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Kinetic Mechanism of Orotate Phosphoribosyltransferase from *Salmonella typhimurium*†

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ABSTRACT: The chemical mechanism of the phosphoribosyltransferases (PRTases), although largely unknown, may proceed either via a concerted direct-transfer mechanism or with a two-step mechanism involving a carboxonium-like intermediate. To study this question, we have cloned the *Salmonella typhimurium pyrE* gene, coding for the enzyme orotate phosphoribosyltransferase (EC 2.2.4.10, OPRTase), and developed a bacterial strain that overproduces the enzyme, which we have purified to homogeneity. Initial velocity and product inhibition studies indicated that *S. typhimurium* OPRTase follows a random sequential kinetic mechanism. This result was further confirmed by equilibrium isotope exchange studies on two substrate–product pairs, PRPP–PP_i and OMP–orotate. In addition, the rates of the individual equilibrium isotope exchanges allowed us to conclude that PP_i release and PRPP release were the rate-determining steps in the forward and reverse reactions, respectively. Although partial reactions between the two substrate–product pairs, PRPP–PP_i and OMP–orotate, were observed, further studies revealed that these exchanges were a result of contaminations. Our results are significant in that *S. typhimurium* OPRTase, like most PRTases but in contrast to its yeast homologue, follows sequential kinetics. The artifactual partial isotope exchanges found in this work may have implications for similar prior work on the yeast enzyme. In view of the careful isotope effect studies of Parsons and co-workers [Goitein, R. K., Chelsky, D., & Parsons, S. M. (1978) *J. Biol. Chem. 253*, 2963-2971] and the results obtained by us, we propose that PRTases may involve a direct-transfer mechanism but with low bond order to the leaving pyrophosphate moiety and attacking base.

Orotate phosphoribosyltransferase (EC 2.2.4.10, OPRTase)[1] catalyzes the formation of an N-glycosidic bond between α-D-5-phosphoribosyl pyrophosphate (PRPP) and orotate to form orotidine 5-phosphate (OMP, see Scheme I), a step in the de novo biosynthesis of the pyrimidine nucleotides (Neuhard & Nygaard, 1987). The transferase reaction is accompanied by anomeric inversion at the C1 of the ribosyl group (Chelsky & Parsons, 1975). OPRTase is one of a family of

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[1] Abbreviations: PRPP, α-D-5-phosphoribosyl pyrophosphate; HGPRTase, hypoxanthine–guanine phosphoribosyltransferase; OPRTase, orotate phosphoribosyltransferase; UPRTase, uracil phosphoribosyltransferase; IPTG, isopropyl β-D-thiogalactopyranoside; TEA, triethylammonium; TSK-DEAE, diethylaminoethyl-substituted Toyo-Pearl resin.

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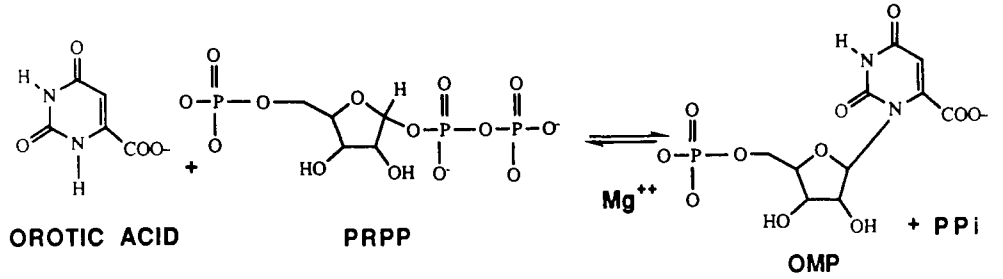
Scheme I: OPRTase Reaction



10 phosphoribosyltransferases (PRTases) that catalyze similar reactions between PRPP and a nitrogenous base (Musick, 1981). The enzymes are of considerable importance in macromolecular precursor biosynthesis and have received attention as targets for rational chemotherapy.

The chemical mechanism of phosphoribosyl transfer by the PRTases remains largely unknown. Two mechanisms are feasible. The first is a direct-transfer mechanism involving a nucleophilic attack by orotate at C1 of PRPP and concomitant displacement of pyrophosphate. A second possibility is a two-step mechanism consisting of an initial elimination of the pyrophosphate from enzyme-bound PRPP and the formation of a carboxonium-like intermediate, followed by attack by orotate to yield OMP. Such enzyme-stabilized pyranosyl- and furanosyl-based carboxonium-type intermediates have been proposed by lysozyme (Kirby, 1987) and sucrase–isomaltase (Cogoli & Semenza, 1975). A third mechanism involving a covalent phosphoribosylated enzyme appears unlikely, on the basis of the stereochemical inversion at C1 (Chelsky & Parsons, 1975).

The mechanistic problem has been approached from several directions. Isotope effect studies on bacterial ATP-PRTase, yeast OPRTase, and human HGPRTase (Goitein et al., 1978) strongly indicate that carboxonium-type intermediates exist for those PRTase reactions. Supporting these results are initial velocity and product inhibition studies on yeast OPRTase (Victor et al., 1979) and yeast UPRTase (Natalini et al., 1979), which demonstrated Ping-Pong-type kinetic mechanisms with PP_i dissociation from the active site preceding binding of the base. Under conditions of elevated Mg^{2+}, human HGPRTase has also been shown to follow a Ping-Pong mechanism (Krenitsky & Papaioannou, 1969). Partial isotope exchanges reflective of an enzyme-bound 5-phosphoribose intermediate have been demonstrated in the case of yeast OPRTase (Victor et al., 1979). In addition, Sloan and coworkers have shown that yeast HGPRTase also catalyzes partial exchanges between PRPP and PP_i but not between the nitrogenous bases and their respective nucleotide products (Ali & Sloan, 1982). However, most PRTases display sequential kinetic mechanisms (Musick, 1981), which require that both substrates bind to enzyme before any product is released. Sequential kinetics are compatible with both chemical mechanisms. Thus, though present knowledge seems to favor a carboxonium-type transition state for PRTases, the direct-transfer-type mechanism has not been ruled out.

