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The Role of Divalent Magnesium in Activating the Reaction Catalyzed by Orotate Phosphoribosyltransferase[1]

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Orotate phosphoribosyltranferase (OPRTase) catalyzes the formation of orotidine 5'-monophosphate from the nitrogenous base orotate and α-D-5-phosphoribosyl-1-pyrophosphate (PRPP). While it is known that Mg^{2+} is necessary for catalysis, the mechanism of activation of the phosphoribosyl transfer by Mg^{2+} remains unclear. The divalent cation may activate the phosphoribosyl transfer by binding to either or both substrates PRPP and orotate or/and the enzyme. In this work we chose to explore the role of divalent magnesium in activating the phosphoribosyl transfer in bacterial OPRTase. Studies on the effect of Mg^{2+} on the OPRTase-catalyzed reaction indicated that the divalent metal was necessary for catalysis. A maximal rate of 70 units/mg was achieved at 2 mM $MgCl_2$. Mn^{2+} could replace Mg^{2+} as the divalent metal. Orotate methyl ester (OAME) and uracil, neither of which form chelates with divalent metal, were found to be substrates for OPRTase. The K_M for OAME and uracil were 190 μM and 2.63 mM and k_{cat}/K_M were 0.91 × 10^5 and 6 M^{-1} s^{-1}, respectively. These values compare with a K_M of 27 μM for orotate, 44 μM for PRPP, and a k_{cat}/K_M of 1.3 × 10^6 M^{-1} s^{-1} for orotate. Spectroscopic studies failed to reveal the existence of Mg^{2+}–orotate complexes. Thus we have concluded that an orotate–metal complex is not necessary for OPRTase catalysis. Metal–enzyme binding studies indicate that only weak metal–enzyme complexes may form in bacterial OPRTase. Thus the role of divalent metal in bacterial OPRTase must be to bind PRPP.

Orotate phosphoribosyltransferase (OPRTase),[4] one of the family of 10 PRTases, catalyzes the Mg^{2+}-dependent formation of orotidine 5'-monophosphate (OMP) from the nitrogenous base orotate and α-D-5-phosphoribosyl-1-pyrophosphate (PRPP). Direct studies on a number of PRTases have shown that Mg^{2+} is necessary for catalysis. The Mg^{2+} requirement was demonstrated in the case of APRTase from monkey liver (1), yeast APRTase and HGPRTase (2), *Salmonella typhimurium* anthranilate PRTase (3) and *S. typhimurium* ATP–PRTase (4). Ali and Sloan (5) showed that the Mg^{2+} requirement in yeast HGPRTase could be substituted by Mn^{2+}, Co^{2+}, or Zn^{2+}. Although all PRTases that have been studied require Mg^{2+}, the actual mechanism of Mg^{2+} activation of the phosphoribosyl transfer is not known.

Activation of the phosphoribosyl transfer could result from Mg^{2+} binding to the nitrogenous base, PRPP, the enzyme, or all three. There remains considerable controversy as to which interactions occur. Like other phosphate containing compounds, PRPP can form complexes with Mg^{2+} in solution. Gadd and Henderson (6) suggested three possible complexes of PRPP with Mg^{2+} and proposed that the monomagnesium complex of PRPP (Scheme I) dominated at concentrations of Mg^{2+} below 10 mM. Thompson *et al.* (7) employed pH titration methods to determine association constants for Mg^{2+}–PRPP complexes and concluded that the monomagnesium complex of PRPP with Mg^{2+} bound at the pyrophosphate moiety of PRPP

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[4] Abbreviations used: APRTase, adenine PRTase; HGPRTase, hypoxanthine–guanine PRTase; OAME, orotic acid methyl ester; OMP, orotidine 5'-monophosphate; OPRTase, orotate PRTase; PEI, polyethyleneimine; PRPP, α-D-5-phosphoribosyl-1-pyrophosphate; PRTase, phosphoribosyltransferase; SDS–PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TEA, triethylamine; UMP, uracil monophosphate; UPRTase, uracil PRTase.

