Kinetic Mechanism of Uracil Phosphoribosyltransferase from *Escherichia coli* and Catalytic Importance of the Conserved Proline in the PRPP Binding Site†

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ABSTRACT: Phosphoribosyltransferases catalyze the formation of nucleotides from a nitrogenous base and 5-phosphoribosyl-α-1-pyrophosphate (PRPP). These enzymes and the PRPP synthases resemble each other in a short homologous sequence of 13 amino acid residues which has been termed the PRPP binding site and which interacts with the ribose 5-phosphate moiety in structurally characterized complexes of PRPP and nucleotides. We show that each class of phosphoribosyltransferases has subtle deviations from the general consensus PRPP binding site and that all uracil phosphoribosyltransferases (UPRTases) have a proline residue at a position where other phosphoribosyltransferases and the PRPP synthases have aspartate. To investigate the role of this unusual proline (Pro 131 in the *E. coli* UPRTase) for enzyme activity, we changed the residue to an aspartate and purified the mutant P131D enzyme to compare its catalytic properties with the properties of the wild-type protein. We found that UPRTase of *E. coli* obeyed the kinetics of a sequential mechanism with the binding of PRPP preceding the binding of uracil. The basic kinetic constants were derived from initial velocity measurements, product inhibition, and ligand binding assays. The change of Pro 131 to Asp caused a 50−60-fold reduction of the catalytic rate (k_{cat}) in both directions of the reaction and approximately a 100-fold increase in the K_M for uracil. The K_M for PRPP was strongly diminished by the mutation, but $k_{cat}/K_{M,PRPP}$ and the dissociation constant ($K_{D,PRPP}$) were nearly unaffected. We conclude that the proline in the PRPP binding site of UPRTase is of only little importance for binding of PRPP to the free enzyme, but is critical for binding of uracil to the enzyme−PRPP complex and for the catalytic rate.

Uracil phosphoribosyltransferase (UPRTase, EC 2.4.2.9)[1] catalyzes the formation of UMP and pyrophosphate (PP_i) from uracil and 5-phosphoribosyl-α-1-diphosphate (PRPP), a step in pyrimidine salvage. UPRTase is a member of the phosphoribosyltransferases (PRTases) that all catalyze transfer of the 5-phosphoribosyl moiety from PRPP to a nitrogenous base (usually a nucleobase) with the release of PP_i. This general reaction is a common step in salvage and de novo pathways for nucleotide synthesis as well as the biosynthetic pathways for histidine and tryptophan *(1, 2)*.

UPRTase was discovered in *Lactobacillus* by Kornberg and co-workers *(3)*. The enzyme was later detected in many microorganisms, but has never been found in mammals, where the weak UPRTase activity is catalyzed by the orotate phosphoribosyltransferase part of UMP synthase *(4)*. UPRTase has been partly characterized from a variety of organisms, including *Escherichia coli (5−8)*, thermophilic bacteria and archea *(9, 10)*, baker's yeast *(11)*, and the pathogenic organisms *Neisseria meningitidis*, *Giardia intestinalis*, *Candida albicans*, *Mycoplasma mycoides*, and *Crithidia luciliae (12−16)*. In addition, UPRTase homologous genes have been cloned and sequenced from several organisms. Cloned genes encoding UPRTase from *E. coli* and *Bacilli* have been expressed in *E. coli* and the recombinant prokaryotic UPRTases characterized to varying extents *(6, 7, 9, 17−20)*. The UPRTase from *E. coli* itself is activated by GTP and regulated at the level of quaternary structure by its ligands *(7)*. Activation by GTP has also been observed for other UPRTases *(10, 13)*, but it does not seem to be a general property of this type of PRTase. Based on analysis of the initial velocity kinetics, it has been proposed that UPRTase from baker's yeast catalyzes the reaction by a ping-pong mechanism *(11)*. However, more recent investigations have revealed sequential mechanisms for three other PRTases *(21−24)*.

PRPP synthase and the type 1 PRTases, which appear to constitute all PRTases with the exceptions of nicotinate and quinolinate PRTases *(25)*, share a homologous sequence motif of 13 amino acid residues, which has been termed the PRPP binding site *(26, 27)*. It has been shown that two neighboring acidic residues in the PRPP binding site are very important for the catalytic function of *E. coli* PRPP synthase *(28)* and *S. typhimurium* OPRTase *(29)*; and in the published structures of PRTases with PRPP or nucleotides bound in

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[1] Abbreviations: PRPP, 5-phosphoribosyl-α-1-pyrophosphate; UPRTase, uracil phosphoribosyltransferase (EC 2.4.2.9); HGPRTase, hypoxanthine-guanine phosphoribosyltransferase; OPRTase, orotate phosphoribosyltransferase; PRTase, phosphoribosyltransferase; PP_i, pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; $K_{M,PRPP}$, K_M for PRPP; $K_{M,uracil}$, K_M for uracil.

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the active sites, these acid residues are in the vicinity of the 2′,3′-hydroxyl groups of the ribose ring *(30−32)*. The UPRTases have an unusual PRPP binding site, which contains a proline residue at a position where all other type 1 PRTases and PRPP synthases have aspartate (see Results). We wanted to test the significance of this proline for the activity of UPRTase from *E. coli*. For that purpose, we changed the residue to an aspartate to make the PRPP binding site be more similar to the "standard" PRPP binding motif. The kinetics of the wild-type and P131D mutant enzymes were characterized. We found that the UPRTase of *E. coli* obeyed the kinetics of a sequential reaction mechanism and that the proline residue in the PRPP binding site was important for the catalytic rate and the K_M for uracil, rather than for PRPP binding to the free enzyme.