To approach this question, we have begun a study of bacterial OPRTase. We have cloned the *Salmonella typhimurium pyrE* gene and developed a bacterial strain that overproduces OPRTase, which we have purified to homogeneity. Initial velocity, product inhibition, and equilibrium isotope exchanges show that *S. typhimurium* OPRTase, like most PRTases but in contrast to its yeast homologue, follows sequential kinetics. In agreement with work on the yeast enzyme (Victor et al., 1979) and in contrast to the predictions of sequential kinetics, we were also able to detect partial exchange reactions between the two substrate–product pairs. In preliminary reports (Bhatia et al., 1989; Bhatia & Grubmeyer, 1990) we concluded that the kinetic mechanism was at least partially Ping-Pong. However, further investigation reported here has revealed that the OMP–orotate exchange arose via contamination of commercial OMP. The PRPP–PP_i exchange reflected a tenacious contamination of the enzyme, probably by OMP, that was removed by gel filtration of the urea-denatured enzyme, followed by renaturation. Thus, our kinetic results confirm that *S. typhimurium* OPRTase proceeds via a purely random kinetic mechanism with no Ping-Pong component.

Table I: Bacterial Strains and Plasmids

name	description	source
LT2	(S.t.) wild type	lab strain
SA2434	(S.t.) *pyrE125 rfa-3079*	K. E. Sanderson
BL21(DE3)	(E.c.B.) *F⁻ ompT rB⁻mB⁻*	J. Dunn, Brookhaven
MB13	BL21(DE3) pCG13	this work
pAV002	pBR328, *pyrE*	C. Miller library
pCG11	pUC18, *AvaI–PstI pyrE*	this work
pCG13	pT7-1, *AvaI–PstI pyrE*	this work

Materials and Methods

Cell growth media were obtained from Difco. PEI–cellulose-precoated chromatography plates (Macherey-Nagel) were from Brinkmann. [6-^{14}C]Orotic acid and [^{32}P]pyrophosphate were products of New England Nuclear. TSK-DEAE resin was from Supelco. Sephadex G-50 resin was from Pharmacia. Liquiscint was from National Diagnostics. Reactive red 120 agarose, protein molecular weight markers, potassium orotate, OMP (Sigma product O1376), PRPP (Sigma product P8296), and other biochemical reagents were from Sigma. Geneclean is a product of BIO 101, La Jolla, CA.

Cloning of pyrE. Bacterial strains and plasmids used are described in Table I. The *pyrE* gene of *S. typhimurium* was subcloned from a genomic library in pBR328 kindly donated by Dr. C. Miller, Case Western Reserve University. The library was transduced into the *S. typhimurium* pyrimidine auxotroph SA2434 (Δ*pyrE*) with phage P22 (Davis et al., 1980), and transduction mixtures were plated on M9 minimal agar (Davis et al., 1980) containing ampicillin to select for *pyrE*$^+$, *amp*^{r}. A P22-transducing stock prepared on a purified colony was used to retransduce SA2434, and all tranductants were both ampicillin resistant and *pyrE*$^+$. Plasmid pAV002 purified from one of these transductants was digested with *Ava*I and *Pst*I, and a 1-kb fragment of DNA containing the *pyrE* gene (Neuhard et al., 1985) was isolated with Geneclean from a 1% agarose gel and ligated into pUC18 cut with the same enzymes. The ligation mixture was used to transform SA2434, and ampicillin-resistant colonies were selected for *pyrE*$^+$ on minimal plates containing ampicillin. The resultant plasmid, pCG11, was maintained in SA2434 and directed constitutive overexpression of OPRTase. To increase ex-

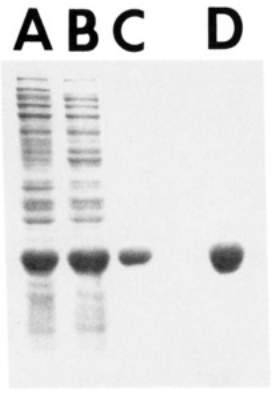


FIGURE 1: 10% SDS–PAGE analysis of *S. typhimurium* OPRTase large-scale purification. A 10% gel is shown: (lane A) crude extract of MB13 after PEI precipitation; (lane B) protein after ammonium sulfate precipitation; (lane C) protein following DEAE chromatography; (lane D) same sample as in lane C but overloaded (10 μg) to show purity of enzyme preparation.

pression further, the *Ava*I–*Pst*I fragment was also ligated into the T7 promoter vector pT7-1 (U.S. Biochemicals, Cleveland, OH) previously cut with *Ava*I and *Pst*I to give pCG13. This plasmid was used to transform the T7 RNA polymerase host BL21(DE3), as suggested by Studier et al. (1990). The resulting strain was MB13 (Table I).

