Locations and Functional Roles of Conserved Lysine Residues in *Salmonella typhimurium* Orotate Phosphoribosyltransferase†

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ABSTRACT: *Salmonella typhimurium* orotate phosphoribosyltransferase (OPRTase) catalyzes the formation of orotidine 5′-monophosphate (OMP) from orotate and α-D-5-phosphoribosyl-1-pyrophosphate (PRPP). There are five highly conserved lysine residues (Lys-19, -26, -73, -100, and -103) in *S. typhimurium* OPRTase. Here, we report the results of mutagenesis and substrate analog studies to investigate the functional roles of these lysines. Together with information from X-ray crystallography [Scapin, G., Grubmeyer, C., & Sacchettini, J. C. (1994) *Biochemistry 33*, 1287−1294; Scapin, G., Ozturk, D. H., Grubmeyer, C., & Sacchettini, J. C. (1995) *Biochemistry 34*, 10744−10754], sequence comparisons, and chemical modification [Grubmeyer, C., Segura, E., & Dorfman, R. (1993) *J. Biol. Chem. 268*, 20299−20304], this work permits the assignment of functions of the five conserved lysines. Lys-19 is external to the active site, and its mutation to glutamine had little effect on enzyme activity. Lys-26 forms a hydrogen bond to OMP at the 3′-hydroxyl group, and its mutation produced 3−10-fold decreases in k_{cat}. Lys-73 extends into the active site, and a conformational change allows it to interact with either the 5′-phosphate of OMP or the 2-hydroxyl and α-phosphoryl oxygen of PRPP in their respective substrate complexes. Mutation of Lys-73 produced a 50−100-fold decrease in k_{cat} and an 8−12-fold increase in the K_M value for PRPP. Mutation of Lys-100 produced a 5-fold decrease in k_{cat} and a 3-fold increase in the K_M for PRPP, consistent with its location within the active site, near the pyrophosphate moiety of PRPP. Lys-103, which is conserved among all known OPRTases, does not show any interaction with PRPP or OMP in enzyme·ligand complexes, and K_M values for these ligands exhibited relatively minor perturbations in Lys-103 mutants. However, mutation of Lys-103 produced 600−1000-fold decreases in k_{cat}, implying an essential role in catalysis.

Cellular nucleotides arise from the action of phosphoribosyltransferases (PRTases), a group of 10 enzymes that use PRPP[1] and a nitrogenous base to form a nucleoside monophosphate and pyrophosphate. Separate PRTases exist for salvage and *de novo* syntheses of purine, pyrimidine, and pyridine nucleotides, as well as for the biosynthesis of histidine and tryptophan (Musick, 1981).

In *de novo* pyrimidine synthesis, the enzyme orotate PRTase (OPRTase, EC 2.4.2.10) produces orotidine 5′-monophosphate, which is subsequently decarboxylated to UMP. OPRTase binds the highly anionic substrates PRPP and orotate, which together have six negative charges at pH 8.0. We have found that the Mg^{2+} requirement in catalysis is the result of binding of the substrate PRPP as its monomagnesium complex (Bhatia & Grubmeyer, 1993). To determine how the additional charges are accommodated at the active site, we initially examined sequence conservation of lysine and arginine residues among six OPRTases: a total of seven positions were identified in which a positive charge was conserved. Lysine modification studies were also performed to identify potential active site lysines (Grubmeyer et al., 1993). Three lysines, Lys-26, Lys-100, and Lys-103, were modified by 2,4,6-trinitrobenzenesulfonic acid (TNBS) and protected by PRPP and OMP. All three were found to be conserved among six sequenced OPRTases. The detailed protection kinetics showed that Lys-26 was associated with the OMP site, whereas Lys-100 and Lys-103 were associated with the pyrophosphate site.

The crystal structure of OPRTase complexed with OMP was recently reported (Scapin et al., 1994), and a high-resolution structure of the enzyme·PRPP·orotate complex is also now available (Scapin et al., 1995). The homodimeric, 213-residue enzyme contains a doubly wound α/β structure which resembles a classical nucleotide binding fold (Rossmann et al., 1975), with a "hood" that covers one end of the sheet and forms part of the orotate binding site. As shown in the preceding paper (Scapin et al., 1995), PRPP binds across the C-terminal side of the β-sheet, with its ribose hydroxyls and 5-phosphate interacting with residues of a previously proposed (Hershey & Taylor, 1986; Hove-Jensen

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[1] Abbreviations: OPRTase, orotate phosphoribosyltransferase; PRTase, phosphoribosyltransferase; HGPRTase, hypoxanthine−guanine phosphoribosyltransferase; OMP, orotidine 5′-monophosphate; OAME, orotic acid 6-methyl ester; PRPP, α-D-5-phosphoribosyl-1-pyrophosphate; PP_i, pyrophosphate; PNP, imidodiphosphate; PCH_2P, methylenebisphosphonate; PAA, phosphonoacetate; dCMP, 2′-deoxycytidine 5′-monophosphate; dUMP, 2′-deoxyuridine 5′-monophosphate; DTT, dithiothreitol; PEI, (polyethylene imine); PAGE, polyacrylamide gel electrophoresis.

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et al., 1986) PRPP-binding sequence motif. The pyrophosphate moiety interacts with a surface loop which contributes basic residues to active sites on both subunits. An interesting feature of the two structures now available is that the ribose moiety is found in a significantly different location in the OMP and PRPP structures (Scapin et al., 1995).

From analysis of the three-dimensional structure it is now possible to identify interactions between the lysine residues and substrate molecules; a wider protein sequence bank comprised of 16 OPRTase sequences has permitted the identification of additional conserved lysine residues. In the current work, we have used residue conservation, crystal structures, and modification studies as a guide for mutagenesis. In this study, we report that mutations of Lys-19, Lys-26, Lys-73, Lys-100, and Lys-103 to Ala and/or Gln result in changes in kinetic parameters (K_M and k_{cat}), which are consistent with the X-ray structure, and demonstrate an essential role for Lys-103 in catalysis.

EXPERIMENTAL PROCEDURES

Materials. Buffers, reagent grade chemicals, substrate analogs, and bacterial culture media were obtained from Sigma and Aldrich. Materials for mutagenesis procedures were purchased from U.S. Biochemicals and Promega Inc. *S. typhimurium* OPRTase was isolated from the overexpressing strain MB13 (Bhatia et al., 1990).