MATERIALS AND METHODS

Materials. Nucleotides, uracil, and PRPP were purchased from Sigma. DEAE-cellulose (DE52) and DEAE (DE81) chromatographic paper were from Whatman Biosystems Ltd.; Blue-Dextran-Sepharose was prepared as described *(33)*. Radioactive nucleotides, [^{32}P]pyrophosphate, and [2-^{14}C]-uracil were obtained from DuPont NEN. [β-^{32}P]PRPP was prepared from [γ-^{32}P]ATP and ribose 5-phosphate as described previously *(7)*. [2-^{14}C]UMP was a gift from H. K. Jensen. Restriction endonucleases and T4 DNA ligase were from New England Biolabs. Alkaline phosphatase from calf intestine was bought from Boehringer (Mannheim). Antibiotics were from Sigma, and other fine chemicals were either from Sigma or from Merck.

Determination of PRPP Concentrations. The precise concentrations of PRPP in stock solutions were determined by means of UPRTase by monitoring the formation of [^{14}C]-UMP from [2-^{14}C]uracil in reactions, where [^{14}C]uracil was present in excess over PRPP.

Construction of Mutant and Host Strains. The plasmid pBM10 contains the wild-type *upp* gene from *E. coli* under the control of the strong $P_{A1/04/03}$ promoter *(7)*. We used this plasmid as a template for a two-step mutagenic PCR reaction. Mutagenesis was carried out by "overlap extension" with four primers *(34)*. The two mutant primers used to produce the P131D UPRTase mutant gene were 5′-cgctgatcgttgac-GACatgctggcaac-3′ and the complementary 5′-gttgccagcat-GTCgtcaacgatcagcg-3′, where capital letters indicate the nonmatching nucleotides. Two primers flanking the cloning region of the plasmid were used together with the mutant primers to amplify the *upp* gene as two separate fragments. The resulting two "megaprimers" were used as templates in a second round of PCR (including the flanking primers) to obtain a continuous copy of the mutant gene. A 339 bp *Kpn*I−*Hin*dIII DNA fragment containing the mutation was used to replace the wild-type *Kpn*I−*Hin*dIII DNA fragment of pBM10 to produce the mutant expression vector pCL6. The cloned region was verified by DNA sequencing using Thermo Sequenase dye terminator cycle sequencing premix kit from Amersham Life Science, and run on a Perkin-Elmer ABI PRISM 377 DNA Sequencer. Only the expected change of sequence was seen. The host strain SØ6710 (*E. coli* K12 F$^-$ *upp udp thi bio argA lysA car-96::*Tn*10*) was constructed by using strain SØ1140 (*upp udp thi bio argA lysA*) *(35)* as the recipient for transduction with P1 phages grown on strain NK6034 containing the *car-96::*Tn*10* mutation (*E. coli* Stock Center Database strain #6181). Transductants were selected on LB-agar plates containing tetracycline (4 mg/L), and tested for growth on minimal agar plates supplemented with glucose (0.2%), casamino acids (0.2%), thiamin (1 mg/L), biotin (1 mg/L), tetracycline (8 mg/L), and uridine (20 mg/L) or uracil (20 mg/L) as source of pyrimidine. Due to the *upp* mutation, the pyrimidine requirement of strain SØ6710 can be satisfied by uridine, but not by uracil.

Expression and Purification. The UPRTases were overexpressed in and purified from strain SØ6710 which was freshly transformed either with plasmid pBM10 *(7)* to produce wild-type enzyme or with plasmid pCL6 to isolate the P131D mutant enzyme. The transformation mixtures were added to 5 mL of LB-broth *(36)* supplemented with ampicillin (100 mg/L) and grown overnight at 37 °C. The cells were harvested by centrifugation, resuspended in 5 mL of fresh LB-broth, and added to a 5 L culture flask containing 1.5 L of LB-broth supplemented with ampicillin (100 mg/L) and grown to stationary phase overnight at 37 °C with vigorous shaking. UPRTase was purified at 4 °C or on an ice bath as previously described *(7)* except that the final ammonium sulfate precipitation and gel-filtration steps were omitted. The fractions from the Blue-Dextran-Sepharose column, which contained most of the UPRTase activity, were pooled and dialyzed exhaustively against 10 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.3. The enzymes, which were about 90% homogeneous as judged from electrophoresis in polyacrylamide gels, were dispensed in aliquots of 0.5 mL (≈5 mg/mL) and stored at 4 °C for immediate use or frozen at −20 °C for long-time storage.

Determination of the Absorption Coefficient of UPRTase. Three milliliters of a solution of wild-type UPRTase was dialyzed overnight against 1 L of 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.5. After dialysis, the enzyme solution had the absorbance $A_{280} = 3.25$. Ten aliquots of 150 μL each were pipetted into carefully weighed Eppendorf tubes and dried at 60 °C overnight. Ten aliquots of the dialysate were treated equally. From the weight of the dried tubes that contained either protein plus salts or only the salts, it appeared that 1.5 mL of enzyme solution contained 7.42 mg dry weight of protein, and thus it was calculated that UPRTase (1 mg/mL) has an absorbance $A_{280} = 0.65$.

Enzyme Assays. (A) Assay A. The standard assay was used to measure UPRTase activity in the direction of UMP synthesis. The assay mixture (50 μL) contained 20 mM Tris/HCl, pH 8.5, 5 mM $MgCl_2$, 1 mM GTP, 1 mM PRPP, and 100 μM [2-^{14}C]uracil (≈5000 cpm/nmol), and the temperature was 37 °C. The enzyme was preincubated for 5 min at 37 °C with all substrates except uracil. Reactions were started by the addition of [^{14}C]uracil (time = 0). Aliquots (10 μL) were added to 5 μL of 0.33 M formic acid at 0 min (i.e., <10 s), 5, 10, and 15 min to stop the reaction, and the entire samples were applied to the centers of 1.5 cm × 1.5 cm squares drawn on a sheet of DE81 chromatographic paper. The sheet was washed with running deionized water to remove unreacted [2-^{14}C]uracil, and the radioactivity retained, i.e., [2-^{14}C]UMP, was determined as described *(9)*. The preparation of [2-^{14}C]uracil that we used contained 0.8% of a radioactive contaminant, which was retained on the ion exchange paper and disturbed determination of the reaction product under conditions, when the concentration of PRPP

was low relative to the concentration of uracil. Therefore, for some measurements, sampling was more frequent, and [2-^{14}C]UMP was separated from [2-^{14}C]uracil and from the application spot by chromatography on polyethylene-impregnated cellulose thin-layer plates in 0.9 M acetic acid−0.1 M LiCl. In these cases, the radioactivity in UMP and uracil was determined using a Packard Instant Imager 2024. The concentrations of PRPP and uracil were varied as described in the text.

