied substrate, although there was considerable overlap among the clinical subgroups.

The apparent $K_m$ values for either hypoxanthine or PRPP for Hprt were obtained using either variable hypoxanthine with fixed PRPP at 1 mM or variable PRPP with fixed hypoxanthine at 200 $\mu$M (Fig. 2 and Table 3). The $K_m$ for hypoxanthine ranged from 6 to 285 $\mu$M, whereas the $K_m$ for PRPP varied from 17 $\mu$M to greater than 1 mM. There were no significant differences among the clinical subgroups for the $K_m$ for either hypoxanthine or PRPP. Most mutants had only slightly reduced the affinity toward hypoxanthine; 31 of 38 mutants had less than 10-fold increases in $K_m$ values. Two mutants showed $K_m$ increases of 32-fold (I136K) and 37-fold (Y195S). The $K_m$ values of PRPP displayed greater variability, with 0.7–215-fold differences from normal. Eight of 38 mutants had at least 10-fold increases in the $K_m$ for PRPP (L68P, G71V, Y72C, D194E, Y195S, R200T, D201N, D201Y). All of the amino acids involved for these mutants were located at the enzyme site for PRPP recognition or binding. These results suggest that $K_m$ values do not correlate with clinical severity but that mutations close to the active site have significant effects on $K_m$ values, especially for PRPP.

Enzyme Kinetics of Mutant HGprt toward Guanine—Apparent $k_{cat}$ values for the mutant HGprt enzymes ranged from null to 22.2 s$^{-1}$ with guanine as the varied substrate (Table 3). There were significant differences among the clinical subgroups for the $k_{cat}$ with guanine as the varied substrate.
groups ($p < 0.005$), with median residual activities of 4% for LND, 39% for HND, and 95% for HRH (Fig. 2). The $k_{cat}$ values ranged from null to 21.8 s$^{-1}$ with PRPP as the varied substrate, again with significant differences among the clinical subgroups ($p < 0.003$). Median residual enzyme activities were 8% for LND, 61% for HND, and 89% for HRH. Like the results for $\text{Hprt}$ toward hypoxanthine, these results suggest a correlation between clinical severity and residual $k_{cat}$ for $\text{Gprt}$ when using either guanine or PRPP as the varied substrate but again with considerable overlap among the clinical subgroups.

**Apparent $K_m$ constants for guanine** ranged from 3.6 to 153 $\mu$M, whereas $K_m$ values for PRPP in the $\text{Gprt}$ reaction varied from 26 $\mu$M to greater than 1 mM. There were no significant differences among the clinical subgroups for either guanine or PRPP as the varied substrate. The affinity toward guanine was only slightly decreased for most mutants with only two mutants having relatively large increases in $K_m$: 13-fold for I136T and 27-fold for I136K. On the other hand, 9 of 38 mutants exhibited greater than 10-fold increases of $K_m$ values for PRPP (E47G, L68P, G71V, Y72C, D194E, Y195S, R200T, D201N, D201Y). Except E47G, all of these were close to the enzyme active site for PRPP recognition or binding. Similar to that observed for the $\text{Hprt}$ reaction, these results suggest that substrate affinity does not correlate with a clinical subgroup. However, a critical role of the enzyme active site in substrate affinity was again evident for the $\text{Gprt}$ reaction, especially toward PRPP.

**Correlation between $\text{Hprt}$ and $\text{Gprt}$ Reactions**—Most mutants had parallel reductions in $k_{cat}$ values toward hypoxanthine and guanine with a correlation coefficient of $R = 0.74$ with PRPP as a fixed substrate. A similar correlation of $\text{Hprt}$ and $\text{Gprt}$ with $R = 0.71$ was observed when the purine was the fixed substrate. Mutants associated with the severe clinical phenotype of LND generally had both low $\text{Hprt}$ and $\text{Gprt}$ activities, whereas mutations associated with HRH or HND had relatively higher $\text{Hprt}$ and $\text{Gprt}$ activities. However, some mutants showed a more severe loss of activity toward only one purine substrate (Table 3 and Fig. 3, A and B).