Construction and Purification of OPRTase Mutants. A 1 kb *Eco*RI−*Pst*I fragment from pCG13 (Bhatia et al., 1990), encoding the entire *pyrE* gene of *S. typhimurium* OPRTase was ligated with M13mp19 DNA (replicative form) previously digested with the same endonucleases. The ligation product, designated DOM13PE, was used to transfect *Escherichia coli* strain CJ236 to produce uracil-containing single-stranded DNA (Ausubel et al., 1987). *In vitro* oligonucleotide-directed mutagenesis was carried out according to Kunkel (1985) using the Bio-Rad Muta-Gene kit, version 2.0, along with T7 DNA polymerase and T4 DNA ligase (U.S. Biochemicals). Mutagenic primers were synthesized at the DNA Synthesis Facility of Temple University School of Medicine. Initial blue-white plaque screening was done by transfecting the *E. coli* strain JM109. Confirmation of the desired mutation was assessed by single-stranded DNA sequencing using the Sequenase 2.0 kit (U.S. Biochemicals). Overexpression of the mutant proteins was achieved by ligating the 1 kb *Eco*RI−*Pst*I fragment from DOM13PE plasmid carrying the desired mutation into previously digested T7 promotor vector pSP72 (Promega Inc.). Subsequently, the recombinant plasmid product was used to transform the *E. coli* host BL21(DE3) containing T7 RNA polymerase (Studier et al., 1990). Transformants were selected and purified on LB plates containing 50 μg/mL ampicillin. Colonies were checked for overexpression of mutant OPRTase by IPTG induction followed by analyses of cell lysates on 15% SDS−PAGE (Laemmli, 1970). The entire *pyrE* gene from the final construct was sequenced to ensure that the nucleotide sequence encoded the desired mutant protein without secondary mutations. Table 1 describes the mutants constructed.

Isolation and purification of the wild-type and mutant enzymes were achieved following the procedure of Bhatia et al. (1990) as modified by Grubmeyer et al. (1993) except that 8% glycerol was included in all buffers to stabilize the mutant proteins. Purified enzyme samples were stored as 65% saturation $(NH_4)_2SO_4$ suspensions at 4 °C. Protein concentrations were measured at 280 nm using $E^{0.1\%} = 0.46$ (Bhatia et al., 1990). For the calculation of k_{cat}, a subunit M_r value of 23 000 was used. Recent sequence data (Scapin et al., 1993) gave an M_r of 23 561.

Table 1: Plasmid Constructs Overexpressing OPRTase Mutants[a]

plasmid	mutation	mutagenic primer
pDOK19Q	K19Q	5′ AGGTACTACAGTTTGGCGA 3′
pDOK26A	K26A	5′ TTTACGCTGGCATCCGGGC 3′
pDOK26Q	K26Q	5′ TTTACGCTGCAATCCGGGC 3′
pDOK73A	K73A	5′ CGCGTACGCAGGTATTC 3′
pDOK73Q	K73Q	5′ CGCGTACCAAGGTATTC 3′
pDOK100A	K100A	5′ TAACCGCGCAGAGGCAAAG 3′
pDOK103A	K103A	5′ AGAGGCAGCAGATCATGGT 3′
pDOK103Q	K103Q	5′ AGAGGCACAAGATCATGGT 3′

[a] For overexpression of the mutant enzymes, the recombinant plasmids listed were used to transform *E. coli* strain BL21(DE3). The underlined codon in each mutagenic primer represents the site where mutation was introduced.

Enzymatic Assay. Prior to assay, enzyme samples were desalted by column centrifugation (Penefsky, 1979) in Sephadex G-50 equilibrated in 100 mM sodium Tricine buffer, pH 8.5. Pyrophosphorolysis of OMP was monitored spectrophotometrically (SLM-Milton Roy 3000 diode array spectrophotometer) as the increase in absorbance (orotate formation) at 303 nm. In this system, E_{303nm} (orotate) = 2.2×10^3 M^{-1} cm^{-1}. Assays were carried out in quartz cuvettes at 30 °C, in 1 mL final volume containing 80 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 0.1 mM OMP, and 2 mM $NaPP_i$. Typically, 2 μg of wild-type enzyme was used in the assays. For the mutants, the amount of enzyme assayed was increased to produce a reliably quantifiable rate. One unit of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of OMP to orotate/min at 30 °C.

Kinetic Parameters of Wild-Type and Mutant OPRTases. The kinetic constants, K_M and k_{cat}, for each enzyme type were determined from the steady-state initial rate measurements for the forward reaction with orotate and PRPP as substrates and the reverse reaction with OMP and PP_i as substrates. For the forward reaction, 1.0 mL assay mixtures contained 75 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, and 10−80 μM orotate at 1 mM PRPP or 20−160 μM PRPP at 0.3 mM orotate for the wild-type enzyme. In the case of mutant enzymes, the PRPP concentrations were increased to 5 mM. For the reverse reaction with wild-type enzyme, assay components were 80 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 2−30 μM OMP at 2 mM PP_i, and 10−100 μM PP_i at 0.1 mM OMP, respectively. For the mutant enzymes, the upper limit of substrate concentrations was increased as required. For K_M determinations of PP_i and PRPP, the amount of $MgCl_2$ was adjusted to keep a constant molar excess over the substrates (1 and 5 mM excess over PP_i and PRPP concentrations employed, respectively). The amount of wild-type enzyme in the assays was 2 μg, and that of the mutant enzymes ranged from 2 to 70 μg depending on the specific activity of the particular mutant. All measurements were carried out at 30 °C. The kinetic data were fit to the hyperbolic form of the Michaelis−Menten equation with the aid of the program HYPER (Cleland, 1979).

Physical Properties of the Mutants. Prior to gel filtration, enzyme samples of 1 mg/mL dissolved in 100 mM sodium

Tricine buffer, pH 8.5, were desalted by the centrifuge column technique (Penefsky, 1979), using 5 mL disposable syringes plugged with glass wool and containing Sephadex G-50 equilibrated in 100 mM sodium Tricine, pH 8.5. The eluate was loaded on a column of HiLoad Superdex-75 Prep Grade (Pharmacia LKB Biotechnology Inc., 1.6 × 60 cm) equilibrated in buffer O (50 mM Tris-HCl, pH 8.0, 1 mM DTT) and eluted at 1 mL/min. Molecular weight standards used were α-chymotrypsin (M_r = 21 600) which eluted at 69.5 mL, ovalbumin (M_r = 44 000) at 54.8 mL, and histidinol dehydrogenase (M_r = 2 × 46 000) at 49.0 mL chromatographed under the same conditions.