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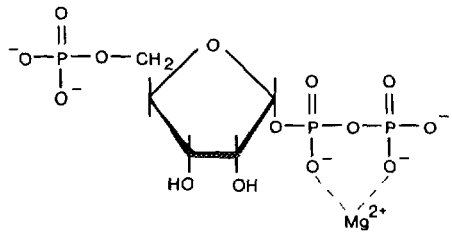


SCHEME I. Mg^{2+}–PRPP complex with metal bound at the pyrophosphate group.

dominates at low Mg^{2+} concentrations (below 10 mM). Since Mg^{2+}–PRPP complexes do exist in solution, at least one role of Mg^{2+} in the reaction catalyzed by PRTases may be to form a complex with PRPP. Further, since the monomagnesium complex of PRPP dominates at low concentrations of Mg^{2+}, it may be the preferred substrate.

The possibility that enzyme–metal complexes are important in activating the reaction catalyzed by PRTases was studied in yeast OPRTase and HGPRTase. Sloan and co-workers concluded from electron paramagnetic resonance and water proton relaxation studies (8) that two allosterically interacting metal binding sites per subunit existed in yeast OPRTase with a dissociation constant of 50 μM for the binding of the first Mn^{2+}. In another work (9), Sloan and co-workers carried out kinetic studies on yeast HGPRTase to define a kinetic mechanism in which a Mg^{2+}–HGPRTase complex was formed. To this complex, PRPP would bind either as a complex with Mg^{2+} or as free as PRPP. Yeast OPRTase and yeast HGPRTase are the only two cases in which $E \cdot Mg^{2+}$ complexes have been suggested.

The possibility that binding of metal to the nitrogenous base in PRTases could be the basis of the metal requirement has been suggested in yeast OPRTase. Tucci *et al.* (10) performed an extensive study of transition metal ion complex formation in 5-substituted orotate analogs. Their results indicated that strong complexation of orotate with transition metals occurred and that the metal chelated to the carboxyl moiety of orotate (Scheme II, 1). Thus, since orotate can form complexes with transition metals in solution, it may also form a Mg^{2+}–orotate complex that might be the preferred substrate for the OPRTase-catalyzed reaction. Dodin and co-workers (11) interpreted their kinetic and binding studies to propose that a Mg^{2+}–orotate complex was the preferred substrate for yeast OPRTase.

In this work we chose to explore the role of Mg^{2+} in *S. typhiurium* OPRTase. The enzyme has previously been overproduced and is known to follow a random sequential kinetic mechanism (12). The dependence of enzyme activity on Mg^{2+} concentration is shown to follow the concentration of the MgPRPP complex. We also show that two analogs of orotate that are unable to bind metal are substrates for the OPRTase-catalyzed reaction, indicating that an orotate–metal complex is not the required substrate. We have also employed spectroscopic studies to demonstrate that although the well-documented orotate–Mn^{2+} complexes do exist, orotate–Mg^{2+} complexes do not form. Finally, binding studies indicate that strong metal–enzyme complexes of the type proposed by Sloan's group (8, 9) do not form in bacterial OPRTase. Thus the role of metal in bacterial OPRTase must be to bind PRPP and form the monomagnesium complex which serves as the substrate for the PRTase-catalyzed reaction.

MATERIALS AND METHODS

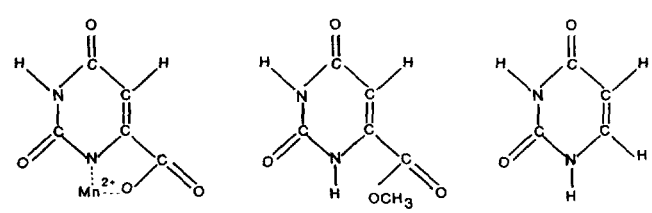
Materials. ^{54}Mn radionuclide and [2-^{14}C]uracil were obtained from NEN Research Products. $MnCl_2$ tetrahydrate (Gold Label) was a product of Aldrich Chemical Co. PEI–cellulose precoated chromatography plates (Macherey-Nagel) were from Brinkmann. Anion-exchange resin DE-52 was a product of Whatman. Potassium orotate, PRPP, orotodine 5'-monophosphate, orotic acid methyl ester, uracil, inorganic pyrophosphatase, and other biochemicals were from Sigma.