(B) Assay B. This assay was designed to determine UPRTase activity in the direction of PRPP formation. The assay mixture (100 μL) contained 20 mM Tris/HCl, pH 8.5, 5 mM $MgCl_2$, 1 mM GTP, 100 μM PRPP, 1 mM UMP, and 1 mM $^{32}PP_i$. The enzyme (5 μg/mL) was incubated for 5 min at 37 °C with all substrates except PP_i. Reaction was started by addition of $^{32}PP_i$ (time = 0). Ten microliter aliquots were mixed with 5 μL of 0.33 M formic acid at 0, 5, 10, and 15 min to stop the reaction, and the samples were loaded on poly(ethylenimine)-impregnated cellulose thin-layer plates and chromatographed in 0.85 M potassium phosphate, pH 3.4, to separate PP_i from PRPP *(37)*. The amount of radioactivity in the PP_i and PRPP spots on the chromatograms was quantified with a Packard Instant Imager 2024.

Isotope Exchange Reactions. Studies of $^{32}PP_i$−PRPP exchange were performed as described under assay B, but with omission of UMP.

Ligand Binding Studies. Radioactively labeled ligands were mixed with UPRTase (≈1.2 nmol) in a total volume of 135−150 μL of 50 mM Tris/HCl, pH 8.5, 8 mM $MgCl_2$, 1 mM GTP (in the case of PRPP binding, additional [^{3}H]-deoxyglucose was added for volume correction). The binding assays were performed at room temperature (23 °C) as described by Ormö and Sjöberg *(38)*. Aliquots (30 μL) of the mixtures were withdrawn for determination of radioactivity in the total concentration of ligand (L_t). The majority (100 μL) of each mixture was transferred to Millipore Ultrafree-MC Centrifugal Filter Units (Polysulfone PTTK 30 000) and centrifuged for 5 min at 5000*g* at room temperature (23 °C). The concentration of free ligand (L_f) was determined by counting the radioactivity in aliquots (30 μL) of the buffer that had passed through the filter.

Heat Stability. The enzymes at a concentration of 100 μg/mL were incubated at different temperatures in 20 mM Tris/HCl, 5 mM $MgCl_2$, 1 mM GTP, pH 8.5. After heating for 10 min, the samples were cooled in an ice bath and assayed, and the remaining activity was assayed as described in assay A.

Differential Scanning Calorimetry. The microcalorimeter, MC2, was used according to the instructions from the manufacturer (MicroCal, Inc.). The protein samples (approximately 1.5 mg/mL) were dialyzed against 10 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5. Before loading the protein sample in the cell (1.2 mL), it was degassed and stirred in an evacuated chamber for 10 min. Degassed dialysate was loaded in the reference cell. The scan rate was 1 °C/min, and a pressure of 2 Bar of nitrogen was kept over the cells. The program Origin 3.1, also from MicroCal Inc., was used to evaluate the scans.

Data Treatment. Initial velocity patterns and data from product inhibition studies were fitted to the following equations using the program HYPER of Cleland *(39)*.

$$v_o = V_{max}\{K_{is}K_{M,uracil}/[PRPP][uracil] + K_{M,PRPP}/[PRPP] + K_{M,uracil}/[uracil] + 1\}^{-1} \quad (1)$$

$$v_o = V_{max}\{1 + K_M(1 + [I]/K_i)/[S]\}^{-1} \quad (2)$$

$$v_o = V_{max}\{1 + [I]/K_{ii} + K_M/[S](1 + [I]/K_{is})\}^{-1} \quad (3)$$

Equation 1 is the rate equation for sequential bi-bi mechanisms, eq 2 is the rate equation for competitive inhibition, and eq 3 is the rate equation for noncompetitive inhibition.

Data from single saturation experiments and the ligand binding data were fitted with the BIOSOFT program UltraFit 3.0 for Macintosh using the following equations:

$$EL = nE_tL(K_D + L)^{-1} \quad (4)$$

$$v_0 = V_{max}S(K_M + S)^{-1} \quad (5)$$

Equation 4 is the single site binding equation, where *EL* is the concentration of the enzyme−ligand complex, E_t is the total concentration of enzyme monomer, *L* is the concentration of free ligand, K_D is the dissociation constant, and *n* is the number of binding sites per monomer. Equation 5 is the Michaelis−Menten equation.

RESULTS

Unusual PRPP Binding Site in UPRTases. The type 1 phosphoribosyltransferases and PRPP synthases share a sequence motif of 13 amino acid residues, which has been named the PRPP binding site *(26, 27)*. We have aligned all currently known sequences of UPRTases to determine the consensus sequence of the PRPP binding site of this enzyme (Figure 1A). The sequence was found to be: VIIV**DPM**-L**ATG**GS, with boldface letters indicating completely conserved residues and lightface upper case letters showing the most abundant residue in that position. The consensus PRPP binding sites for other type 1 PRTases and the PRPP synthases were determined in a similar way and are compiled in Figure 1B. From the analysis, it appears that the consensus sequence for each class of PRTase has characteristic, conserved deviations from the general consensus PRPP binding site (ΦΦΦΦDDΦΦXTGGT, with Φ indicating hydrophobic residues and X any amino acid), and that these variations thus correlate with the substrate specificity of the enzyme. The PRPP binding site of UPRTase is unusual, because it contains a fully conserved proline residue (Pro 131 in the *E. coli* enzyme) at a position in the sequence where other type 1 PRTases and the PRPP synthases have an aspartate residue. We wanted to analyze the importance of the conserved proline for the activity of UPRTase from *E. coli*. Therefore, we constructed and purified the P131D mutant enzyme (as described under Materials and Methods) and compared its kinetic behavior with the kinetics of the wild-type enzyme. The substitution of Pro 131 with aspartate did not affect the stability of the protein, judged from inactivation studies which showed that 50% of the activity was lost by heating for 10 min at 56 and 57 °C for wild type and mutant enzyme, respectively, and from differential scanning calorimetry, which revealed denaturation temperatures of 57.5 and 58.7 °C for the wild-type and mutant proteins (not shown).