Nondenaturing 15% PAGE gels (8 × 10 cm) were prepared according to Laemmli (1970) except that SDS was omitted and 10% glycerol was included in all buffers. Typically, samples containing about 2 μg of OPRTase in 100 mM sodium Tricine, pH 8.5, containing 1 mM DTT, were loaded on the gels. The loading dye contained 50 mM Tris-HCl, pH 6.8, 10% glycerol, 1 mM DTT, and 0.025% bromophenol blue. Gels were electrophoresed at 90 V for 2.5−3 h at room temperature.

Thermostability studies of the mutants were conducted at 60 °C in 100 mM sodium Tricine buffer, pH 8.5, employing a thermostated heating block with mineral oil in the depressions. Aliquots were removed at 10−15 min intervals and assayed at 30 °C for residual activity over the 1 h incubation period.

Kinetics with Substrate Analogs. Various pyrophosphate analogs were tested as substrates and/or inhibitors of the wild-type enzyme and with some of the mutant enzymes at 30 °C, in assays employing the reverse reaction. To test the inhibitory effects of the analogs, 2 μg of enzyme and analog concentrations up to 10 mM were included in the assays. In the cases where the ability of the analogs to serve as substrates was tested, analog concentrations up to 10 mM and enzyme amounts up to 2 mg were included in the assays. Analog concentrations of 0.25−20 mM were included in the assays for K_M and K_I determinations of the analogs.

The orotate analog orotate 6-methyl ester (OAME, λ_{max} = 288 nm, E_{288nm} = 5300 M^{-1} cm^{-1}) was tested as a substrate for mutants K26Q and K103A. The rates were monitored as the decrease in OAME absorbance at 302 nm (E_{302nm} = 4200 M^{-1} cm^{-1}) where the product orotidine 6-methyl ester 5′-monophosphate (λ_{max} = 264 nm) has no appreciable absorbance. K_M determinations were conducted in the range of 60−400 μM OAME at 1 mM PRPP.

Uracil, an analog of orotate which lacks the 6-carboxylate group, was also tested as a substrate for mutants K26Q and K26A and wild type as a control. For the assays, the method employed by Bhatia and Grubmeyer (1993) was followed using radiolabeled uracil. Incubation mixtures (50 μL total) contained 30 μg of protein, 1 mM [2-^{14}C]uracil (200 000 cpm), and 1 mM PRPP. The reaction was monitored on PEI-cellulose plates developed in 0.1 M LiCl (Bhatia & Grubmeyer, 1993).

RESULTS

Structural Locations of Conserved Lysine Residues. S. typhimurium OPRTase contains 13 lysine and 12 arginine residues. Previously, we used the sequences of six OPRTases, available in GenBank, to locate four conserved lysine residues (Grubmeyer et al., 1993). We have extended this analysis to include additional sequences present in the Swiss Protein Data Base, allowing comparisons among 16 sequences. The reported sequences span a broad phylogenetic spectrum and include Gram-negative and -positive bacteria, fungi, protozoa, insects, and mammals. The sequences appear to fall into three groups (Table 2). The nine members of group I, representing bacteria and fungi, are monofunctional OPRTases. Group II contains five examples (slime mold, protozoa, fruit fly, bovine, and human) of bifunctional OPRTase/OMP decarboxylases (UMP synthases), and group III contains two divergent, monofunctional OPRTase sequences from the genus *Bacillus*. From this analysis, five lysine residues of the *S. typhimurium* enzyme appear to be highly conserved, as judged by an identity in at least eight of the sequences.

Table 2: Sequence Comparisons among Conserved Lysine Residues[a]

	group I		group II		group III	
residue	Lys	Arg	Lys	Arg	Lys	Arg
Lys-19	7	1	5	0	0	0
Lys-26	8	0	5	0	0	0
Lys-73	8	0	0	0	0	0
Lys-100	9	0	5	0	0	0
Lys-103	9	0	5	0	2	0

[a] Conservation of lysine residues among 16 OPRTases: nine monofunctional (group I), five bifunctional (group II), and two divergent sequences from the genus *Bacillus* (group III). Residue numbers refer to the sequence of the *S. typhimurium* protein. Sequences used were as follows: group I, *Salmonella typhimurium* (Scapin et al., 1993), *Escherchia coli* (Poulsen et al., 1984), *Podospora anserina* (Turcq & Begueret, 1987), *Saccharomyces cerevisiae* (de Montigny et al., 1989), *Sordaria macrospora* (le Chevanton & Leblon, 1989), *Cryptoccoccus neoformans* (Edman & Kwon-Chong, 1990), *Trichoderma reesei* (Berges & Barreau, 1991), *Rhizobium trifolii* (Fennington, 1994), and *Colletotrichum graminicola* (Rasmussen et al., 1992); group II, human (Suttle et al., 1988), fruit fly (Eisenberg et al., 1990), bovine (Schoeber et al., 1993), *Naegleria gruberi* (Remillard et al., 1993), and *Dictyostelium discoideum* (Boy-Marcotte et al., 1984); group III, *Bacillus subtilis* (Quinn et al., 1991) and *Bacillus caldolyticus* (Ghim et al., 1994).

Each of the five conserved lysines was examined in the two available three-dimensional structures of OPRTase. Figure 1 gives a schematic representation of the residues and solvent molecules forming hydrogen bonds with OMP and PRPP in both complexes. Figure 2 presents ribbon diagrams showing the lysine residues considered here. Lys-19 is outside the active site (Scapin et al., 1994) and appears to interact only with solvent in the two structures (Figure 2). For the other conserved lysine residues, the crystal structures suggest functional roles. Lys-26 is at the tip of a sharp turn between two β-strands, termed the "hood", found at one end of the active site. In the OMP complex, the ε-amino group of Lys-26 forms a 3.2 Å hydrogen bond with the 3′ hydroxyl group of the nucleotide (Scapin et al., 1995). In the enzyme•PRPP•orotate complex, in which the ribosyl group has moved into the PRPP subsite, Lys-26 is no longer close to the hydroxyl group and instead hydrogen bonds with the 5-phosphoryl group of PRPP (Scapin et al., 1995). In addition, the backbone nitrogen of Lys-26 forms a hydrogen bond (3.1 Å) with the carboxylate of orotate in both enzyme•OMP and enzyme•PRPP•orotate complexes (Scapin et al., 1995).