Enzyme preparation. The enzyme was purified by methods described earlier (12). To remove any contaminating ligands from OPRTase, the enzyme was denatured in 8 M urea, treated by gel filtration chromatography in 8 M urea, and subsequently renatured by methods also described in Bhatia *et al.* (12). The urea-treated enzyme had a specific activity of 60 units/mg (forward OPRTase-catalyzed reaction) and was used for all experiments carried out in this work.

Effect of divalent metal on the forward OPRTase-catalyzed reaction. A series of 1-ml reaction mixtures containing 300 μM orotate, 1 mM PRPP, in 75 mM Tris–HCl, pH 8.0, was prepared. To these reaction mixtures 0–10 mM $MgCl_2$ or 0–10 mM $MnCl_2$ was added. Reaction mixtures were incubated for 5 min at 30°C before being assayed for OPRTase activity by the method described earlier (12).

Determination of kinetic constants. For determination of kinetic constants for orotic acid methyl ester (OAME) and orotate, a 1-ml reaction mixture contained 100–300 μM OAME or 10–40 μM orotate, 1 mM PRPP in 75 mM Tris-HCl, pH 8.0, at 30°C. Reactions were initiated by the addition of 0.5 μg of OPRTase for the OAME and 0.2 μg for the orotate experiments. The spectroscopic assay was performed by methods described earlier (12). The V_{max} and K_M values obtained in these experiments were obtained at a single PRPP level approximately 20-fold greater than the K_M for PRPP. Data were analyzed by the program HYPER (13) and gave less than 10% error on derived values.

Synthesis of OAME mononucleotide from OAME. A 10-ml reaction mixture containing 1 mM PRPP, 0.8 mM OAME in 75 mM Tris–HCl, 6 mM $MgCl_2$, pH 8.0, was incubated at 30°C. To this reaction mixture, 40 μg of OPRTase and 30 μg of yeast inorganic pyrophosphatase was added. The reaction was allowed to incubate at 30°C for 2 h before being frozen at −70°C and subsequently freeze-dried. The freeze-dried reaction mixture was dissolved in 1.0-ml water and applied to a DEAE-cellulose (HCO_3 form) column (1.0 × 20 cm). The column was washed with 10 ml of water, to remove unreacted OAME, and then eluted with 10 ml



SCHEME II. (1) Binding of transition metal such as Mn^{2+} to orotate as proposed by Tucci *et al.* (10). (2) Orotic acid methyl ester. (3) Uracil.

of 0.1 M triethylamine (TEA)–HCO_3, pH 8.0. Fractions with a maximal absorbance at 264 or 278 nm were collected, pooled separately, and lyophilized to dryness. The dried products were repeatedly dissolved in water and lyophilized until a slight yellow color, resulting from contamination by TEA, was removed.

Synthesis of OAME from its mononucleotide. The product absorbing at 264 nm obtained and purified from the reaction of OAME with PRPP (described above) was added to a reaction mixture containing 1 mM PP_i in 75 mM Tris–HCl, 6 mM $MgCl_2$, pH 8.0. The reaction was initiated by the addition of 40 μg of OPRTase and was allowed to incubate at 30°C for 2 h. Procedures for the purification of the products were the same as those described for the mononucleotide product.