A

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MYCGE     VIILDPMLATGTT
MYCPN     VIILDPMLATGTT
SYNY3     LLLVDPMLATGNT
LACLA     IFLVDPMLATGGS
STRSL     IFVVDPMLATGGS
YEAST     VFLLDPMLATGGS
BACCL     FIIVDPMLATGGS
BACSU     FIVVDPMLATGGS
ECOLI     ALIVDPMLATGGS
HAEIN     SIVVDPMLATGGS
MYCHO     IIIVDPMLATGSS
TOXGO     VMLLDPMCATAGS
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B

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pyr5      CLIVEDVV-TSGS
pyrE      VLIVDDVI-TAGT
hprt      VLIVEDIIDTGKT
upp       VLLVDPMLATGGS
apt       VVIVDDLLATGGT
prs       AILVDDMIDTGGT
pur1      VVLVDDSIVRGTT
his1      ADAIVDLVESGXT
trpD      XXXVVDIVGTGGD
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FIGURE 1: (A) Alignment of the hypothetical PRPP binding site in deduced amino acid sequences homologous to *E. coli* UPRTase. Alignments were done using the PILEUP program in the GCG package (University of Wisconsin, Madison, WI). White letters on black background indicate the central highly conserved part of the motif. Black letters on gray indicate positions with similar types of amino acid residues, I/L/V or S/T. The organisms were the following: BACCL, *Bacillus caldolyticus* (P70881); BACSU, *Bacillus subtilis* (P39149); ECOLI, *Escherichia coli* (P25532); HAEIN, *Haemophilus influenzae* (P43857); LACLA, *Lactococcus lactis* (P50926); MYCGE, *Mycoplasma genitalis* (P47276); MYCHO, *Mycoplasma hominis* (P43049); MYCPN, *Mycoplasma pneumonia* (P75081); STRSL, *Streptococcus salivarius* (P36399); SYNY3, *Synechocystis sp*. (P72753); TOXGO, *Toxoplasma gondii* (Q26998); YEAST, *Saccharomyces cerevisiae* (P18562). The shading highlights the most conserved parts of the PRPP binding site. (B) Alignment of PRPP binding site consensus sequences from different, some hypothetical, type 1 PRTases and PRPP synthase. The consensus sequences originate from alignments (not shown) of the deduced amino acid from the following: pyr5, the OPRTase part of 7 UMP synthases; pyrE, 25 OPRTases; hprt, 17 HGPRTases; upp, 16 UPRTases; apt, 23 adenine PRTases; prs, 27 PRPP synthases; xgpt, 4 xanthine−guanine PRTases; pur1, 11 amido PRTases; his1, 9 ATP−PRTases. Shading indicates the minimal degree of conservation of each residue within the single families: black, 100%; light gray, ≥70%; dark gray, 100% D or E. Unshaded residues indicate the most abundant residue at the position.

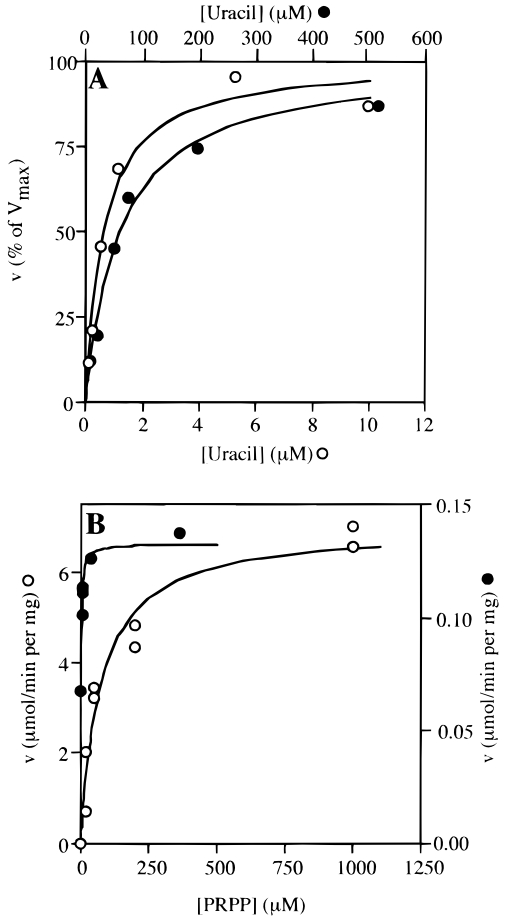


FIGURE 2: Saturation of UPRTase with uracil (A) and PRPP (B) in the presence of fixed (almost) saturating concentrations of the second substrate. Wild-type UPRTase, open symbols; P131D mutant UPRTase, closed symbols. (A) The assay mixtures contained 20 mM Tris-HCl, 1 mM GTP, 1 mM PRPP, 5 mM $MgCl_2$, pH 8.5, and the indicated concentrations of [^{14}C]uracil. Different scales were used to indicate the uracil concentration used for the two enzymes. For clarity reasons, the initial velocities are given as percent of V_{max}, which was 7 μmol min^{-1} mg^{-1} for the wild-type enzyme and 0.13 μmol min^{-1} mg^{-1} for the mutant protein. (B) The assay mixtures contained 20 mM Tris-HCl, 1 mM GTP, 5 mM $MgCl_2$, pH 8.5, the indicated concentrations of PRPP, and either 100 μM [^{14}C]uracil for the wild-type or 500 μM for the mutant protein. Both different abscissa and ordinate axes were used for the two enzymes. Assay procedure A was used, and the curves represents the best fit to the Michaelis−Menten equation (eq 5).