Purification of OPRTase. The strain MB13, which directs the overproduction of OPRTase was grown in LB medium (Davis et al., 1980). For the purification procedures, each of 12 2-L flasks containing 500 mL (total 6 L) of LB medium, supplemented with 50 μg/mL ampicillin, was innoculated with 2 mL of an overnight culture of MB13. The 12 flasks were shaken at 37 °C in a New Brunswick Scientific G25 environmental incubator shaker until an A_{600nm} = 0.6 was reached. At this time IPTG (0.5 mM) was added to the cultures. Cells were harvested 3 h later by centrifugation at 10000*g* for 5 min; they were then resuspended in buffer O (50 mM Tris-HCl, pH 8.0, containing 2 mM EDTA and 2 mM mercaptoethanol) and centrifuged again. Washed harvested cells, typically 30 g, were suspended in 60 mL of buffer O and frozen at −20 °C. To purify OPRTase from the MB13 cells, cell suspension containing 60 g of cells was sonicated with the full-size probe of a Heat Systems (Plainsville, NY) W185 sonicator for 5 min at 4 °C and then centrifuged (10000*g* for 10 min) to remove debris. The turbid supernatant was clarified by addition of 35 μL/mL 10% PEI solution [poly(ethylenimine), Sigma product P3143, brought to pH 8.0 by addition of HCl]. The precipitate that formed was removed by centrifugation at 10000*g* for 10 min. PEI precipitation was followed by $(NH_4)_2SO_4$ fractionation (40–65% saturation) of the supernatant. The redissolved precipitate (about 80 mL) was then dialyzed overnight against three changes of buffer O (2 L each) and the slightly turbid solution centrifuged at 22000*g* for 20 min. The clear supernatant was applied to a TSK-DEAE column (5 × 40 cm) previously equilibrated in buffer O. The protein was eluted by a linear gradient of 0–0.3 M Na_2SO_4 in buffer O over a volume of 5.6 L, using Pharmacia FPLC pumps P 500 and Gradient Programmer GP 250. Fractions containing high levels of OPRTase activity were pooled, and protein was precipitated with 75% saturated $(NH_4)_2SO_4$. The enzyme obtained from this procedure was homogeneous as determined by 10% SDS–PAGE (Laemmli, 1970; Figure 1) and was used for most studies carried out in this work. The enzyme, stored as an $(NH_4)_2SO_4$ precipitate, was stable for many months. OPRTase obtained by this purification procedure was subjected to amino-terminal peptide sequencing on a Porton Instruments 2020/2090 microsequencer.

Although the protocol above provided us with highly purified enzyme, a step based on dye affinity chromatography was also developed. The stored enzyme was centrifuged at 10000*g* for 10 min. The pellet was dissolved in a minimal amount of buffer O and desalted on a column of Sephadex G-50 (2.5 × 20 cm) which had previously been equilibrated in buffer O. The desalted protein was applied to a reactive red 120 agarose dye affinity column (2 × 22 cm) previously equilibrated with buffer O. Following a 120-mL wash of the column with buffer O, protein was eluted with a linear gradient 0–0.7 M NaCl in buffer O, over a volume of 480 mL. Fractions containing OPRTase activity were pooled and stored as described earlier.

Enzyme Assays. OPRTase was assayed by employing methods modified from Kornberg et al. (1957) and Umezu et al. (1971). The assay is based on the observation that orotate consumption results in a decrease in absorption at 290–300 nm. Assays were performed on a Hewlett-Packard 8452A diode array spectrophotometer thermostated at 30 °C and employed the data acquisition strategy suggested for this instrument by Bednar and Hadcock (1988). Absorbance measurements were taken every 2 s for 60 s. Absorbance measurements in the range of 440–450 nm (five diodes) were subtracted from those of five diodes covering the range 290–300 nm. We have determined the extinction coefficient of orotate to be 4400 M^{-1} with these instrument settings. OMP does not absorb light in the 290–300-nm range. A 1.00-mL reaction mixture contained 300 μM orotate and 1 mM PRPP in 75 mM Tris-HCl, pH 8.0, and 6 mM $MgCl_2$ at 30 °C. Assay mixtures minus the enzyme were stored in quartz cuvettes in a 30 °C dry bath for at least 5 min prior to assaying. For an assay, 10 μL of the $(NH_4)_2SO_4$ suspension of the enzyme was centrifuged at 14000*g* for 2 min in a Eppendorf microfuge. The supernatant was removed, and the precipitate was dissolved in 10 μL of buffer containing 75 mM Tris-HCl, pH 8.0, and 6 mM $MgCl_2$. This enzyme preparation was then further diluted with the same buffer to suit the experiments to be performed. Under the described conditions, 0.1 μg of enzyme displayed a linear rate of orotate consumption over 1 min. One unit of enzyme activity is defined as the amount of OPRTase required to convert 1 μmol of orotate to OMP per minute at 30 °C. Protein was measured by the biuret method (Gornall et al., 1949) with bovine serum albumin as a standard.

Steady-State Kinetics. Initial velocity experiments were carried out with the spectrophotometric assay on the forward (phosphoribosyltransferase) reaction and the reverse (pyrophosphorolysis) reaction. For the forward reaction, a 1.0-mL reaction mixture contained 12–80 μM orotate, 15–120 μM PRPP, 75 mM Tris-HCl, pH 8.0, and 6 mM $MgCl_2$. The reverse reaction mixture (1.0 mL) contained 5–40 μM OMP and 25–240 μM PP_i in 75 mM Tris-HCl, pH 8.0, and 6 mM $MgCl_2$. Addition of 0.06–0.1 μg of enzyme provided a linear rate of orotate consumption over a min. Where product inhibition studies were performed on the forward and reverse reactions, the product was tested at four levels, keeping one substrate at saturating levels and varying the other from $0.5K_m$ to $3K_m$.

Kinetic data were analyzed by the programs of Cleland (1979). The data for the initial velocity experiments were fitted with a BASIC translation of the program SEQUEN. Data obtained from the product inhibition studies were fitted to lines by the programs COMP and NONCOMP. Each complete experiment was performed several times to ensure that the de-

viations of data from the fitted lines were random and not systematic.