**TABLE 2**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ $\mu$M</th>
<th>PRPP $\mu$M</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ $\mu$M</th>
<th>PRPP $\mu$M</th>
<th>Source</th>
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</thead>
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<td>Recombinant</td>
<td>10.1 ± 0.5</td>
<td>7.7 ± 1.5</td>
<td>25.7 ± 5.2</td>
<td>15.2 ± 0.4</td>
<td>5.6 ± 0.7</td>
<td>56.4 ± 5.2</td>
<td>This study</td>
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<tr>
<td>Recombinant</td>
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<td>2.4 ± 0.6</td>
<td>35 ± 6</td>
<td>18</td>
<td>3.5 ± 1.2</td>
<td>65 ± 6</td>
<td>(71)</td>
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<tr>
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<td>1.0</td>
<td>32.9</td>
<td>10.5</td>
<td>4.5</td>
<td>73.1</td>
<td>(72)</td>
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<tr>
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<td>11.8 ± 1</td>
<td>13.8</td>
<td>1.9 ± 0.4</td>
<td>8.3 ± 0.7</td>
<td>(73)</td>
</tr>
<tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>(74)</td>
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<tr>
<td>Erythrocytes</td>
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<td>7.7 ± 0.4</td>
<td>66 ± 2</td>
<td>ND</td>
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<td>(75)</td>
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**FIGURE 1.** Michaelis-Menten plots for human HGprt. Each data point was determined in triplicate, and the symbols reflect average ± S.D. Panels A and C show the $\text{Hprt}$ reaction, whereas panels B and D show the $\text{Gprt}$ reaction. Panels A and B were plotted at varied purine concentration with PRPP fixed at 1 mM, whereas panels C and D were plotted at varied PRPP concentration with fixed purine at 200 $\mu$M.
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To more readily identify mutants with relative loss of activity toward only one purine substrate, the \( k_{\text{cat}} \) ratios for Hprt to Gprt were examined. The normal enzyme had a ratio of 0.66, indicating higher catalytic activity toward guanine in comparison with hypoxanthine. The HGprt mutants had ratios ranging from 0.2 to 137, but no statistically significant differences were noted among the clinical subgroups (\( p = 0.5 \)). A few mutants revealed significant skewing toward one or the other reaction. For example, the G71V mutant had 42% residual activity toward hypoxanthine but null activity toward guanine. Similarly, the D194E mutant had 69% residual Hprt activity but only 6% residual Gprt activity. Conversely, the V133M mutant had 7% residual Hprt activity with 22% residual Gprt activity. All three mutants were associated with the intermediate phenotype of HND.

Most mutants also had roughly parallel changes in \( K_m \) values toward hypoxanthine and guanine (Table 3 and Fig. 3C). The correlation coefficient for \( K_m \) values toward hypoxanthine and guanine were \( R = 0.76 \). To identify mutants with relative loss of affinity toward only one purine substrate, the \( K_m \) ratios for hypoxanthine to guanine were examined. The normal enzyme has a ratio with 1.4, indicating a relative preference toward guanine in comparison with hypoxanthine. The HGprt mutants had ratios ranging from 0.6 to 10.9, but no statistically significant differences were noted when these ratios were examined according to clinical subgroups (\( p = 0.215 \)).

The affinities toward PRPP when either hypoxanthine or guanine as substrate were less strongly correlated, with \( R = 0.53 \) (Fig. 3D). The \( K_m \) ratio for PRPP between the Hprt and Gprt reaction for the normal enzyme was 0.5, and the mutants had ratios ranging from 0.1 to 4.9 without significant differences among the clinical subgroups (\( p = 0.80 \)).

To determine if total purine recycling capacity, regardless of purine substrate, might show a better correlation with the clinical phenotype, we calculated the combined Hprt + Gprt activities of each mutant in relation to the normal enzyme (Fig. 2). There were significant differences among the clinical subgroups (\( p = 0.003 \)). The median combined residual enzyme activities were 8% for LND, 53% for HND, and 93% for HRH. The correlation between total enzyme activity and clinical phenotypes also was observed using PRPP as the varied substrate (\( p < 0.001 \)). The median combined residual enzyme activities were 7% for LND, 49% for HND, and 85% for HRH. These results show that total purine recycling correlates with clinical severity, but examining total recycling does not eliminate the overlapping values among the patient subgroups.

\textit{Stability of Native and Mutant HGprt—}The kinetic studies revealed mutant enzymes from some of the severely affected
LND patients to have high residual activities, including some with activity higher than native HGprt (Table 3). This observation is not consistent with the concept that clinical phenotype results from significantly reduced HGprt activity, suggesting that some additional factors may affect actual enzyme activities under more natural conditions in vivo. Isolated reports have suggested that protein stability may vary among different HGprt mutants (20, 24–27) so the stability of each of the mutant enzymes with at least 10% residual enzyme activity was compared with the native enzyme after extended preincubations at 37 °C.