Uracil as a substrate. An assay method utilizing radiolabeled uracil was used. A 50-μl reaction mixture containing 1 mM PRPP, 1 mM uracil (100,000 cpm) in 75 mM Tris–HCl, 6 mM $MgCl_2$, pH 8.0, was incubated at 30°C. To three such reaction mixtures 0, 15, and 30 μg of OPRTase was added. From these reaction mixtures 5-μl samples were removed after appropriate times and applied to a PEI–cellulose plate. The plate was developed for 8 cm in 0.1 M LiCl. The R_f for uracil under these conditions was 0.9 and that for UMP was 0.4. The spots were located with a uv lamp (265 nm) and scraped into vials for liquid scintillation counting. For determination of kinetic constants, the 50-μl reaction mixture contained 1 mM PRPP, 1–3 mM uracil (100,000 cpm), in 75 mM Tris–HCl, 6 mM $MgCl_2$, pH 8.0. Data were analyzed by the program HYPER (13).

To investigate the effect of OMP upon the OPRTase-catalyzed conversion of uracil to UMP, a 50-μl reaction mixture containing 1 mM PRPP, 2 mM uracil (100,000 cpm), 0–40 μM OMP in 75 mM Tris–HCl, pH 8.0, was incubated at 30°C. Enzyme (10 μg) was added to initiate the reaction. From these reaction mixtures 5 μl was applied at 0, 5, 15, and 30 min to PEI–cellulose plates. Chromatography and liquid scintillation counting was as described in Bhatia *et al.* (12).

Effect of divalent metal upon the orotate spectrum. Solutions (1.0 ml) containing 60 μM orotate, $MnCl_2$ (0–2 mM) or $MgCl_2$ (0–5 mM) in 75 mM Tris–HCl, pH 8.0, were prepared. The absorption spectrum for each solution was measured over 250–350 nm on a HP8452 diode array spectrophotometer. The spectrum obtained in the presence of divalent metal was subtracted from that obtained without divalent metal to provide a difference spectrum, and the value of the difference at 320 nm was used to quantitate binding. To study the effect of Mg^{2+} upon the Mn^{2+}–orotate spectrum, a 1.0-ml solution contained 0.5 mM $MnCl_2$, 60 μM orotate, and 0–5 mM $MgCl_2$ in 75 mM Tris–HCl, pH 8.0.

Enzyme–metal binding studies. To investigate the binding of Mn^{2+} to OPRTase, a 100-μl reaction mixture containing 40 μM OPRTase (subunit concentration based on M_r 23,000), 40 μM $^{54}MnCl_2$ (500,000 cpm) in 75 mM Tris–HCl, pH 8.0, was prepared. The reaction mixture was then centrifuged in a Millipore Ultrafree MC filter unit for 15 min at 600*g*. From the top and bottom chambers of the filter units, 30-μl samples were removed for determination of ^{54}Mn by liquid scintillation counting.

RESULTS

Effect of Mg^{2+} and Mn^{2+} on the OPRTase-catalyzed reaction. The metal requirement for the reaction catalyzed by bacterial OPRTase was investigated. In the absence of added Mg^{2+}, a residual OPRTase activity of 1 unit/mg was measured. Upon addition of 1 mM EDTA, pH 8.0, to the assay mixture (no Mg^{2+}), residual OPRTase activity was reduced to 0.01 units/mg. In a second experiment, [Mg^{2+}] was varied at 1 mM PRPP (Fig. 1). OPRTase activity increased with increasing [Mg^{2+}]. A maximal rate of 70 units/mg was achieved at 2 mM $MgCl_2$. A theoretical curve was drawn in which the known K_A for the MgPRPP complex (14) was used to predict the concentration of