Equilibrium Isotope Exchanges. The equilibrium constant for the OPRTase reaction was measured by the method suggested by Purich and Allison (1980). Exchanges were carried out between two substrate–product pairs, PRPP–PP_i and OMP–orotate. For the preparation of the reaction mixtures, the method recommended by Purich and Allison (1980) was used. For each substrate–product exchange pair two stock solutions were prepared, one containing PRPP and PP_i and the other containing OMP and orotate, in appropriate ratios so as to maintain equilibrium. A 100-μL exchange reaction mixture contained final concentrations of 75 mM Tris-HCl, pH 8.0, and 6 mM $MgCl_2$ and appropriate amounts of each stock solution to yield the desired concentrations (see legend to Figure 4). To this reaction mixture, 0.01–0.06 μg of OPRTase was added, and the mixture was incubated at 30 °C for 30 min to assure equilibration. [^{32}P]PP_i (80000 cpm) or [6-^{14}C]orotic acid (10000 cpm) was added to initiate the exchange reaction. After 3 min for the OMP–orotate exchange reactions and 5 min for the PRPP–PP_i exchange reactions, 5 μL of reaction mixture was applied to a PEI–cellulose plate. For the OMP–orotate exchange, a 10-cm TLC plate was developed in 0.75 M LiCl for 8 cm. The R_f for orotate under these conditions was 0.75 and that for OMP was 0.3. The spots were located with a UV lamp (265 nm), scraped into scintillation vials containing 0.3 mL of 1 N HCl, and mixed. Liquiscint (2.7 mL) was added, and samples were counted in a Beckman LS8100 scintillation counter. In the case of the PRPP–PP_i exchange, a 20-cm TLC plate was developed for 18 cm in 2 M LiCl and 0.75 M ammonium formate at pH 5.0. R_f values were determined to be 0.37 for PP_i and 0.65 for PRPP. Spots were located with a molybdate spray reagent (Dawson et al., 1986) and quantitated as in the OMP–orotate exchange reactions.

Rates for the equilibrium isotope exchanges were calculated with eq 1 (Purich & Allison, 1980), where V is the initial velocity of the isotope exchange measured in units/mg of enzyme, X and Y are the concentrations of the exchanging substrates in mM, F is the fractional attainment of equilibrium, and t is the time in min. The double-reciprocal plots for the exchanges were fitted to lines by the program HYPER (Cleland, 1979).

$$V = \frac{[X][Y][\ln (1 - F)]}{([X] + [Y])t} \quad (1)$$

Partial Isotope Exchange Reactions. For the OMP–orotate exchange, 50-μL reaction mixtures contained 800 μM OMP and 800 μM [6-^{14}C]orotate (100000 cpm), in 75 mM Tris-HCl, pH 8.0, and 6 mM $MgCl_2$. Exchanges were carried out at 30 °C with 0.2 μg of OPRTase. A sample of the reaction mixture (5 μL, 10000 cpm) was spotted on a PEI–cellulose TLC plate. The method used for the PRPP–PP_i exchanges was similar. A 50-μL reaction mixture contained 2 mM [^{32}P]PP_i (400000 cpm), 2 mM PRPP, 75 mM Tris-HCl, pH 8.0, and 6 mM $MgCl_2$. A sample (5 μL) of the reaction mixture (80000 cpm) was spotted on a 20-cm PEI–cellulose TLC plate. The TLC quantitation methods were the same as those used in the equilibrium exchange reactions. Rates were calculated by the method of Grubmeyer et al. (1987).

Purification of OMP. The method was modified from that of Cho et al. (1988). A 0.5 × 8 cm DEAE-cellulose (Whatman DE52) column in the HCO_3 form was prepared. In a control experiment a 100-μL mixture containing 5 mM OMP and PRPP and 50 μL of a 10 mM [^{32}P]PP_i (50000 cpm) solution was applied to the column. The column was then washed with 20 mL of water. A step gradient from 0.1 to 0.5 M triethylamine (TEA)–HCO_3, pH 8.0, was used to elute the substrates from the column. Each step of the gradient was 20 mL with increments of 0.1 M TEA-HCO_3. Fractions of 1 mL were collected. PRPP and PP_i were eluted in the 0.3–0.4 M TEA-HCO_3 range, and OMP was eluted at 0.1 M TEA-HCO_3, thus providing an efficient method of separation of OMP from PP_i and PRPP. To purify commercial OMP, 100 μL of a 10 mM OMP solution was applied to a freshly prepared DEAE-HCO_3 column and eluted by the above method. The OMP obtained by this procedure was lyophilized. The powder obtained was repeatedly dissolved in 0.5 mL of water and freeze-dried until all the yellow color, which results from traces of TEA, was removed. The OMP from this procedure was applied to a second freshly prepared DEAE column, and the elution and drying procedures were repeated. The OMP obtained was used in the partial exchange reactions.

Urea Treatment of Enzyme. A $(NH_4)_2SO_4$ precipitate of OPRTase containing 19 mg of protein was centrifuged at 14000*g* for 3 min in an Eppendorf microfuge. The supernatant was discarded, and the precipitate was dissolved in 100 μL of buffer O. The protein sample was then passed through a 0.5 × 8 cm G-50 column preequilibrated with buffer O. The protein-containing fractions were pooled and incubated at room temperature for about 1 h in 50 mM Tris-HCl, pH 8.0, containing 5 mM DTT, 1 mM EDTA, and 8 M urea. The protein sample was then applied to a 1.5 × 40 cm G-50 column preequilibrated and eluted with the same buffer. The protein-containing fractions were once again pooled and chromatographed on a G-50 (0.5 × 8 cm) column preequilibrated in buffer O. The protein-containing fractions were pooled and frozen at −70 °C.

Results

Cloning of pyrE. As the amount of OPRTase protein produced by *S. typhimurium* is insufficient for enzymological studies, we decided to clone the *pyrE* gene in an efficient expression system. Clones of *Escherichia coli* and *S. typhimurium pyrE* genes had previously been mapped and fully [*E. coli* (Poulsen et al., 1983)] or partially [*S. typhimurium* (Neuhard et al., 1985)] sequenced, which helped us in our subcloning. The *pyrE* gene was readily cloned from an existing *S. typhimurium* library in pBR328 (pAV002). Because the overexpression of OPRTase from pAV002 was only about 70-fold, relative to wild type, we sought to increase the protein production with a subclone made in the high copy number *lac* promoter plasmid pUC18. This plasmid, pCG11, when present in *S. typhimurium* strain SA2434, directed an approximate 20-fold additional overexpression of the protein and provided enzyme for many of the experiments in this work. Because the *pyrE* gene is under regulation by an upstream attenuator (Poulsen et al., 1983) that is present in pCG11, overexpression was still limited. To increase overexpression further, we used the T7 promoter/polymerase strategy described by Studier et al. (1990). Since T7 RNA polymerase does not recognize bacterial transcription termination signals, we reasoned that the attenuator would be ineffective in this construct. The vector employed was pT7-1 (U.S. Biochemicals), and the plasmid construct pCG13 (Figure 2) directed a further increase in expression when present in BL21(DE3) (Studier et al., 1990) and induced with IPTG. This represents an approximate 5000-fold overproduction compared with the wild type LT-2.