Initial Comparison of Wild-Type and P131 Mutant UPRTases. The activity of UPRTase from *E. coli* is stimulated by GTP *(5−8)*. The concentration of GTP needed to give half-maximal activation of both mutant and wild-type enzymes was found to be 80 μM (data not shown). All kinetic experiments described in this work were therefore performed in the presence of 1 mM GTP to have maximal activity.

The curves in Figure 2A show the saturation of wild-type and mutant UPRTases with uracil in the presence of 1 mM PRPP, which is saturating for both enzymes (see below). The P131D mutation resulted in a 54-fold reduction of the V_{max} of the enzyme from 7 μmol min^{-1} mg^{-1} for the wild-type protein to 0.13 μmol min^{-1} mg^{-1} for the mutant, and to our surprise the K_M for uracil ($K_{M,uracil}$) had increased dramatically (about 100-fold), from 0.63 ± 0.12 μM for the wild type to 60 ± 8 μM for the mutant enzyme. Figure 2B shows the saturation of the enzymes with PRPP at fixed, almost saturating concentrations of uracil, i.e., 100 μM for the wild type and 500 μM for the mutant protein. The K_M for PRPP was 73 ± 19 μM for the wild-type enzyme, in agreement with previous determinations *(6, 7)*, but the K_M for PRPP had become very low for the mutant enzyme and difficult to determine accurately. The saturation curve shown in Figure 2B was made with PRPP concentrations in the interval 1.8−360 μM in the presence of 0.5 mM uracil, which is 8−9-fold higher than $K_{M,uracil}$. It indicated that $K_{M,PRPP}$ is 1.3 ± 0.4 μM and that V_{max} is 132 ± 6 nmol min^{-1} mg^{-1}, in agreement with several other measurements (not shown). Thus, it appears that $k_{cat}/K_{M,uracil}$ had decreased 3−4 orders of magnitude as a result of the P131D mutation, while $K_{M,PRPP}$ had decreased approximately 56-fold in parallel to k_{cat}, so that $k_{cat}/K_{M,PRPP}$ remained essentially unaffected. To understand the meaning of these changes in the kinetic parameters

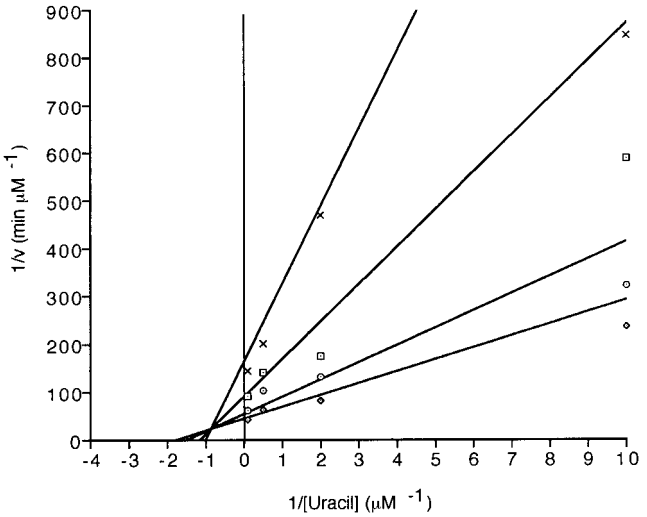


FIGURE 3: Double-reciprocal plots of the forward UPRTase reaction catalyzed by wild-type UPRTase. Experiments were carried out in 20 mM Tris-HCl and 5 mM $MgCl_2$, pH 8.5, as described under Materials and Methods. The concentration of uracil was varied from 0.1 to 10 μM in the presence of different fixed concentrations of PRPP: (crosses) 20 μM; (squares) 50 μM; (circles) 200 μM; and (diamonds) 1000 μM. The lines were fitted to the data by use of eq 1.

Table 1: Kinetic Parameters and Equilibrium Binding Constants for Wild-Type and P131D Mutant UPRTases

kinetic constant	wild-type enzyme	P131D mutant enzyme
k_{cat}, forward (s^{-1})	2.7	0.050 ± 0.002
apparent k_{cat}, reverse (s^{-1})	0.024 ± 0.002	0.00041 ± 0.00003
$K_{M,PRPP}$ (μM)	58 ± 21	1.3 ± 0.3
$K_{M,uracil}$ (μM)	0.53 ± 0.21	60 ± 8
$K_{D,PRPP}$ (μM)	14 ± 3	9 ± 2
$K_{D,uracil}$ (μM)	NB[a]	NB[a]
K_{D,PP_i} (μM)	NB[a]	NB[a]
$K_{D,UMP}$ (μM)	14 ± 4	30 ± 8
$k_{cat}/K_{M,uracil}$ ($mM^{-1}\ s^{-1}$)	5100	0.83
$k_{+1} = k_{cat}/K_{M,PRPP}$ ($mM^{-1}\ s^{-1}$)	47	38
$k_{-1} = K_{D,PRPP} \times k_{+1}$ (s^{-1})	0.7	0.3

[a] NB, no binding could be detected.

in the P131D mutant enzyme, it was necessary to investigate the reaction mechanism for the UPRTase from *E. coli*, which had not been determined previously.

Sequential Reaction Mechanism of Wild-Type UPRTase. The kinetics of the reaction in the direction of UMP synthesis were determined by measuring initial reaction rates at varying concentrations of either uracil or PRPP, using assay A described under Materials and Methods. When the reciprocals of the reaction velocities were depicted as a function of the reciprocal PRPP concentrations at different concentrations of uracil, a series of intersecting lines was observed (Figure 3). Such an initial velocity pattern is characteristic for sequential mechanisms, while ping-pong mechanisms produce parallel lines in similar plots *(40)*. Therefore, the data in Figure 3 were fitted to the rate equation for a sequential reaction (i.e., eq 1 under Materials and Methods). The resulting kinetic constants are in good agreement with those obtained during the initial characterization and are listed in Table 1.