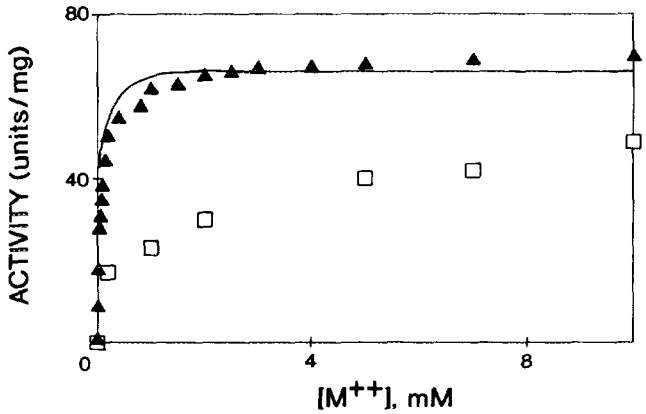


FIG. 1. Effect of Mn^{2+} and Mg^{2+} on OPRTase activity. Reactions were carried out in 75 mM Tris–HCl, pH 8.0, 300 μM orotate, and 1 mM PRPP. (▲) Effect of increasing $MgCl_2$ concentration upon the rate of the forward OPRTase-catalyzed reaction. Experiments were carried out at 1 mM PRPP and 0–10 mM $MgCl_2$. (□) Effect of increasing $MnCl_2$ concentration upon the forward OPRTase-catalyzed reaction. Reactions were carried out in 300 μM orotate, 1 mM PRPP in 75 mM Tris–HCl, pH 8.0. The line used to fit the data for the effect of Mg^{2+} on activity was computed from the Michaelis–Menten equation employing a K_M of 44.1 μM for PRPP (12) and V_{max} of 70 units/mg. The concentration of MgPRPP was computed as described by Salerno and Giacomello (14) employing a K_A of 1700 M^{-1} for the binding of Mg^{2+} to PRPP.

MgPRPP at each Mg^{2+} concentration. The previously determined K_M for PRPP, 44.1 μM (12), was then used to predict the reaction velocity at each Mg^{2+} concentration. The line provides a reasonable fit to the experimental data. In another experiment, [Mn^{2+}] was varied at 1 mM PRPP. A maximal rate of 48 units/mg was measured at 10 mM $MnCl_2$. These experiments indicated that OPRTase required Mg^{2+} for catalysis and that the Mg^{2+} requirement could be replaced by Mn^{2+}, as previously shown for yeast OPRTase (5).

Alternative substrate studies. OAME (Scheme II, 2) and uracil (Scheme II, 3), which lack the C6 carboxyl group, and thus cannot form the metal chelate proposed for orotate, provided a test for the importance of metal–orotate complexes.

OAME exibited spectral properties similar to orotate (λ_{max} at 278 nm). Addition of 6 μg of OPRTase to a 1.0-ml solution containing 100 μM OAME, 1 mM PRPP in 75 mM Tris–HCl, 6 mM $MgCl_2$, pH 8.0, resulted in a linear time-dependent decrease in absorbance at 290–300 nm due to consumption of OAME. This suggested that OAME was a substrate for bacterial OPRTase. An alternative explanation was that OAME was contaminated with orotate or that OAME was being hydrolyzed to orotate and methanol under these assay conditions and that orotate was being converted to OMP. If the alternative explanation were true, then the product of the reaction would be OMP and not the OAME mononucleotide. To characterize the nucleotide formed, a large scale preparation of the product was carried out. The product showed an absorbance maximum at 264 nm, indistinguishable from

TABLE I

Determination of Kinetic Constants for Orotate and Its Substrate Analogs

Substrate	K_M (mM)	k_{cat}[a] (s^{-1})	k_{cat}/K_M ($M^{-1}\ s^{-1}$)
Orotate	0.028	27.5	9.8×10^5
Orotic acid methyl ester	0.19	17.2	0.91×10^5
Uracil	2.63	1.6×10^{-3}	0.6

[a] Calculated on the basis of a subunit molecular weight of 23,000.

OMP. However, when applied to a PEI-cellulose plate and developed for 7 cm in 0.25 M LiCl, a R_f of 0.32 was obtained for that product, while OMP showed a R_f of 0.16. The product was used as a substrate for the reverse OPRTase-catalyzed pyrophosphorolysis reaction in a large scale preparation. When this reaction was carried out, a single product with a λ_{max} at 278 nm was observed and purified. On paper chromatography in *n*-propanol:1 N acetic acid, 3:1, this product showed the same R_f as commercial OAME (0.71) and was readily distinguished from orotate (R_f 0.32), confirming that the original product was OAME mononucleotide. Thus, OAME is a substrate for the OPRTase-catalyzed reaction. Since OAME cannot bind Mg^{2+} in a manner similar to orotate, a Mg^{2+}-orotate complex was not required for bacterial OPRTase. Kinetic constants for OAME were determined and are shown in Table I.