Enzyme Purification and Characterization. As can be seen in the SDS–PAGE analysis (Figure 1), OPRTase is a major protein in the crude extract of BL21(DE3) containing the

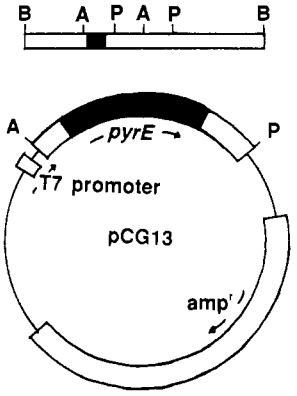


FIGURE 2: Cloning of the *pyrE* gene. (A) *S. typhimurium Sau*3A fragment containing the *pyrE* gene ligated into *Bam*H1 site of pBR328. The *pyrE* gene is indicated by the solid area. Letters refer to restriction sites: (A) *Aval*; (B) *Bam*H1; (P) *Pst*I. The distance between *Aval* and *Pst*I sites is 1 kb. (B) Expression system pCG13. The 1-kb *Aval*–*Pst*I fragment containing the *pyrE* gene was ligated into pT7-1 plasmid as shown.

Table II: Purification of *S. typhimurium* Orotate Phosphoribosyltransferase from MB13

step	vol (mL)	total act. (units)	total protein (mg)[a]	sp act. (units/mg)
PEI treatment	120	39 600	2200	17.8
$(NH_4)_2SO_4$ (40–65%)	85	34 000	1083	34.4
DEAE ion exchange	300	31 000	324[b]	95.7

[a] Protein is measured by the biuret method (Gornall et al., 1949). [b] Purified protein measurement is based on extinction coefficient (see Results).

plasmid pCG13. This fact allowed us to develop a simple and efficient purification procedure for OPRTase (see Materials and Methods and Table II). The protocol provided about 325 mg of OPRTase, which was homogeneous as judged by 10% SDS–PAGE.

S. typhimurium OPRTase comigrates with trypsinogen (M_r 24 000) on 10% SDS–PAGE. OPRTase in buffer O coelutes with monomeric egg albumin (M_r 45 000) on a Superose 6 analytical gel filtration column when run with dimeric *S. typhimurium* histidinol dehydrogenase (M_r 45 823 per subunit; Kohno & Gray, 1981) and monomeric cytochrome *c* (M_r 12 384) as standards. Thus, like *E. coli* OPRTase (Poulsen et al., 1983), *S. typhimurium* OPRTase is dimeric, with a subunit molecular weight of about 23 000.

We determined 17 amino acid residues at the amino terminus of OPRTase: Met-Lys-Pro-Tyr-Gln-Arg-Gln-Phe-Ile-Glu-Phe-Ala-Leu-Asn-Lys-Gln-Val, which corresponds exactly to the published sequence (Neuhard et al., 1985). On the basis of the deduced amino acid sequence of *E. coli* OPRTase (Neuhard et al., 1985), which is highly similar to that from *S. typhimurium* (Poulsen et al., 1983), we have calculated an extinction coefficient $E_{280}^{0.1\%}$ of 0.46 for *S. typhimurium* OPRTase by the method of Gill and von Hippel (1989).

Steady-State Kinetics. Like yeast OPRTase, we found that the *S. typhimurium* OPRTase reaction is freely reversible with the forward phosphoribosyltransferase reaction (85 units/mg) slightly faster than the reverse reaction (55 units/mg). The K_m values for the four substrates and products are indicated in Table III.

Patterns for the forward and reverse OPRTase reactions are shown in Figure 3. The data obtained for the forward reactions are well fit with either a Ping-Pong or a sequential mechanism in which K_i values are lower than K_m; however, the reverse reaction shows a clearly intersecting pattern, diagnostic of a sequential mechanism.

Table III: Summary of Kinetic Constants and Product Inhibition Patterns for the *S. typhimurium* OPRTase

Initial Velocity Studies		
substrate	K_m (μM)[a]	K_i (μM)[a]
orotate	27.5	5.9
PRPP	44.1	9.4
OMP	3.1	8.1
PP_i	31.1	80.1

Product Inhibition Studies		
inhibitor	substrate	inhibition type
OMP	PRPP	competitive
OMP	orotate	competitive
PP_i	PRPP	competitive
PP_i	orotate	noncompetitive
orotate	PP_i	noncompetitive
orotate	OMP	competitive
PRPP	PP_i	competitive
PRPP	OMP	competitive

[a] K_m and K_i values are obtained from initial velocity experiments. Values are those provided by analysis of the data by the Cleland program SEQUEN.