We also attempted to measure the rates of isotope exchange, when single substrate/product pairs (i.e., PRPP plus $^{32}PP_i$ or [2-^{14}C]uracil plus UMP) were incubated with UPRTase, since such exchange reactions are indicative for ping-pong mechanisms *(40)*. No transfer of the radioactive label between the reactants could be detected, even after 2 h incubation with UPRTase at a concentration of 0.5 mg/mL, in agreement with the sequential mechanism defined above.

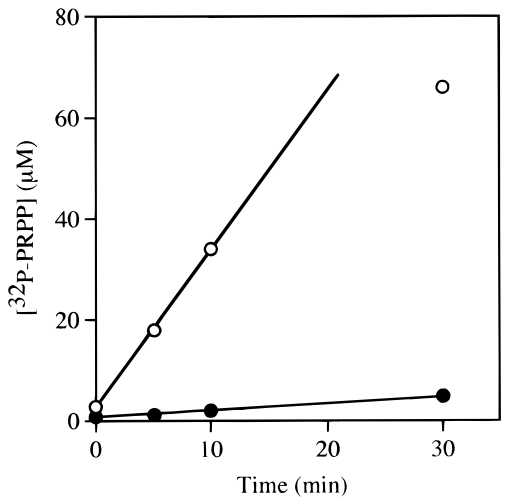


FIGURE 4: Reverse reaction of UPRTase. The assay mix contained 20 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 1 mM GTP, 1 mM $^{32}PP_i$, 1 mM UMP, 100 μM PRPP, and wild type (open circles) or P131D mutant (closed circles) UPRTases to a final concentration of 40 μg/mL in the assay (assay procedure B).

Equilibrium of the Reaction. The equilibrium for the UPRTase reaction was estimated from an experiment where the enzyme was incubated with 1 mM $^{32}PP_i$ and 1 mM UMP. The formation of [^{32}P]PRPP was monitored for 2 h at 37 °C. It had apparently reached equilibrium at a concentration of PRPP equal to 8−9 μM (data not shown), which corresponds to an equilibrium constant, $K_{eq} = [PP_i]_{eq} \times [UMP]_{eq} \times ([PRPP]_{eq} \times [uracil]_{eq})^{-1}$, of $(12-15) \times 10^3$. This equilibrium constant is between that observed for the OPRTase reaction, $K_{eq} = 0.1-0.6$ *(23, 41−44)*, and the equilibrium constant for the guanine phosphoribosyltransferase reaction, $K_{eq} = (1.8-4.3) \times 10^5$ *(21, 45)*.

Reverse Reaction. To determine the kinetics of the reverse reaction, i.e., formation of [^{32}P]PRPP and uracil from UMP and $^{32}PP_i$, we included unlabeled PRPP in the reaction mixtures to prevent back-reaction of the formed [^{32}P]PRPP. The mixtures contained 1 mM UMP, 1 mM $^{32}PP_i$, 100 μM unlabeled PRPP, and 40 μg/mL−0.5 mg/mL of UPRTase, and they were analyzed by chromatography on thin-layer plates after different times of incubation. From the early time points of the curve in Figure 4, we could estimate that the initial velocity of the exchange reaction was 0.070 μmol of [^{32}P]PRPP formed from $^{32}PP_i$ min^{-1} mg^{-1}. This rate corresponds to an apparent value of k_{cat} for the reverse reaction equal to 0.027 s^{-1}, which may differ from the true k_{cat} because the substrate concentrations were not extrapolated to saturation. Figure 4 also shows similar measurements of the reverse reaction for the P131D mutant enzyme. The reaction rate in this direction was 59-fold lower than seen for the wild-type protein.

Product Inhibition. To determine the reaction order of UPRTase, we measured the inhibition by the products UMP and PP_i in the presence of variable concentrations of PRPP