Normal HGprt was quite stable, retaining more than 80% of its original activity after 72 h at 37 °C (Fig. 4). The mutants showed variable changes in enzyme stability. All of the mutants associated with the severe clinical phenotype of LND but with relatively high $k_{cat}$ values appeared extremely unstable, with no detectable activity at 72 h (F74L, H204D, and V189L). Two other mutants associated with the LND phenotype (E47G and D44G) had intermediate stability, retaining less than 40% activity after 72 h.

The mutants associated with the intermediate HND phenotype showed a broad spectrum of stabilities. Some had stabilities comparable with normal (I42T, V133M, V188A, R167T, L65F, and R200T), whereas others ranged from 50% to null activity after 72 h (R48H, Y195C, D80V, M43R/D44N, F199C, D194E, and G71V). For the mildest clinical phenotype of HRH, six of eight mutants displayed stability similar to the normal enzyme (Y72C, T168I, I136T, A161S, R51Q, and S104R). The remaining two mutants had intermediate stability with 40% activity after 72 h (S110L and D31E). These results imply the varied stability of mutants might contribute to the final enzyme activity in vivo.

**DISCUSSION**

HGprt and its deficiency syndromes provide a valuable opportunity for exploring mechanisms responsible for genotype-phenotype relationships. The results of the current studies are important for three reasons. First, they demonstrate that a novel assay can be used to precisely explore the kinetic parameters of mutant forms of HGprt as they may relate to variations in the severity of the corresponding clinical phenotype. This new assay has some obvious advantages over prior assays but has some of its own limitations too. Second, these results demonstrate that there is a good correlation between residual catalytic activity of the HGprt enzyme and overall clinical severity, with several superimposed mechanisms that can explain apparent exceptions. These mechanisms include variations in protein stability, variations in protein expressibility, and/or changes in the affinity of the enzyme toward its substrates that may render it non-functional with concentrations of these substrates available in living cells. Finally, these results provide a method for determining which of the two functions of HGprt, hypoxanthine and guanine recycling, may be more relevant for the clinical manifestations of the disease.

**Methods for Exploring Genotype-Phenotype Correlations**—Prior studies based on measuring the incorporation of radiolabeled purine bases into fibroblasts (13, 14) or erythrocytes (28, 29) in live cultures have shown relatively good but imperfect correlations between residual HGprt activity and clinical severity. The main strength of these cell-based assays is that enzyme activity is measured in a setting likely to resemble the situation in vivo, with a normal cytosolic milieu and PRPP concentrations at physiologically relevant levels. However, these methods suffer a number of weaknesses too. The main weakness is that it is impossible to obtain precise enzyme kinetics with multiple
substrates at different concentrations in a living cell. These assays also are technically challenging because the fibroblast assay requires establishing cultures from a skin biopsy from patients, and live erythrocytes must be studied within 2 days of collection due to rapid deterioration of function. These limitations make it difficult to simultaneously compare samples from multiple patients, especially when they live in different countries or in different decades. The fibroblast assay also suffers uncertainties regarding normal variations in HGprt activity due to patient age, skin biopsy site, the age of the cultures, and other factors such as growth stage in culture and optimal culture conditions. The erythrocyte assays are known to be vulnerable to artificially low activities for enzymes with poor structural stability because they lack a nucleus with ongoing mRNA production and protein synthesis to compensate for protein loss. These technical issues have led many investigators to employ simpler assays based on cell lysates from frozen samples. Some of the results of these simpler assays have questioned the correlation between enzyme activity and clinical severity by providing examples of high residual activity with a severe clinical phenotype or null activity with a mild clinical phenotype (15–18).