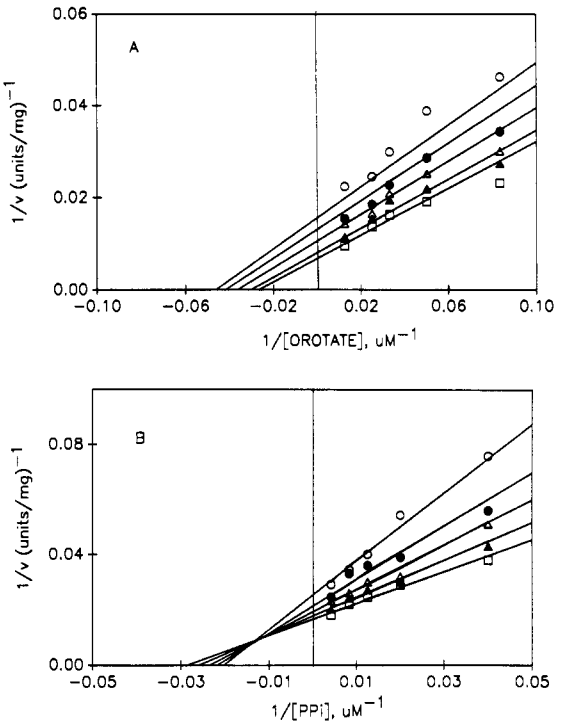


FIGURE 3: Double-reciprocal plots of the forward (A) and reverse (B) OPRTase reactions. Experiments were carried out in 75 mM Tris-HCl and 6 mM $MgCl_2$, pH 8.0, as described under Materials and Methods. (A) Forward reaction. Orotate concentration is varied from 12 to 80 μM at fixed levels of PRPP. PRPP concentrations are (○) 15, (●) 20, (△) 30, (▲) 60, and (□) 120 μM. (B) Reverse reaction. PP_i concentration is varied from 25 to 240 μM at fixed levels of OMP. OMP concentrations are (○) 5, (●) 8, (△) 12, (▲) 20, and (□) 40 μM. For both (A) and (B), lines were fitted to the data by program SEQUEN (Cleland, 1979).

To determine the binding order of substrates and products, we performed product inhibition studies with both products of the forward and reverse OPRTase reactions (Table III). OMP, one of the products of the phosphoribosyltransferase reaction, was competitive versus both orotate and PRPP, indicating a random component to the kinetic mechanism. Further, PP_i, the second product, was competitive versus PRPP and noncompetitive versus orotate. These results are those predicted for a random kinetic mechanism (Cleland, 1977).

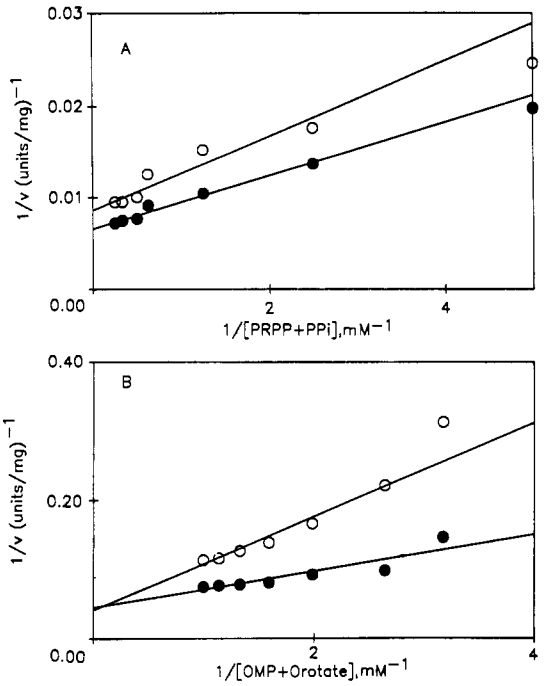


FIGURE 4: Double-reciprocal plots of equilibrium isotope exchanges carried out by OPRTase. Experiments were carried out in 75 mM Tris-HCl and 6 mM $MgCl_2$, pH 8.0. (A) OMP–orotate exchange. Two stock solutions were prepared. One contained OMP and orotate at 0.2 mM and 2 mM, respectively. The other contained PRPP and PP_i at 5 mM each. The ratio of concentrations of PRPP and PP_i was maintained at 1:1, and the total concentration of PRPP plus PP_i was increased from 0.2 to 4 mM. (○) 0.5 mM orotate and 0.05 mM OMP; (●) 1 mM orotate and 0.1 mM OMP. (B) PRPP–PP_i exchange. Two separate stock solutions were prepared. One contained OMP at 75 μM and orotate at 1.5 mM. The other stock solution contained PRPP at 2.5 mM and PP_i at 5 mM. The ratio of concentrations of OMP and orotate was maintained at 1:20, and the total concentration of OMP plus orotate was increased from 0.312 to 1.008 mM. (○) 0.25 mM PRPP and 0.5 mM PP_i; (●) 0.5 mM PRPP and 1 mM PP_i. Lines were fitted to the data by the program HYPERO.

We also performed product inhibition studies on the reverse OPRTase reaction. PRPP, which is a product of the reverse pyrophosphorolysis reaction, was competitive versus both PP_i and OMP. Orotate was competitive versus OMP and noncompetitive versus PP_i, the latter observation indicating that orotate and PP_i bind to distinct sites. The results were consistent with a random mechanism for the reverse reaction, with the dead-end complex E·orotate·PP_i.

Equilibrium Isotope Exchanges. To confirm the random sequential kinetic mechanism, we performed equilibrium isotope exchange studies with two of the substrate–product pairs, PRPP–PP_i and OMP–orotate. The equilibrium constant for the forward OPRTase reaction was determined following the procedure of Purich and Allison (1980) to be 0.108, thus favoring the pyrophosphorolysis reaction. Both exchanges were readily detected, and neither occurred in the absence of Mg^{2+} or upon the addition of a 4 mM excess of EDTA (over Mg^{2+}) to an exchange reaction mixture, suggesting a Mg^{2+} requirement. In addition, neither exchange reaction was observed if enzyme was first denatured by boiling or addition of SDS to a final concentration of 1%. Figure 4A shows the effect of increasing PRPP plus PP_i concentration upon the rate of the OMP–orotate exchange. Even at $20K_m$ concentrations of PRPP and PP_i there was no depression in the exchange rate. A similar linear plot was obtained for the PRPP–PP_i exchange (Figure 4B). No depression in the exchange rates was observed at $20K_m$ concentrations of orotate and OMP. Our failure to detect a depression of either exchange at high substrate levels

Scheme II: Ping-Pong Mechanism for Yeast OPRTase

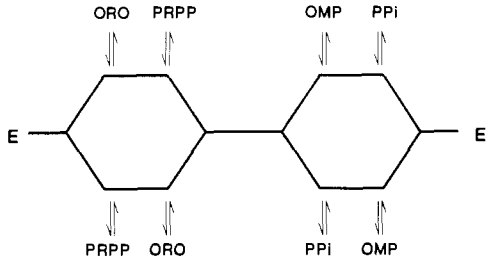
is completely consistent with a random sequential mechanism. The maximal rates obtained under the described conditions were 151 units/mg for the OMP–orotate exchange and 24 units/mg for the PRPP–PP_i exchange.

