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THE ROLE FOR GLUTAMIC ACID AT POSITION 196 IN HUMAN HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE (HPRT) AS INVESTIGATED USING SITE-DIRECTED MUTAGENESIS

B. Canyuk,[1] A. E-Wan,[1] W. Keawwijit,[1] T. Nualnoi,[1] L. Sirisatean,[1] P. Tansakul,[2] and C. Tanthana[1]

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand*
[2]*Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand*

□ *The crystal structure of human HPRT reveals the involvement of E196 side chain at the A-B dimer interface. Interference by valine substitution at this position (E196V), as identified in patients with Lesch-Nyhan disease, nearly abolishes enzymatic activity. Kinetic analysis of the active mutants (E196A, E196D, E196Q, and E196R) suggests that interaction between K68 and E196 side chains contributes to stabilization of cis- configuration during the catalytic cycle. The study also provides further insight into the role of A-B dimer interactions relating to K68 in the regulation of cis- trans isomerization that potentially governs the rate-limiting steps in the HPRT reaction.*

Keywords Human HPRT; LND; glutamic acid 196; E196, functional role

INTRODUCTION

Hypoxanthine phosphoribosyltransferase (HPRT, E.C. 2.4.2.8) is a key enzyme in the purine salvage pathway. HPRT catalyzes the reversible transfer of a phosphoribosyl moiety from phosphoribosylpyrophosphate (PRPP) to 6-oxopurine bases. The enzyme exhibits a sequential kinetic mechanism with PRPP binding first followed by the purine base. After catalysis, pyrophosphate (PPi) is released before the nucleotide. The releases of nucleotide and PRPP are rate-limiting steps in the nucleotide formation and pyrophosphorolysis, respectively.[1] Human HPRT in the crystal structures exists as either a dimer or a tetramer, consistent with a previous report.[2] The A-B dimer interface is conserved in both dimer and tetramer.[3] The

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Address correspondence to B. Canyuk, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand. E-mail: bhutorn.c@psu.ac.th

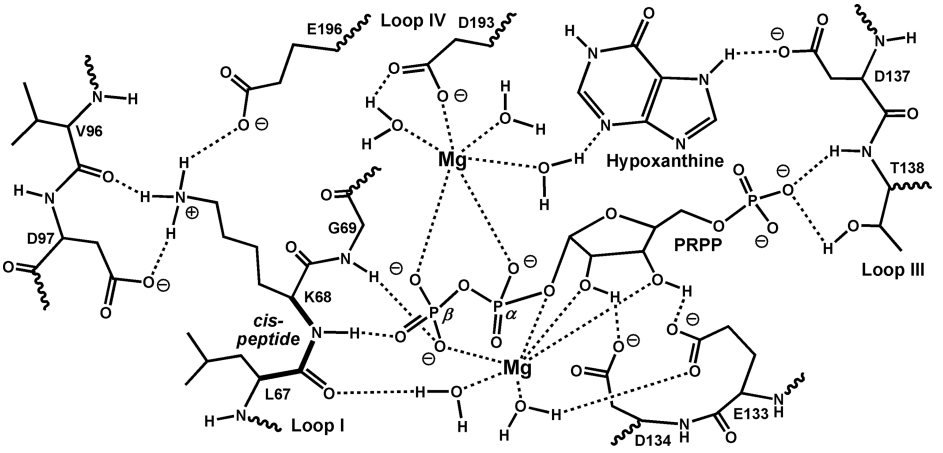

FIGURE 1 Noncovalent interactions (dashed lines) between residues in the active sites of human HPRT with bound substrates and residues in the opposing subunit (V96 and D97) at the A-B dimer interface, as deduced from the crystal structure.[4]

K68 of the active site loop I participates in dimer formation. Loop I is highly conserved as a tripeptide of L67-K68-G69. The peptide between L67-K68 adopts non-proline *cis*-configuration in the crystal structures of human HPRT with fully occupied active site.[4] The *cis*-geometry enables the K68 side chain to extend across the A-B dimer interface and interacts with E196 of the same subunit and V96 and D97 of the opposing subunit. Simultaneously, *cis*-geometry allows the K68 main-chain hydrogen to interact with the PPi moiety (Figure 1). However, the L67-K68 peptide adopts *trans*-configuration in the apo-enzyme which allows the K68 side chain to interact with the re-oriented E133 and D137 that blocks the binding sites of PRPP and 5'-phosphoribosyl portion of nucleotides.[5]