Lys-73, Lys-100, and Lys-103 are located in the solvent-exposed region of the active cleft closer to the subunit

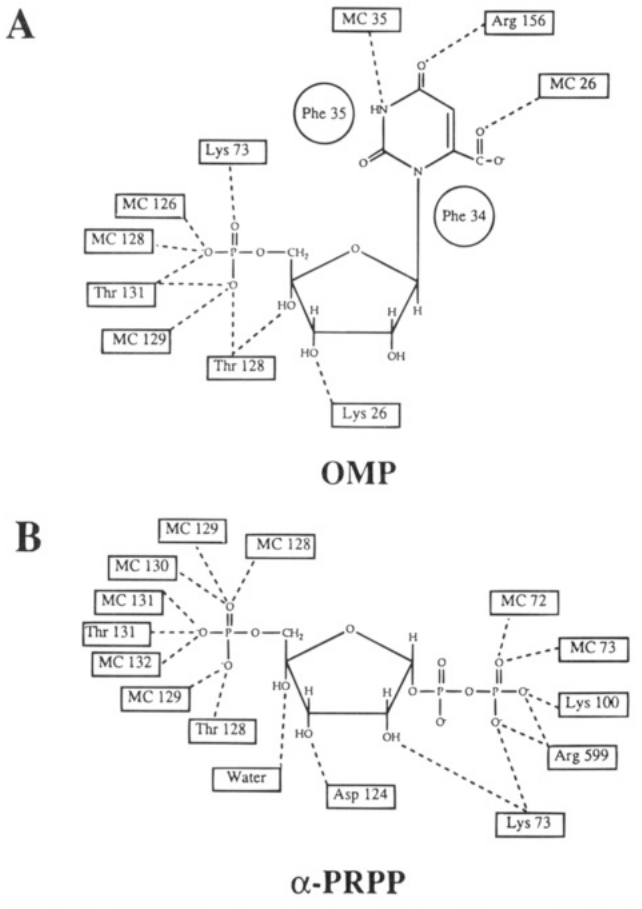

FIGURE 1: Schematic representation of the residues and solvent molecules interacting with bound substrates. (A) OMP in the OPRTase•OMP complex and (B) PRPP in the OPRTase•PRPP•orotate complex. Boxes indicate residues that form hydrogen bonds, and circles indicate van der Waals interactions. Arg-599 in diagram B is Arg-99 from the symmetry-related subunit in the dimeric enzyme (Scapin et al., 1994, 1995).

interface. In the case of Lys-73, both main-chain and side-chain atoms undergo a movement when the enzyme•OMP and enzyme•PRPP•orotate complexes are compared (Figure 2) (Scapin et al., 1995). In the former, Lys-73 extends across the active site to interact with the 5′-phosphate of OMP. In the enzyme•PRPP•orotate complex, the side chain of Lys-73 adopts a less extended conformation and hydrogen bonds with both the 2-hydroxyl and the β-phosphate of PRPP. Although Lys-100 does not interact with substrate in the enzyme•OMP complex, it hydrogen bonds with the β-phosphate of PRPP in the enzyme•PRPP•orotate complex (Figure 2). Lys-103 is solvent exposed as part of a loop (residues 98−119) in which residues 103−107 are poorly resolved in the two complexes. However, Lys-103 is about 6 Å from the pyrophosphoryl moiety of PRPP in the active site on the adjacent subunit and could reach that active site if loop movement occurs in solution or during the reaction (Figure 2B).

Mutagenesis and Physical Properties of Mutant OPRTases. The catalytic and structural importance of the conserved lysine residues was explored by replacing them with glutamine and/or alanine residues. Although glutamine conserves the ability to form hydrogen bonds, alanine was the most frequently tolerated substitution for residues whose solvent accessibility is high (Pakula & Sauer, 1989; Bowie et al., 1990).

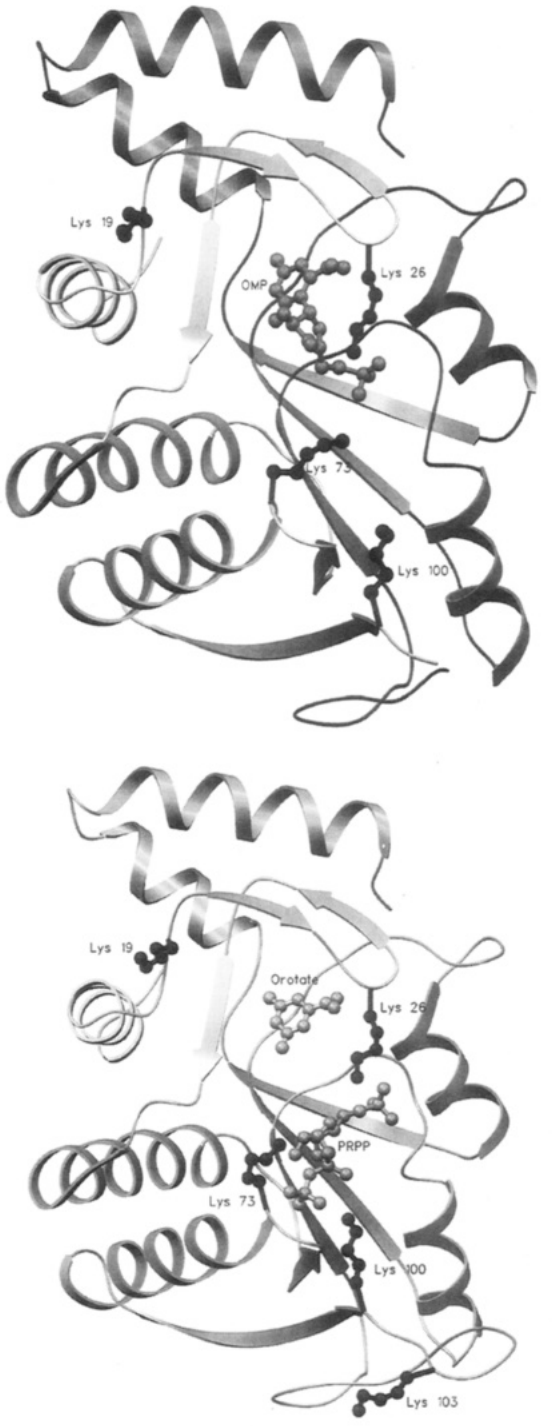

FIGURE 2: Ribbon diagrams of the (A, top) enzyme•OMP complex and (B, bottom) enzyme•PRPP•orotate complex. In the electron density map of the enzyme•OMP complex, residues 103−107 are poorly resolved; hence there is a break in the ribbon diagram of this complex (Scapin et al., 1994).