Partial Isotope Exchanges. The fact that yeast OPRTase displayed Ping-Pong-type kinetics and catalyzed the two partial reactions (Victor et al., 1979) prompted us to perform similar partial isotope exchanges utilizing the *S. typhimurium* OPRTase. Such an experiment tested the ability of the enzyme to catalyze the exchange of radiolabel between one substrate–product pair, either PRPP–PP_i or OMP–orotate, in the absence of elements of the other substrate–product pair. As can be seen in Scheme II, such exchanges would require that a phosphoribosylated enzyme intermediate exist. Initially, we found that *S. typhimurium* OPRTase catalyzed the exchange of label between the two substrate–product pairs. The rate for the OMP–orotate exchanges was approximately 2 units/mg and that of the PRPP–PP_i exchange was about 15 units/mg. Neither of the two exchanges was observed in the absence of Mg^{2+} or the presence of 10 mM EDTA in the exchange reaction mixture. Also, neither exchange was observed if enzyme was absent or denatured by boiling for 5 min or addition of SDS to a final concentration of 1%. These facts indicated that the partial isotope exchanges required divalent Mg^{2+} and were enzyme catalyzed. These observations were repeated with several batches of protein, some purified with the additional dye affinity column described under Materials and Methods.

Artifactual partial exchanges resulting from contamination of any of the substrates or enzyme can often lead to misinterpretation of results (Purich, 1983). We thus explored each element of the two exchange pairs for contamination. It seemed unlikely that commercial PP_i could be contaminated by OMP or orotate. OMP and orotate are highly absorbant at 266 nm (E = 6200 M^{-1}) and 295 nm (E = 3950 M^{-1}; Kornberg et al., 1957), respectively. A 10 mM solution of PRPP did not show detectable absorbance (<0.005) at 266 or 295 nm, indicating the absence of contaminating OMP or orotate in commercial PRPP. We found that Sephadex chromatography of urea-denatured OPRTase resulted in enzyme with a specific activity in the forward reaction of 57 units/mg compared with 85 units/mg before urea treatment. When the urea-treated sample was employed in the partial exchange reactions, it still catalyzed the OMP–orotate exchange at a rate of 2.2 units/mg. However, the urea-treated enzyme no longer catalyzed a detectable exchange of radiolabel between PRPP and PP_i. This suggested a contamination of the normal enzyme preparation, probably by OMP. When 1 μM OMP was added to a PRPP–PP_i exchange reaction containing 1 μM urea-treated OPRTase subunit, the enzyme catalyzed an exchange reaction at a rate of 9 units/mg, about 60% that of control OPRTase. This contamination was tenacious; when a small sample of native OPRTase obtained after DEAE chromatography was purified by gel filtration in buffer O, the PRPP–PP_i exchange rate was only partially inhibited to 4 units/mg. Given the K_i values for OMP (Table III), it is puzzling that bound OMP remained after a purification procedure which involves extensive dialysis as well as chromatography.

The unhampered ability of the urea-denatured enzyme to catalyze the OMP–orotate exchanges could indicate a nonartifactual exchange or a contamination of one of the sub-

Scheme III: Kinetic Mechanism for Bacterial OPRTase



strates. Commercially available orotic acid (Sigma product O2875) is chemically synthesized, and it is unlikely that it would be contaminated by PRPP or PP_i. We repurified OMP by passing it through ion exchange chromatography twice. The repurified OMP showed the same spectroscopic properties (λ_{max} = 266 nm) and R_f value (0.3 in 0.75 M LiCl, see Materials and Methods) as commercial OMP and was a substrate for the overall reverse OPRTase reaction. However, when repurified OMP was employed as a substrate, OPRTase did not catalyze a detectable OMP–orotate exchange (rate less than 0.001 units/mg). To determine whether the contaminating agent in commercial OMP was PRPP or PP_i, we performed OMP–orotate exchanges with commercial OMP in the presence of 15 μg (10.5 units) of yeast inorganic pyrophosphatase to hydrolyze any PP_i. In the presence of pyrophosphatase, OPRTase no longer catalyzed a detectable movement of radiolabel from orotate to OMP (rate less than 0.003 units/mg), confirming a contamination of OMP by pyrophosphate.

Discussion

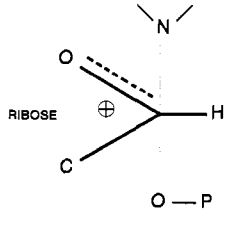
The most important finding arising from this work is that *S. typhimurium* OPRTase follows a random kinetic mechanism (Scheme III); no evidence for a kinetically separate enzyme–phosphoribose species was detected. This conclusion arises from initial velocity and product inhibition studies, equilibrium isotope exchange data, and thorough investigation of partial reactions. Our findings have important implications for the mechanism of phosphoribosyl transfer by PRTases, a subject which has received considerable attention (Goitein et al., 1978; Victor et al., 1979).

Of the two possibilities (see introduction), a chemical mechanism involving a carboxonium ion has been more attractive. The strongest support for a carboxonium-type transition state for PRTases has come from the observation of secondary ^3H isotope effects for three PRTases tested (Goitein et al., 1978). In addition, there have been scattered observations of Ping-Pong-type kinetics in initial velocity experiments (Krenitsky & Papaioannou, 1969; Victor et al., 1979; Natalini et al., 1979) and the demonstration of partial isotope exchange reactions in some PRTases (Ali & Sloan, 1982; Victor et al., 1979). Finally, for a number of other glycosyltransferases (Kirby, 1987; Cogoli & Semenza, 1975) carboxonium-type transition states have been well demonstrated. However, it is noteworthy that most PRTases studied to date utilize sequential kinetic mechanisms, suggesting a direct-transfer-type mechanism. Of the PRTases, human HGPRTase proceeds via Ping-Pong kinetics only in the presence of excess (5 mM) Mg^{2+}, following a sequential mechanism at low Mg^{2+} (Krenitsky & Papaioannou, 1969). Its yeast counterpart follows a sequential mechanism (Ali & Sloan, 1982). Although Ping-Pong kinetics were observed in the initial characterization of yeast UPRTase, the mechanism has not been further investigated (Natalini et al., 1979). The clearest example is yeast OPRTase, which demonstrated Ping-Pong kinetics and also catalyzed both partial reactions (Victor et al., 1979). In contrast to the work on yeast OPRTase, our results on bacterial OPRTase demonstrate a purely sequential mechanism and provide no evidence for partial exchanges.