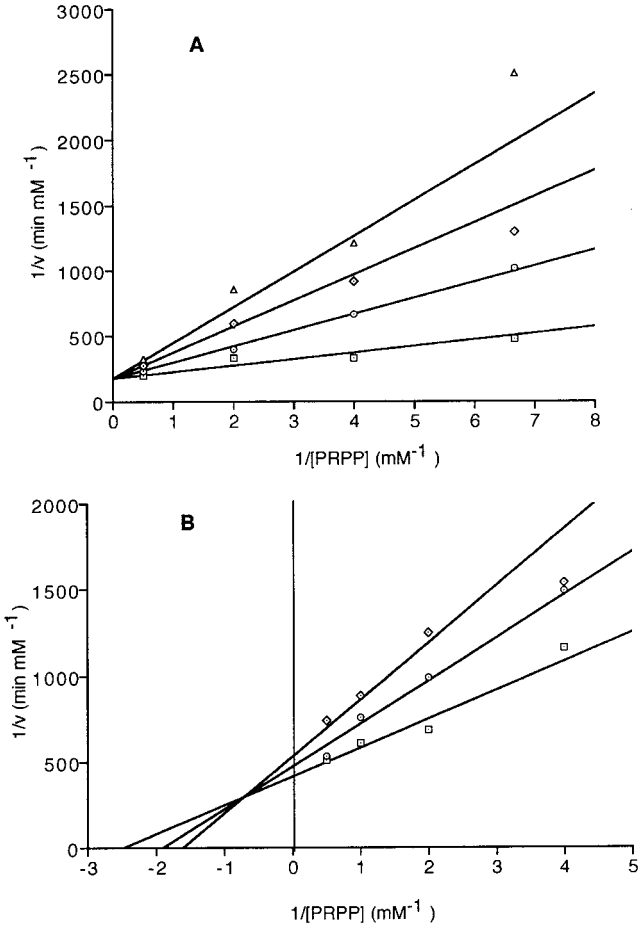


FIGURE 5: Inhibition patterns of products UMP (A) or pyrophosphate (B) against PRPP. The initial rates were measured at variable PRPP concentrations at different fixed inhibitor concentrations. (A) The concentration of PRPP was varied from 125 μM to 2 mM, while the concentration of uracil was kept constant at 100 μM. Different concentrations of UMP were added to the reaction: (squares) 0; (circles) 0.33; (diamonds) 0.66; and (triangles) 1 mM UMP. The lines are a fit to the equation for competitive inhibition (eq 2). (B) PRPP concentration was varied from 0.25 to 2.0 mM at fixed levels of PP_i. PP_i concentrations are (squares) 0, (circles) 0.5, and (diamonds) 1 mM. Lines are a fit to eq 3 for noncompetitive inhibition.

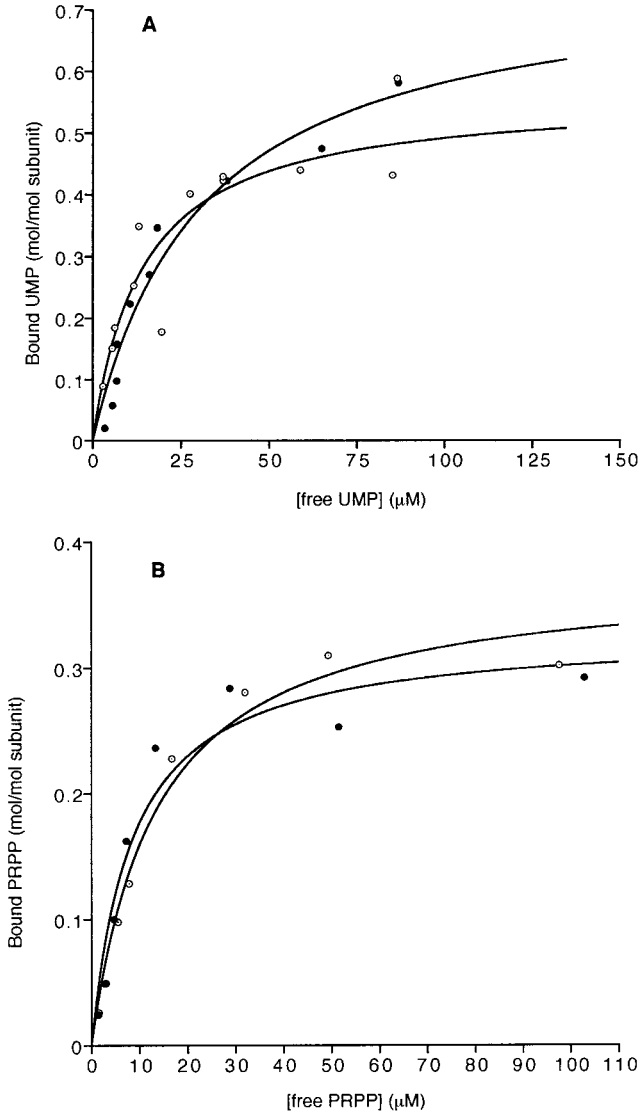


FIGURE 6: Binding of [^{14}C]UMP (A) and [β-^{32}P]PRPP (B) to UPRTase. (A) [^{14}C]UMP was used in the concentration range 5−100 μM. Wild-type enzyme (open circles) or P131D mutant enzyme (closed circles). The subunit concentrations were approximately 40 μM. (B) [β-^{32}P]PRPP was used at 10−250 μM. Wild-type UPRTase (open circles) was used at a subunit concentration of 117 μM; the P131D mutant enzyme (closed circles) was used at a subunit concentration of 88 μM. Lines are best fits to eq 4 for ligand binding.

and a fixed and saturating concentration of uracil (100 μM). From Figure 5A it is seen that UMP acted as a competitive inhibitor versus PRPP, whereas PP_i produced noncompetitive inhibition (Figure 5B). This inhibition pattern agrees best with an ordered mechanism, in which PRPP binds as the first substrate and UMP dissociates as the last product, although such a mechanism is expected to give rise to uncompetitive inhibition between PP_i and PRPP *(40)*. The noncompetitive inhibition might result from formation of a dead-end complex between enzyme, uracil, and PP_i.

Ligand Binding. The reaction order was also investigated by measuring the binding of individual ligands to the free enzyme. We observed binding of UMP and of PRPP to the free enzyme, and the measured dissociation constants are summarized in Table 1. The dissociation constant (K_D) for UMP was about 16 μM (Figure 6A), in agreement with previous findings *(7)*. The K_D for PRPP was 14 ± 3 μM (Figure 6B) and somewhat lower than that observed by Jensen and Mygind *(7)*, who used a much slower technique to measure ligand binding. Absolutely no binding of uracil to the free enzyme could be detected, and binding of pyrophosphate was weak or nonexisting. However, we were unable to use high concentrations of PP_i (>1 mM) in these assays, because $MgPP_i$ precipitated. In the presence of 1 mM UMP, substantial binding of $^{32}PP_i$ occurred, $K_D = 164 \pm 34$ μM (data not shown), and in binding assays, when the enzyme was incubated with [^{14}C]uracil and PP_i, some retention of uracil by the enzyme was seen, but it only correspond to 10% of the enzyme concentration and no convincing binding curves could be made.

The binding data for the mutant enzyme are presented together with the data for the wild-type protein, and the resulting K_D values are listed in Table 1. As for the wild-type enzyme, we did not detect binding of uracil or PP_i, while PRPP and UMP showed good binding to the free enzyme (Figure 6B). The dissociation constants for these interactions were similar to or up to 5-fold higher than the corresponding

constants for the wild-type enzyme (Table 1). Thus, the binding data for the P131D mutant enzyme were in accordance with the sequential reaction mechanism, and the moderate changes of the K_D values cannot explain the large changes in the K_M values.