Each of the mutant enzymes (Table 1) was overexpressed in the *E. coli* strain BL21(DE3). SDS−15% PAGE showed high level expression after induction, and all mutant enzymes were readily purified to homogeneity, using a procedure similar to that used for the wild-type enzyme (Grubmeyer et al., 1993) with 8% glycerol added to all isolation buffers. DNA sequencing confirmed the presence of desired mutations; no secondary mutations were found. In addition, after purification, each mutant enzyme was subjected to 30 cycles of N-terminal peptide sequencing, which in each case showed the asparagine diagnostic of the *S. typhimurium* sequence in

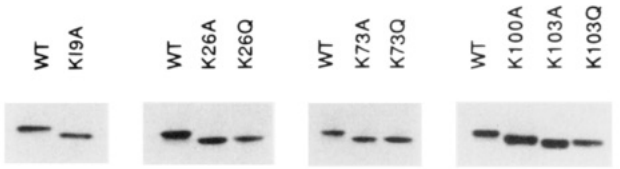

FIGURE 3: Migration patterns of wild-type and mutant OPRTases on nondenaturing 15% PAGE.

Table 3: Thermostability Studies of OPRTase Mutants[a]

enzyme	specific activity (uits/mg) $t = 0$	specific activity (uits/mg) $t = 1$ h	residual activity (%)
WT	52	45	86
K19Q	43	22	49
K26Q	4	3	75
K26A	22	17	77
K73Q	0.9	0.7	78
K73A	1	0.6	60
K100A	13	6	46
K103Q	0.1	0.08	80
K103A	0.09	0.07	67

[a] Stability of the wild-type *S. typhimurium* orotate phosphoribosyltransferase and lysine mutants was monitored as the decrease in activity of the reverse reaction at 60 °C over a period of 1 h.

cycle 14; the *E. coli* enzyme has a serine at this position. The N-terminal peptide sequencing procedure also served to confirm the presence of the K19Q, K26Q, and K26A mutations.

To investigate their physical properties, mutant enzymes were subjected to gel-filtration chromatography on Superdex 75. Wild-type and mutant enzymes behaved as dimers, eluting at 55.6 ± 0.3 mL, as reported previously for wild-type enzyme (Bhatia et al., 1990). Equilibrium and sedimentation ultracentrifugation, as well as ligand binding studies, have implied that different species may be present at extremes of high and low protein concentrations (Dessen de Souza e Silva, 1993). In the current work, all OPRTase forms examined gave symmetrical chromatographic peaks with a peak-width-at-half-height value of 2.7 ± 0.1 mL. This behavior was identical to that of ovalbumin, which eluted at nearly the same volume, and is the expected result for a monodisperse solution of dimeric OPRTase.

To further characterize the physical state of mutant OPRTases, all mutants were electrophoresed on nondenaturing gels (Experimental Procedures). The mutant proteins each migrated as single species, with the slight increase in mobility expected from the loss of two positively charged residues per dimer (Figure 3).

Stability tests were undertaken by incubating the enzymes at 60 °C for 1 h. Wild-type enzyme retained 86% of its activity over this period. The least stable mutant enzymes, K100A and K19Q, retained 46% and 49% of their original activity, respectively, whereas all other mutant OPRTases retained at least 60% and most at least 75% of original activity after heat treatment (Table 3).

Kinetic Characterization of Mutant OPRTases. To examine the functional roles of the conserved lysine residues, kinetic properties of the mutant enzymes were examined in the forward (phosphoribosyltransferase) and reverse (pyrophosphorolysis) reactions. These data are compiled in Table 4. The K19Q mutant was the least affected, with virtually no loss in k_{cat} in either direction and only minor decreases in k_{cat}/K_M.

Table 4: Kinetic Parameters of the Lysine Mutants of *S. typhimurium* Orotate Phosphoribosyltransferase for the Forward and Reverse Reactions[a]

	Kinetic Parameters for the Forward Reaction[a]				
		K_M (μM)		k_{cat}/K_m (mM^{-1} s^{-1})	
enzyme	k_{cat} (s^{-1})	orotate	PRPP	orotate	PRPP
WT	34 ± 0.6	26 ± 1	49 ± 5	1300	690
K19Q	31 ± 0.9	78 ± 10	96 ± 4	400	320
K26Q	3.1 ± 0.03	65 ± 2	55 ± 4	48	56
K26A	8.7±0.2	70 ± 5	50 ± 5	125	175
K73Q	0.6 ± 0.02	31 ± 1	576 ± 96	19	1
K73A	0.3 ± 0.003	29 ± 1	394 ± 16	9	0.7
K100A	12 ± 0.2	72 ± 4	179 ± 11	160	64
K103Q	0.13 ± 0.001	34 ± 2	120 ± 3	4	1
K103A	0.05 ± 0.002	42 ± 3	285 ± 4	1	0.2
	Kinetic Parameters for the Reverse Reaction				
		K_M (μM)		k_{cat}/K_M (mM^{-1} s^{-1})	
enzyme	k_{cat} (s^{-1})	OMP	PP_i	OMP	PP_i
WT	18 ± 0.3	5 ± 0.4	33 ± 1	3600	550
K19Q	12 ± 0.3	18 ± 2	50 ± 2	830	300
K26Q	1.3 ± 0.01	37 ± 1	111 ± 3	34	11
K26A	9.6 ± 0.1	19 ± 1	74 ± 2	500	130
K73Q	0.3 ± 0.004	13 ± 1	102 ± 3	24	3
K73A	0.3 ± 0.006	11 ± 1	182 ± 18	28	2
K100A	12 ± 0.2	13 ± 1	958 ± 40	830	11
K103Q	0.05 ± 0.004	3.4±0.2	144 ± 2	15	0.3
K103A	0.05 ± 0.0005	20 ± 1	146 ± 1	2	0.3

[a] Data were analyzed by the program HYPER (Cleland, 1979).

Mutation of Lys-26 to alanine or glutamine resulted in changes in both k_{cat} and K_M values. Although the K_M value for PRPP was not affected, that for OMP increased 5−8-fold, and values for other substrates increased 2−3-fold (Table 4). Surprisingly, the decrease in k_{cat} was greater in the K26Q mutant: K26Q exhibited about a 10-fold decrease in k_{cat} in both the forward and reverse reactions, but in the case of the K26A mutant, k_{cat} decreased only 2−3-fold in both directions.