The initial velocity plot (Figure 3) obtained when the data for the forward reaction were plotted might be construed as representative of a Ping-Pong mechanism. A similar plot was obtained when the same data were plotted versus PRPP (data not shown). Plots such as these suggest $K_i < K_m$ for orotate and PRPP, and this was shown to be the case (see Table III). The plots obtained for the reverse reaction were clearly intersecting however, indicating a sequential mechanism. Our product inhibition studies on the forward and reverse reactions confirmed our proposition for random binding of substrates. To finally confirm the kinetic mechanism, we performed equilibrium isotope exchange studies. The results clearly indicated a random sequential mechanism.

Previously, Sloan and co-workers demonstrated that yeast OPRTase catalyzes partial isotope exchanges between the two substrate–product pairs (Victor et al., 1979). This prompted us to perform similar experiments on the bacterial enzyme. Although we were able to detect both exchanges, they proved to be the results of tenacious contaminations. The OMP–orotate exchange arose by contamination of commercial OMP by PP_i. We were able to remove the PP_i by repeated purification of commercial OMP by ion exchange chromatography. The enzyme no longer catalyzed a detectable OMP–orotate exchange with the purified OMP. The PRPP–PP_i exchange resulted from contamination of the enzyme, probably by OMP. We were able to remove the contaminant completely only by gel filtration of urea-treated protein. Thus, *S. typhimurium* OPRTase does not exhibit any Ping-Pong component in its kinetic mechanism. It clearly proceeds by a sequential mechanism with random binding of substrates. The difficulties we encountered in revealing the exchange artifacts suggest to us that prior work on the yeast enzyme might have been subject to similar artifacts.

The resolution of the discrepancy between our kinetic and exchange data, which provide clear evidence for a sequential mechanism, and the careful isotope effect studies of Parsons and co-workers (Goitein et al., 1978), suggesting a dissociative chemical mechanism, is not clear. It remains possible that a dissociative-type mechanism occurs at the active site but only between the complete substrate or product complex. We are using positional isotope exchange (PIX) methods (Cohn & Hu, 1978) to explore this possibility. Perhaps a more attractive hypothesis comes from the case of the chemically analogous AMP nucleosidase reaction. Here, hydrolysis of the glycosyl bond occurs by direct transfer to water through an associative transition state with a very high degree of carboxonium character (Mentch et al., 1987). Such an intermediate involving a low degree of bond order between the two ligands has also been suggested for the solvolysis of D-glucopyranosyl derivatives (Sinnott & Jencks, 1980). Perhaps PRTases also involve such an associative transition state:



We experimentally determined K_{eq} for the forward

OPRTase reaction to be 0.1, which agrees with that obtained for mouse Ehrlich ascites cell (Traut & Jones, 1977) and yeast OPRTase (Lieberman et al., 1957). Sloan and co-workers used kinetic constants to calculate a value of 0.49 (Victor et al., 1979). More recently, a value of 0.7 was reported for yeast OPRTase (Tavares et al., 1987). The difference between that value and the one we report could be caused by large differences in the concentration of Mg^{2+} in the experimental protocols.

On the basis of our steady-state kinetic and equilibrium isotope exchange studies, we can make some conclusions as to the rates of the individual reaction steps (Figure 3). From the maximal rates of the forward (85 units/mg) and reverse (55 units/mg) reactions, assuming a subunit molecular weight of 23 000 for OPRTase, we have calculated k_{cat} values of 33 s^{-1} (forward reaction) and 22 s^{-1} (reverse reaction). The maximal rates obtained for the two equilibrium isotope exchange experiments yield rate constants of 58 s^{-1} for the OMP–orotate exchange and 9 s^{-1} for the PRPP–PP_i exchange. Since the OMP–orotate exchange rate was about twice as fast as the overall individual rates of the forward and reverse reactions, it could be concluded that neither OMP release in the forward reaction nor orotate release in the reverse reaction was rate limiting in overall catalysis. Also, from the OMP–orotate exchange rate, it is clear that ternary complex interconversion was at least as fast as 58 s^{-1} and was not the rate-limiting step in overall catalysis. Since ternary complex interconversion was not rate limiting, the overall forward reaction must be limited by the release of PP_i and the overall reverse reaction limited by the release of PRPP. The PRPP–PP_i exchange rate was slower than the overall individual rates of the forward and reverse reactions. The slow rate of the PRPP–PP_i exchange (9 s^{-1}) is expected from the fact that the exchange sequence contains the rate-limiting steps from both forward and reverse catalysis. The formula $1/V_{ex1} + 1/V_{ex2} = 1/V_{for} + 1/V_{rev}$ (Cleland, 1977) predicts a value of 17 s^{-1} for the PRPP–PP_i exchange, close to that observed.

Although a number of PRTases have been kinetically characterized, further studies have been hindered by insufficient yields of purified enzyme. In the current work we have solved this problem by developing an overproducer of bacterial OPRTase. Bacterial OPRTase, unlike its mammalian counterpart, does not occur as a bifunctional complex with OMP decarboxylase (McClard et al., 1980), allowing the study of the OPRTase reaction independently. The application of the techniques of molecular genetics to the bacterial protein promises to reveal structure/function relations in this important group of enzymes.

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