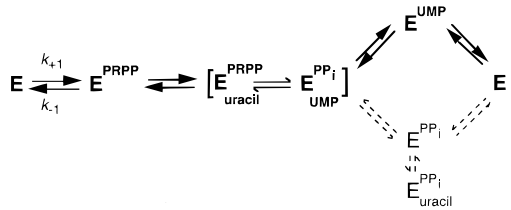
DISCUSSION

The results herein support a sequential mechanism for UPRTase with the obligatory binding of PRPP preceding binding of uracil. The order by which the products are released is less clear. Both the competitive inhibition by UMP against PRPP and the binding assays show that the free enzyme has high affinity for UMP. The binding of PP_i, to the apoenzyme, on the other hand, was at best very weak. These observations might indicate that PP_i, is released before UMP, but such a strictly ordered mechanism is expected to give uncompetitive inhibition by PP_i against PRPP at the high and saturating concentration of uracil that was used ([uracil] = 100 μM $\geq$ 150$K_{M,uracil}$). The observed noncompetitive inhibition by PP_i against PRPP may indicate that PP_i can bind to the free enzyme with low affinity and form a dead-end complex with the nucleobase (uracil). Our binding assays gave only weak support for the existence of such a complex. However, similar dead-end complexes have been observed for both the orotate and hypoxanthine−guanine and phosphoribosyltransferases (*21, 46*) and, interestingly, in the latter case, it was formed even without detectable binding of the individual ligands (hypoxanthine or PP_i) to the apoenzyme (*21*). These considerations about the mechanism of UPRTase are summarized in Scheme 1.

The sequential mechanism is consistent with the inversion of the configuration at the anomeric carbon-1 of the pentose moiety, from an α-configuration in PRPP to a β-configuration in UMP, since it implies that reaction takes place as a direct substitution between the two substrates, while they are both bound to the enzyme. However, the mechanism deviates from the ping-pong mechanism, which was proposed for the UPRTase from baker's yeast *(11)*. We do not know the reason for this discrepancy, but will emphasize that intersecting lines for sequential mechanisms may sometimes appear almost parallel *(23, 47)* and that studies of isotope exchange reactions, which are indicative for ping-pong mechanisms, were not performed for the yeast enzyme *(11)*.

It appears that *E. coli* UPRTase is kinetically more closely related to HGPRTase than to OPRTase, even though UPRTase and OPRTase both participate in biosynthesis of pyrimidine nucleotides, and although orotate only deviates from uracil by the presence of a carboxylate group in the 6-position. OPRTase follows a random sequential mechanism *(23)*, while the human HGPRTase follows a strictly ordered mechanism *(21, 45)*. Both HGPRTase and OPRTase crystallize as dimers, but a significant difference between the two enzymes is that two (shared) active sites are formed in the interface between the subunits of the dimer of OPRTase *(29, 48)*, while the two active sites of the dimer of HGPRTase point away from the subunit interface *(30)*. The recently determined crystal structures of the UPRTases from *Bacillus caldolyticus* (A. Kadziola and S. Larsen, personal communication) and *Toxoplasma gondii (49)* have revealed that these enzymes also form dimers with the two active sites pointing away from the subunit interface and show that the oligomeric state of UPRTase thus more closely resembles HGPRTase than OPRTase.

Scheme 1: Possible Sequential Reaction Mechanism for UPRTase from *E. coli*[a]



[a] The reactions indicated by solid lines are believed to constitute the predominant pathway.

Our primary reason for studying the reaction mechanism of UPRTase was to test the catalytic importance of the unusual proline in the PRPP binding site in this type of PRTase. We expected that changing Pro 131 to an aspartic residue might increase the affinity for PRPP, since the PRPP binding site would become more similar to the consensus sequence of the general PRPP binding site. Instead, we observed that this amino acid substitution caused a 54−59-fold reduction in the catalytic rate in both directions of the reaction and resulted in an approximately 100-fold increase in the K_M for uracil. The K_M for PRPP was reduced 45−56-fold by the mutation, and, provided that the mutation does not interfere with PRPP binding to the free enzyme, this change of the K_M for PRPP is an expected consequence of the reduced k_{cat} for the mutant enzyme, since the K_M for the first substrate for enzymes with ordered sequential reaction mechanisms generally is k_{cat}/k_{+1} (k_{+1} being the association rate constant for binding of the first substrate to the free enzyme *(39)*).

Product release limits the reaction rate of some PRTases *(21, 24)*, but since both the k_{cat}/K_M for PRPP (=k_{+1}) and the equilibrium constant K_D for dissociation of the enzyme−PRPP complex were essentially unaffected by the P131D mutation, which leads to the conclusion that the dissociation rate constant (k_{-1}) for release of PRPP from the enzyme−PRPP complex is also unchanged (Table 1), it appears that the P131D mutation has primarily interfered with the binding of uracil to the enzyme−PRPP complex and interactions that are necessary for the overall catalytic rate. Although known ground-state crystal structures show residues in the P131 position interacting only with the ribose hydroxyls, with the base some distance away, we hypothesize that the P131D mutation has disturbed catalytic interactions in the transition state in UPRT and that both the increase in $K_{M,uracil}$ and the reduction in k_{cat} may result from a misalignment of the bound PRPP with residues that are involved in contacting the incoming uracil and stabilizing the transition state of the reaction.

In conclusion, we have shown that UPRTase from *E. coli* works by a sequential mechanism with PRPP binding prior to uracil. We believe that we have also shown that the unusual proline in the PRPP binding site of the UPRTases is critical for the catalytic rate and for saturation of the enzyme with uracil, but not important for binding of PRPP to the free enzyme. In more general terms, these results may suggest that different groups of PRTases have acquired the conserved sequence variations in the PRPP binding site in order to optimize the interaction with the second substrate

and the transition state of the specific reaction that they catalyze.

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