Lys-73 hydrogen bonds with both the pyrophosphate and 2-hydroxyl of PRPP, and its mutation has profound effects on the K_M values for PRPP and, to a lesser extent, PP_i, increasing the former 8−12-fold and the latter by a factor of 3−6 (Table 4). In the forward reaction, k_{cat} for K73Q was decreased about 50-fold, whereas for K73A, k_{cat} was decreased 100-fold. In the OMP complex, Lys-73 hydrogen bonds with the 5′-phosphate, and K73Q or K73A mutations caused only a 2-fold increase in the K_M value for OMP. In the pyrophosphorolysis reaction, k_{cat} values for both mutants were 50-fold less than the wild-type value (Table 4). The kinetic results suggest an important role for Lys-73 in PRPP binding in the ground state of the enzyme and also involvement in catalysis.

The role of Lys-100, which hydrogen bonds with the β-phosphate of PRPP in the enzyme·PRPP·orotate complex, was assessed by the K100A mutation. This change resulted in a 3-fold decrease in k_{cat} and an approximate 3-fold increase in the K_M value for PRPP in the foward direction. For the pyrophosphorolysis reaction, k_{cat} decreased only 2-fold, but the K_M value for PP_i increased 30-fold (Table 4), consistent with the three-dimensional structure.

Mutation of Lys-103 resulted in the most striking decreases in k_{cat}/K_M values among all the lysine mutants prepared, despite the fact that the K_M values for both mutants were not substantially different from those of the wild-type

Table 5: Pyrophosphate Analogs as Substrates or Inhibitors of Wild-Type OPRTase[a]

	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)	K_I (mM)
pyrophosphate	18	33	550	0.08[b]
imidodiphosphate	35	940	38	ND[e]
phosphonoacetic acid	0.04	1850	0.02	*c*
phosphonoformic acid	*d*	*d*	*d*	0.22 ± 0.02

[a] In addition to the compounds listed, methylene diphosphonate, oxalic acid, phosphonomycin, phosphonoglycolate, malonic acid, ketomalonic acid, tartronic acid, sodium phosphate, and tripolyphosphate were also tested for their ability to act as substrates or inhibitors. No significant enzymatic activity or inhibitory effect was observed in the presence of these compounds. Data were analyzed by the Cleland program HYPER (1979). [b] Bhatia et al. (1990). [c] No inhibitory effect observed at 10 mM. [d] No reliably detectable enzymatic activity (rates < 0.010 ΔA/min) was observed up to 200 μg of enzyme. [e] ND: not determined.

enzyme. K103Q and K103A exhibited k_{cat}/K_M values 1800–3500-fold less than the wild-type enzyme with the most extreme decrease observed for K103A (Table 4). In the crystal structure, Lys-103 appears to be interacting with solvent.

Pyrophosphate Analogs. To further investigate the role of lysine residues in the mechanism of OPRTase, we examined a variety of possible analogs of pyrophosphate as alternative substrates and inhibitors. Previously, 5-phosphoribosyl 1-*O*-(1-thiodiphosphate) (PRPPαS), 5-phosphoribosyl 1-*O*-(2-thiodiphosphate) (PRPPβS), and 5-phosphoribosyl-1-methylenediphosphonate (PRPCP) have been shown to act as alternative substrates for OPRTase (Murray et al., 1969; Smithers & O'Sullivan, 1984; McClard et al., 1984); and 5-phospho-2-deoxyribosyl-1-phosphonyldiphosphate is a good inhibitor of this enzyme (Witte & McClard, 1993). In the present studies, the dicarboxylic acid compounds malonic acid, tartronic acid, and oxalic acid exhibited no substrate activity or inhibitory effect, nor did the phospho-containing compounds tripolyphosphate and phosphate up to concentrations of 10 mM in the assays (data not shown). The phosphonate compounds phosphonomycin [(1*R*,2*S*)-(1,2-epoxypropyl)phosphonic acid], a broad spectrum antibiotic (Hendlin et al., 1969), and phosphonoglycolate also were ineffective as substrates or inhibitors up to concentrations of 10 mM in the assays (data not shown).

Table 5 summarizes the results with analogs which served as substrates or inhibitors. Imidodiphosphate (PNP) was a good substrate for OPRTase, with k_{cat}/K_M 10-fold lower than with PP_i. The methylenediphosphonate analog of PP_i showed no detectable activity as a substrate and also failed to demonstrate any inhibitory properties. Phosphonoacetic acid (PAA) was a poor substrate, with k_{cat}/K_M 4 orders of magnitude below that of PP_i. The K_M value for phosphonoacetate was about 40-fold higher than that for PP_i, and k_{cat} for this alternative substrate was 500-fold less than for PP_i. Phosphonoformic acid (foscarnet), an antiviral agent (Helgstrand et al., 1978), was not a substrate but was an inhibitor (K_I = 220 μM).

When the mutant enzymes were evaluated for their interaction with PP_i, PNP, and PAA (Table 6), two features were immediately evident. First, when PNP was compared with PP_i as substrate, all the mutant enzymes used PNP with reduced k_{cat}, except for the K73A mutant enzyme where no reliable activity measurements could be made. In contrast, the wild-type enzyme showed a 2-fold increase in k_{cat} with PNP. Second, the mutant and wild-type enzymes showed very similar k_{cat} and K_M values with PAA. No enzymatic activity was reliably detected with the K73A and K103Q mutant enzymes when PAA was used as a substrate.

Lysine-26 hydrogen bonds through its amino group with OMP at the 3′-hydroxyl and through its main-chain nitrogen with the exocyclic carboxylate moiety of orotate. To test the effects of the K26Q mutation, we also employed the alternative base orotate 6-methyl ester (OAME) (Bhatia et al., 1990) as substrate. Wild-type enzyme discriminates against OAME with a 10-fold decrease in k_{cat} and a 6-fold increase in the K_M value (Bhatia & Grubmeyer, 1990). Table 7 shows that the K26Q mutation reversed this discrimination. The k_{cat} value for the ester was 5-fold higher than for orotate itself, and the K_M value was virtually identical for the two substrates. k_{cat}/K_M for orotate methyl ester is thus 4-fold higher than for orotate with K26Q, making the mutant enzyme a methyl orotate phosphoribosyltransferase with a catalytic efficiency almost as good as that of wild-type enzyme as an orotate phosphoribosyltransferase. K103A, a mutation not affecting the orotate site, did not show this change in base specificity.

Previously, uracil was shown to be a very poor substrate for wild-type OPRTase (Bhatia & Grubmeyer, 1993). Compared to wild-type OPRTase, the mutant enzymes K26A and K26Q did not exhibit any substantial change in kinetic parameters with uracil (Table 7).