The assay described here bypasses some of the limitations of the live fibroblast and erythrocyte assays while introducing other limitations. Engineering the enzymes and producing them in bacteria is feasible as HGprt is a cytosolic enzyme that does not undergo any post-translational modifications that significantly alter its enzymatic activity. This assay permits a very precise determination of the kinetic properties toward each relevant substrate: hypoxanthine, guanine, and PRPP. This assay also eliminates the technical challenges associated with obtaining skin biopsies or fresh blood from patients. It also is possible to obtain information on enzyme expression and stability by monitoring the production of the enzyme and the behavior of the enzyme over time. The main weakness of this assay is that in vitro conditions do not replicate the exact intracellular environment, leading to potential discrepancies between what the enzyme does in vitro and what it does in vivo. For example, a mutant HGprt enzyme with high apparent \( k_{\text{cat}} \) may have null activity in vivo if it also has a high substrate \( K_m \) that exceeds substrate concentrations available in vivo. These limitations can be overcome to some extent by knowledge of the typical concentrations of hypoxanthine and guanine available in vivo (16). However, the concentrations of PRPP in vivo appear to vary inversely with residual HGprt activity, making it difficult to estimate physiological PRPP levels for each mutant. The attempts to correlate residual enzyme activity with clinical severity below must be considered with the limitations of the enzyme assay in mind.

Genotype–Phenotype Correlations and Discordance—Prior studies comparing the type and location of mutations in the HPRT1 gene have shown that the majority of cases with milder phenotypes have missense mutations predicted to lead to substitution of single amino acids (1, 2). It is quite rare for these

![FIGURE 3. Correlation of Hprt and Gprt reactions. Panel A and B compares \( k_{\text{cat}} \) values for Hprt and Gprt, whereas panels C and D compares \( K_m \) values for purine and PRPP. The latter are shown in a log scale due to the large variation in absolute values. The normal enzyme is shown as a solid circle in each panel. The mutants are shown as open circles (LND), open squares (HND), or open triangles (HRH). The dotted line shows the predicted relation of normal Hprt to Gprt across different enzyme levels, and the solid line shows the empirically derived correlation of Hprt to Gprt by linear regression. Some mutants with skewed loss of activity toward one or the other reaction were evident (arrows). There were good correlations between \( k_{\text{cat}} \) values for Hprt and Gprt with fixed PRPP and for \( k_{\text{cat}} \) values for PRPP with fixed hypoxanthine or guanine. There also were correlations between \( K_m \) values with fixed PRPP or for PRPP with fixed hypoxanthine or guanine, although these correlations were driven largely by a small number of mutants with very high \( K_m \) values.](image-url)
milder variants to have nonsense mutations, deletions, duplications, insertions, or other complex mutations. The majority of patients with these mutations typically exhibit the severest clinical phenotype. These observations are consistent with the idea that clinical severity correlates with residual enzyme activity, because missense mutations are more likely to maintain overall protein structure and thereby retain some residual enzyme function. In contrast, the other mutations are more likely to seriously disrupt the expression of functional protein because of early truncation or a shift in the amino acid reading frame.

Despite the limitations of the new method described above, there appears to be a good correlation between overall clinical severity and residual catalytic activity ($k_{\text{cat}}$) of the HGprt mutants toward hypoxanthine, guanine, combined hypoxanthine + guanine, and PRPP. However there was substantial overlap among the three patient groups, indicating that $k_{\text{cat}}$ alone does not provide a precise predictor of the phenotype. There was no correlation between clinical severity and the affinity ($K_m$) of the HGprt mutants toward any substrate, but information regarding affinity was useful for identifying mutants requiring very high substrate concentrations. Mutants with significant decreases in substrate affinity are likely to be poorly functional or even non-functional with available substrate concentrations in vivo. These include mutants with greater than 10-fold increases in the $K_m$ values for hypoxanthine (I136K, Y195S), guanine (I136T, I136K), or PRPP (E47G, L68P, G71V, Y72C, D194E, Y195S, R200T, D201N, D201Y).

Enzyme stability also varied among the mutants but did not correlate with overall clinical severity. However, information regarding stability again was useful by permitting the identification of mutants with high apparent $k_{\text{cat}}$ values but likely to lose activity rapidly in vivo. Examples included H204D, V189L, F74L, D44G, and E47G. All of these mutants were associated with the severe LND phenotype even though some had high $k_{\text{cat}}$ values. Furthermore, an unstable mutant might cause varied clinical phenotypes in different patients, as in vivo stability could be influenced by age, health, and other variables. In fact, variable residual enzyme activity due to protein instability has been proposed to underlie the varied phenotypes associated with R48H, which has been associated with both HND and HRH (20).