The *cis-trans* isomerization of HPRT has been proposed to regulate substrate binding and product release throughout the catalytic cycle.[6] The involvement of K68 at the A-B dimer interface is proposed to provide communication between subunits. The molecular mechanism for such communication in the catalysis is still unclear. Interference of the native microenvironment of the K68 side chain, such as valine substitution at the position 196 (E196V), leads to Lesch-Nyhan disease (LND) in humans.[7] This study is aimed to clarify the functional contribution of E196 in HPRT catalysis by site-specific mutagenesis and functional analyses using a functional complementation assay and steady state kinetic analysis. Understanding the contribution of E196 would provide further insight into the functional role of A-B dimer interactions relating to K68.

MATERIALS AND METHODS

Construction of Recombinant Plasmids Harboring cDNA Encoding Human WT-HPRT and E196 Mutant HPRTs

The cDNA of WT-HPRT was prepared by RT-PCR using specific primers and mRNA extracted from human white blood cells. The cDNA was inserted into pBAce plasmid.[8] The mutagenic cDNAs encoding E196A, E196D, E196Q, E196R, and E196V HPRTs were prepared by overlap extension using PCR with mutagenic primers and inserted into pBAce plasmid.[9]

Functional Complementation Assay

Catalytic activity of each mutant was individually assessed by measuring growth of purine auxotroph *Escherichia coli* SØ609 transformants expressing the recombinant mutant HPRT, in semi-defined liquid media supplemented with hypoxanthine and guanine at 37°C.[10] The growth curves were plotted between incubation time (hours) versus OD600 of cultures.

Expression and Purification of Recombinant HPRTs

Recombinant WT and E196 mutant HPRTs were expressed in *E. coli* SØ606 transformants in low phosphate induction media.[10] The WT HPRT was purified by affinity chromatography with GMP-agarose (Sigma, St. Louis, MO, USA). Mutant HPRTs could not be purified by affinity chromatography because they bound very weakly to GMP-agarose. They were isolated from crude lysates by heating at 80°C. After heating, the soluble fractions containing catalytically active mutant HPRT were further purified by size exclusion chromatography.

Determination of Steady State Kinetic Parameters of WT and Active E196 Mutant HPRTs

Kinetic parameters for the nucleotide formation and pyrophosphorolysis were measured by spectrophotometric methods.[1] The K_m and k_{cat} values were determined by non-linear regression analysis using the KaleidaGraph software, version 3.5b5 (Synergy Software, Reading, PA, USA).

RESULTS

Functional Complementation Assay

Growth rates of *E. coli* SØ609 transformants reflect the activity of recombinant HPRT.[10] Growth rates of those cultures expressing the recombinant WT and all mutant HPRTs except E196V were similarly exponential and

reached stationary phase with OD600 values of 0.6–0.7, within 10–12 hours. In contrast, the growth rate of the *E. coli* SØ609 transformant expressing the recombinant E196V mutant HPRT was extremely slow and OD600 value reached only 0.05 after incubation for 10 hours (results not shown).

Binding Affinity of Recombinant WT and E196 Mutant HPRTs to GMP-Agarose

The WT HPRT was purified to near homogeneity by using its high affinity to GMP-agarose. Affinities to GMP-agarose of all E196 mutants were very low (results not shown). HPRT is highly thermostable, so all highly active E196 mutants could be partially purified from bacterial crude lysates by heating at 80^{o}C. The mutant HPRTs constituted major component in the soluble fractions and their activities were retained. Subsequent purification of the soluble fractions by size-exclusion chromatography provided highly purified mutant HPRTs.

Steady State Kinetic Parameters

Steady state kinetic parameters for nucleotide formation and pyrophosphorolysis of WT HPRT and highly active E196 HPRT mutants are summarized in Table 1. Kinetic parameters of the E196V mutant were not determined due to extremely low enzyme activity.