DISCUSSION

The work presented in this paper establishes an essential role for Lys-103 in the phosphoribosyl transfer reaction of *S. typhimurium* OPRTase and additionally delineates active site functions of Lys-26, Lys-73, and Lys-100. Previously, we reported that OMP and PRPP protected Lys-103 from modification by TNBS (Grubmeyer et al., 1993). These results indicated that Lys-103 must be located at the active site. An important role for Lys-103 was also likely because of its conservation among all sequenced OPRTases. However, the three-dimensional structures (Scapin et al., 1994, 1995) show Lys-103 to be about 6 Å from bound PRPP and do not clearly indicate how an active site role might be exercised or what that role might be.

An initial concern with the Lys-103 mutants was whether the low activity resulted from a structural, rather than a functional defect. Gel filtration and thermal denaturation studies showed that the two mutants were similar to wild-type enzyme. As shown in the following paper (Ozturk et al., 1995), Lys-103 mutants can also form substantially active heterodimers with other mutant subunits, again demonstrating that the mutant enzymes do not suffer severe physical derangement.

Lys-103 is located in a loop comprised of residues 98–119. The loop is at the subunit interface and is near both active sites of the OPRTase dimer (Scapin et al., 1995). In both enzyme·OMP and enzyme·PRPP·orotate complexes, residues 103–107 of the loop display weak electron density and are poorly resolved, suggesting that the loop may be mobile. Alternatively, the loop may adopt several distinct conformations within the crystal (discrete disorder). A third possibility is that the loop normally interacts more directly

Table 6: Kinetic Evaluation of Pyrophosphate Analogs with Lysine Mutants[a]

	k_{cat} (s^{-1})			K_M (mM)			k_{cat}/K_M (mM^{-1} s^{-1})		
mutant	PP_i	PNP	PAA	PP_i	PNP	PAA	PP_i	PNP	PAA
WT	18 ± 0.4	35 ± 0.04	0.04 ± 0.04	0.033 ± 0.001	0.9 ± 0.12	1.9 ± 0.2	1400	98	0.06
K26Q	1.3 ± 0.04	0.12 ± 0.02	0.13 ± 0.004	0.11 ± 0.003	1.1 ± 0.3	3.6 ± 1.4	30	0.3	0.09
K73A	0.3 ± 0.004	ND[b]	ND	0.18 ± 0.012	ND	ND	2	ND	ND
K100A	11 ± 0.1	0.7 ± 0.08	0.09 ± 0.02	0.96 ± 0.04	0.4 ± 0.3	1.8 ± 0.7	150	4	0.1
K103Q	0.05 ± 0.001	0.05 ± 0.001	ND	0.15 ± 0.002	0.4 ± 0.1	ND	2.4	0.3	ND

[a] K_M and k_{cat} values and specific activities of lysine mutants of *S. typhimurium* OPRTase with pyrophosphate analogs. Data were fit to the program HYPER (Cleland, 1979). [b] ND: not determined; could not be discriminated from background drift.

Table 7: Kinetic Constants of Orotate, Orotate 6-Methyl Ester, and Uracil in Wild-Type and Lysine Mutants of *S. typhimurium* Orotate Phosphoribosyltransferase[a]

	k_{cat} (s^{-1})			K_M (μM)			k_{cat}/K_M (mM^{-1} s^{-1})		
enzyme	orotate	OAME	uracil	orotate	OAME	uracil	orotate	OAME	uracil
WT	32 ± 0.8	3.5 ± 0.04	0.9 ± 0.04	28 ± 2	182 ± 8	3160 ± 25	1140	19	0.3
K26Q	3.3 ± 0.2	16 ± 2	0.7 ± 0.04	70 ± 6	82 ± 17	1810 ± 45	48	192	0.4
K26A	10 ± 0.4	ND[b]	0.7 ± 0.04	67 ± 5	ND	3710 ± 37	150	ND	0.2
K103A	0.06 ± 0.004	0.02 ± 0.001	ND	43 ± 1	41 ± 0.2	ND	1.3	0.4	ND

[a] The incubation and the assays were carried out at 30 °C. Data were analyzed by the Cleland (1979) program HYPER. [b] ND: not determined.

with the active site than is seen in current structures but that crystal packing interactions favor the solvent-exposed loop conformation.

What role does Lys-103 play in the OPRTase reaction? The most striking effect of Lys-103 mutations was on k_{cat}, with only minor perturbations of K_M values, indicating that Lys-103 is less important for ground-state enzyme−substrate interactions but is involved in the catalytic process. One possibility is that Lys-103 serves as an acid to protonate the leaving pyrophosphate group or as a base to deprotonate the incoming orotate in the forward reaction. In order for Lys-103 to serve either of these functions the ϵ-amino group must move 7−15 Å from its position in the crystal structures. Support for a potential role for Lys-103 as an active site base comes from pH titrations which show a basic group with a pK of about 9 being required for forward catalysis (J. Blanchard, personal communication). However, the pH profiles for the enzyme are complex, and at this time it is not possible to assign any of the observed pK values to particular residues or reaction steps.

A second possible role for Lys-103, not excluding its possible action as a base, is that the loop containing Lys-103 moves during catalysis to shield the active site. OPRTase active sites are significantly exposed to solvent in both substrate complexes. If catalysis occurs with either a labile oxocarbonium intermediate or an oxocarbonium-like transition state (Bhatia et al., 1990), solvent molecules would need to be excluded. A related observation from the crystal structures of enzyme•OMP and enzyme•PRPP•orotate complexes is that the ribose ring moves in an arcing fashion about the 5-phosphoryl group, carrying ribosyl C1 about 7 Å between its positions in the enzyme•OMP and enzyme•PRPP•orotate complexes. Ribosyl movement may be coupled to loop movement. Peptide loops which function to occlude the active site from bulk solvent are well precedented. Examples include triose phosphate isomerase (Lolis & Petsko, 1990), HIV-1 protease (Wlodawer & Erickson, 1993), and soybean β-amylase (Mikami et al., 1994). In those cases, a mobile loop adopts a fixed closed position in enzyme−substrate or enzyme−inhibitor complexes. However, in the 2.7 Å crystal structure of *E. coli* B glutathione synthetase (Kato et al., 1989; Yamaguchi et al., 1990), a flexible loop atop the proposed ATP binding site was poorly resolved in both apoenzyme and enzyme•ATP complexes. It was proposed that this loop may prevent water from hydrolyzing the γ-Glu-Cys phosphate intermediate during catalysis (Tanaka et al., 1992). As shown in the following paper (Ozturk et al., 1995), a novel feature of the Lys-103 loop in OPRTase is that it functions in the active site of the adjacent subunit.