Enzyme expressibility also varied among the mutants. Among the mutants that did not express well were four from the LND group, three from the HND group, and one from the HRH group. The reasons for poor expression could not be determined but may reflect poor expression levels, poor protein stability or solubility, or incompatibility with the purification protocol. This phenomenon suggests a third mechanism that may influence the final activity of mutant HGprt in vivo and might also lead to varied clinical phenotypes. Indeed, prior studies of individual cases have occasionally shown normal mRNA transcripts yet nearly undetectable protein in immunoblots from cells derived from patients, suggesting a mutant that either is poorly expressed or rapidly degraded (24–27). In these cases enzyme activity in vivo may be influenced by variations in total protein expression, protein degradation, or even chaperone proteins that aid in maintaining or repairing the structural integrity of HGprt.

DUAL FUNCTIONS OF HGPRT—Because HGprt recycles both hypoxanthine and guanine, clinical abnormalities may result from failure of recycling of one or both substrates. A prior study using the live fibroblast assay suggested that hypoxanthine recycling was more important because clinical severity correlated better with residual Hprr than Gprr (13, 14). On the other hand, another study based on mathematical modeling of the biochemical consequences of the enzyme deficiency suggested that guanine recycling was more important (19). In keeping with this alternative idea are theoretical issues regarding the relevance of guanine-related pathways for neural function (30).
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The current studies shed light on this controversy by providing a means to precisely and separately delineate the two reactions of HGprt. The relative importance of the two pathways is best illustrated by mutants with skewed loss of activity toward only one substrate. In theory, a mutant enzyme with null activity toward one substrate yet preserved activity toward the other substrate in association with severe disease would imply that the defective pathway is responsible for disease, whereas the preserved pathway is incapable of protecting against disease. Conversely, a mutant with similarly skewed activity toward one substrate in association with mild disease would imply that the defective pathway is not relevant for causing disease. Thus, focusing on mutants with extremely skewed activity toward hypoxanthine or guanine may point to the enzymatic function that is more relevant for causing disease.

In this regard the G71V mutant displayed high residual Hprt activity (42%) but no detectable Gprt activity in association with the intermediate HND phenotype. This observation may suggest that apparently complete loss of Gprt activity may not cause severe disease. The relatively high Hprt activity of the G71V mutant might also suggest that hypoxanthine recycling alone cannot protect against the manifestation of disease. However, this mutant was very unstable, making it likely that Hpirt activity in vivo may be lower and potentially responsible for the clinical manifestations. Although the G71V mutant does not provide unequivocal information on the relative importance of hypoxanthine or guanine recycling due to its instability, it suggests residual hypoxanthine recycling may be partly protective against disease manifestations. The D194E mutant also displayed skewed activity with 69% residual Hpirt activity but only 6% residual Gprt activity in association with HND. This observation might suggest that high residual Hpirt activity does not prevent disease. However, the D194E mutant showed significantly reduced affinity toward PRPP, requiring substrate levels not likely to be achieved in vivo. Thus this mutant also does not provide unequivocal information regarding the relative importance of hypoxanthine or guanine recycling. Conversely, the V133M mutant showed 7% residual Hpirt activity with 22% residual Gprt activity in association with the HND phenotype. Enzyme stability and substrate affinities were only modestly affected. This mutant, therefore, suggests that high residual Gprt does not protect against the development of disease manifestations, with the implication that hypoxanthine recycling is more relevant for causing disease.

Although the mutants with skewed activity provide suggestive evidence supporting hypoxanthine recycling as the clinically relevant biochemical function, the number of mutants examined that showed relatively skewed loss of Hpirt or Gprt activity is too small to make definitive conclusions regarding the relative importance of hypoxanthine or guanine recycling. Of course it remains feasible that preservation of recycling in either pathway may protect against disease, because hypoxanthine-based and guanine-based purines may be interconverted by reactions in the purine metabolic pathways downstream from the recycling step.

Conclusions—The results of these studies demonstrate that a novel assay can be used to precisely determine the kinetic properties of mutant HGprt enzymes toward each of its substrates. The loss of HGprt activity can be attributed to four different mechanisms. These mechanisms include loss of catalytic activity, reduced affinity for one or more substrates, poor stability, or poor expressivity. These mechanisms are not mutually exclusive. Although the predominant defect for some mutants might involve only one mechanism, most mutants provided evidence for combined defects. Overall, there is a good correlation between residual catalytic activity of the enzyme and clinical severity, although the other mechanisms may disrupt this correlation. It seems likely that these principles, evident from studies of HGprt, are not limited to genotype-phenotype correlations in LND and its variants, as they may apply to almost any protein with measurable function. The results may be helpful for understanding why genotype-phenotype correlations based on protein function often are challenging to establish for other human genetic diseases.

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