DISCUSSION

Removal of the carboxylate side chain at position 196 by alanine substitution (E196A) or replacement with polar or charged residues at position 196 (E196D, E196Q, and E196R) did not affect the activity of mutant HPRTs, as demonstrated in a functional complementation assay. This result indicated that E196 did not directly participate in the reaction chemistry. However, substitution at E196 with bulky and hydrophobic residue (E196V) resulted in marked decrease of the activity and is consistent with the finding that the E196V mutation in human leads to LND. The E196 side chain together with V96 and D97 in the opposing subunit (Figure 1), potentially stabilize K68 in the *cis*-configuration. Thus, removal of the *cis*-stabilizing contribution by E196 mutations likely enhances propensity for *trans*-isomerization. The *trans*-configuration apparently obstructs the binding site of PRPP and 5′-phosphoribosyl portion of IMP which is consistent with marked increase in K_m values of all substrates (6-432 fold of WT) except hypoxanthine, for mutant HPRTs. The large increase in k_{cat} for mutants (4 to 13-fold of WT) is likely the result of the marked increase in K_m values of products (IMP or PRPP), whose releases exert rate-limiting steps in the WT enzyme. Increase in the K_m of IMP is consistent with low affinity of all mutants to

TABLE 1 Steady state kinetic parameters

Nucleotide formation	Variable hypoxanthine			Variable PRPP		
Enzyme	k_{cat} (S^{-1})	K_m (μM)	k_{cat}/K_m (S^{-1} μM^{-1})	k_{cat} (S^{-1})	K_m (μM)	k_{cat}/K_m (S^{-1} μM^{-1})
WT	2.6 ± 0.6	3.8 ± 0.3	0.7	2.5 ± 0.05	19.1 ± 1.6	0.1
E196A	20.3 ± 0.8	6.3 ± 0.9	3.2	20.3 ± 1.2	1156 ± 167	0.02
E196D	13.8 ± 0.5	4.9 ± 0.8	2.8	14.2 ± 0.5	185.0 ± 26.5	0.08
E196Q	13.7 ± 0.4	3.6 ± 0.5	3.8	10.9 ± 0.3	199.6 ± 18.8	0.05
E196R	3.8 ± 0.1	2.8 ± 0.4	1.4	2.8 ± 0.1	659.3 ± 59.9	0.004
IMP pyrophosphorolysis	Variable IMP			Variable PPi		
Enzyme	k_{cat} (S^{-1})	K_m (μM)	k_{cat}/K_m (S^{-1} μM^{-1})	k_{cat} (S^{-1})	K_m (μM)	k_{cat}/K_m (S^{-1} μM^{-1})
WT	0.038 ± 0.001	2.41 ± 0.26	0.016	0.041 ± 0.0006	17.45 ± 1.16	0.002
E196A	0.23 ± 0.05	1043 ± 295	0.0002	NA	NA	NA
E196D	0.18 ± 0.004	72.6 ± 7.3	0.0024	0.33 ± 0.08	967.8 ± 369	0.0003
E196Q	0.51 ± 0.01	14.7 ± 1.5	0.034	NA	NA	NA
E196R	NA	NA	NA	NA	NA	NA

NA: not determined due to saturation concentration could not be achieved.

GMP-agarose. Weakened stabilization of *cis*-configuration in the K68R HPRT of *Trypanosoma cruzi* also resulted in marked decrease in affinity for IMP (280-fold K_m of WT and >20-fold K_D of WT) and GMP.[11] Weakened *trans*-stabilization is potential in the D137A HPRT of *T. cruzi* which imposed no effect on K_m for hypoxanthine but resulted in 22-fold reduction in the K_m for PRPP.[10]

The result presented herein suggests the role of A-B dimer interactions relating to K68 in the regulation of *cis-trans* isomerization that governs the rate-limiting step in the HPRT reaction. Valine substitution (E196V) almost abolished the enzymatic activity, indicating that the mutation imposed a more deleterious structural consequences that needs to be further clarified.

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