Previous OPRTase sequence alignments (Grubmeyer et al., 1993) did not suggest any functional importance for Lys-73, but inclusion of additional fungal sequences from the Swiss Protein Data Base showed that in all but one group I (monofunctional) OPRTase Lys-73 is present as an identity (Table 2). Crystal structures proposed an important role for Lys-73, which adopts different conformations in the two substrate complexes, resulting in a hydrogen bond with the 5′-phosphate of bound OMP but with the β-phosphoryl moiety of bound PRPP (Scapin et al., 1994, 1995). Mutation of Lys-73 to alanine or glutamine resulted in 50−100-fold decreases in k_{cat}. These perturbations were second only to those resulting from mutations at Lys-103. The K_M values for PRPP and PP_i were perturbed in Lys-73 mutants as expected from the crystal structure. The 2-fold effect on the K_M value of OMP implies a modest energetic contribution to OMP binding by the hydrogen bond between OMP and Lys-73, whereas the 10-fold effect on the K_M for PRPP demonstrates a more important interaction. The conserved Lys-68 of human HGPRTase (Eads et al., 1994) and Lys-328 in glutamine-amido-PRTase (Smith et al., 1994) appear to be positional homologs of Lys-73 of OPRTase.

Kinetic studies with pyrophosphate analogs further defined the roles of Lys-73 and Lys-100. Among the 12 compounds tested, only PNP and PAA were utilized by OPRTase as substrates for the pyrophosphorolysis reaction, and PAA was a very poor substrate. The results thus demonstrate a high degree of substrate specificity for PP_i, and the crystal structures suggest that Lys-73 and Lys-100 should have an important role in this specificity. The behavior of the analogs might be expected to reflect their inherent chemical reactivity, geometry, and ability to interact in solution and on the

enzyme with the required divalent metal ion. The chemical reactivity of the phosphoryl group of phosphonates is similar to that of anhydrides (Yount, 1975), indicating that reactivity is not a determining factor. It has been reported that OPRTase interacts with Mg^{2+} by binding the MgPRPP substrate (Bhatia & Grubmeyer, 1993), although two cases involving pyrophosphoryl substrates have been identified recently in which a second metal ion is required for productive catalysis or substrate binding (Frick et al., 1994; Bertagnolli & Cook, 1994). The structural features of mono- or dimagnesium complexes of PNP and PAA may interfere with their ability to bind or act as good substrates. With the wild-type enzyme, k_{cat} values were 2-fold higher with PNP than for PP_i. The 25-fold increase in the K_M values for this good geometric analog of PP_i (Yount, 1975) again implies a high degree of specificity. Isotope-exchange data previously indicated that PP_i release is rate-limiting for the forward reaction (Bhatia et al., 1990), and poorly bound PNP might not suffer from this limitation. The ability of wild-type enzyme to accept PAA as a substrate was unexpected since the enzyme fails to utilize other analogs, such as PCH_2P which is structurally similar to PP_i. Catalysis with PAA was 500-fold slower than with PP_i but was quantifiable.

From the crystal structure, the interaction of OPRTase with the β-phosphoryl group of PRPP and PP_i would be expected to be disrupted in complexes of Lys-73 and Lys-100 mutant enzymes with the analogs. Mutations at either of these positions resulted in substantial increases in the K_M values for PP_i. The K73A mutant protein failed to give reliably quantifiable product with either alternative substrate, possibly because of high K_M values. However, K100A was surprisingly unperturbed in the K_M values for PP_i analogs, yielding values that were close to those for PP_i itself. The results imply that Lys-100 does not make an important interaction with either of the two analogs, in clear contrast to native enzyme interacting with PP_i. It is thus likely that the high K_M values for PNP and PAA originate in a defective interaction with Lys-100 and that mutagenesis at that position fails to perturb their binding.

The three-dimensional structure of the OPRTase•OMP complex shows that Lys-26 forms a hydrogen bond with the 3′-hydroxyl of OMP. In the same complex, the backbone amide of Lys-26 forms a hydrogen bond to the exocyclic carboxylate of the orotate moiety. It was thus possible that positioning of the Lys-26 side chain by the ribosyl group might affect base specificity. This was indeed found to be the case with the 6-methyl ester of orotate (OAME). In the K26Q mutant, k_{cat}/K_M was shown to be 4-fold greater with the ester than with orotate itself. Recently, Liu and Santi (1993) have reported that the conserved Asn-229 in thymidylate synthase is responsible for exclusion of 2′-deoxycytidine 5′-monophosphate (dCMP), a nucleotide structurally very similar to the substrate 2′-deoxyuridine 5′-monophosphate (dUMP). Mutation of Asn-229 to glutamine generates an enzyme that utilizes dCMP better than dUMP. In both OPRTase and thymidylate synthase a residue−substrate interaction in the ground state appears to function by excluding alternative substrates rather than forming an essential interaction with the actual substrate. However, the continued discrimination against uracil by K26A and K26Q mutant enzymes showed that the importance of the 6-carboxylate group in orotate for substrate recognition by OPRTase was not eliminated by the mutations of Lys-26.

A number of tools exist to help enzymologists decipher the roles of individual residues in catalysis. Many of them have proven useful with OPRTase; only together can they give definitive answers. Chemical modification studies, often plagued by specificity problems, in our case showed that Lys-26, Lys-100, and Lys-103 are at or near the active site, but crystallography and mutagenesis were required to determine their relative importance or exact roles. Comparisons among OPRTase sequences demonstrated a gradation of apparent evolutionary conservation but failed initially (Grubmeyer et al., 1993) to reveal the importance of Lys-73, which is not conserved in only one monofunctional and in all bifunctional OPRTases and yet occupies a key position in both enzyme−substrate complexes of *S. typhimurium* OPRTase. Further support for the importance of Lys-73 in OPRTase came from recent crystal structures of human HGPRTase and glutamine-amido-PRTase in which there are structurally homologous conserved lysines (Eads et al., 1994; Smith et al., 1994). Alternatively, as crystallography and mutagenesis showed with Lys-19, it is possible that a highly conserved residue is not important for structure or function. Mutagenesis, which might be expected to clarify roles definitively, is dependent on the methods used to evaluate the proteins produced. In OPRTase, the effects of Lys-26 mutation on substrate specificity demonstrate that the side chain is important for formation of a correct interaction of orotate with the backbone. However, this role was only revealed by results with an alternative substrate, OAME.

Crystallographic observations alone would not suggest any role for Lys-103 which is about 6 Å from the bound substrates. However, mutagenesis confirmed the chemical modification data (Grubmeyer et al., 1993) in demonstrating an essential role for this residue. In the following paper, we show that the role of Lys-103 is carried out in the adjacent active site.

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