Uracil was also employed as a potential substrate for the OPRTase-catalyzed reaction. An assay utilizing radiolabeled uracil was used. When 6 μg of OPRTase was added to a reaction mixture containing 1 mM PRPP, 1 mM uracil (100,000 cpm), 6 mM $MgCl_2$ in 75 mM Tris-HCl, pH 8.0, two radiolabeled spots were observed with R_f values of 0.4 and 0.9 corresponding to UMP and uracil, respectively. There was no conversion of uracil to UMP in the absence of OPRTase or Mg^{2+}. The possibility that the conversion of uracil was being carried out by a contaminating UPRTase activity was tested. The enzyme preparation was homogeneous as determined by SDS-PAGE and no other proteins were observed upon overloading the SDS-PAGE gel. However, minimal contamination of OPRTase by UPRTase might have escaped detection. OMP is a potent and specific competitive inhibitor of the forward OPRTase-catalyzed reaction (K_i = 8.1 μM, 12) and thus the conversion of uracil to UMP, if catalyzed by OPRTase, should be subject to potent inhibition by OMP. As shown in Fig. 2, the addition of 10 μM OMP caused a 50% inhibition of the conversion of uracil to UMP. Kinetic constants for uracil are compiled in Table I.

Metal-orotate binding studies. The fact that transition metal complexes of orotate exist in solution was partly responsible for the proposal (10) that an orotate-metal complex was the true substrate for the OPRTase. Thus,

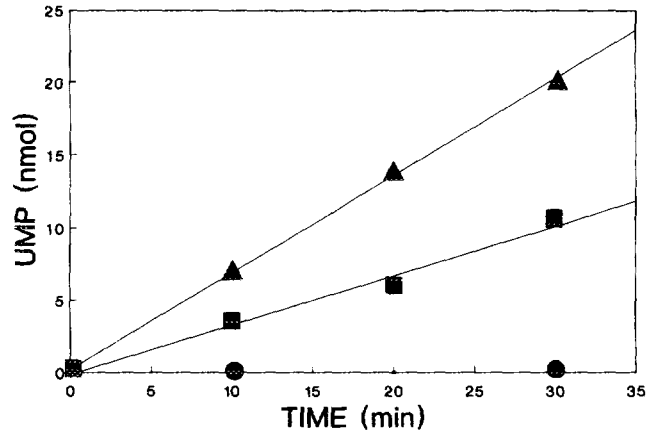


FIG. 2. Effect of OMP upon the OPRTase-catalyzed conversion of uracil to UMP. Assays were carried out in 75 mM Tris-HCl, pH 8.0, containing 6 mM $MgCl_2$. Time points were 0, 5, 15, 30 min. OMP concentrations were (▲) no OMP, (■) 10 μM, and (●) 40 μM.

spectroscopic studies were carried out to determine if Mg^{2+}-orotate complexes exist in solution. The Mn^{2+}-orotate complex shows peak absorbance at 310 nm (15), and at 320 nm, where free orotate does not absorb, the complex was readily quantitated (Fig. 3). From these results a dissociation constant (K_D) of 510 μM for the binding of Mn^{2+} to orotate was calculated. Using the same method, the addition of up to 5 mM $MgCl_2$ to a 60 μM orotate solution had no effect on the spectrum of orotate. Thus, either the orotate does not form a complex with Mg^{2+} or the complex does not absorb in the uv range. If Mg^{2+} could bind to orotate then the addition of $MgCl_2$ to a solution containing Mn^{2+}-orotate complexes should result in the decrease of the Mn^{2+}-orotate peak at 320 nm. The addition of high concentrations of $MgCl_2$ (5 mM) to a solution containing $MnCl_2$ (0.5 mM) and orotate (60

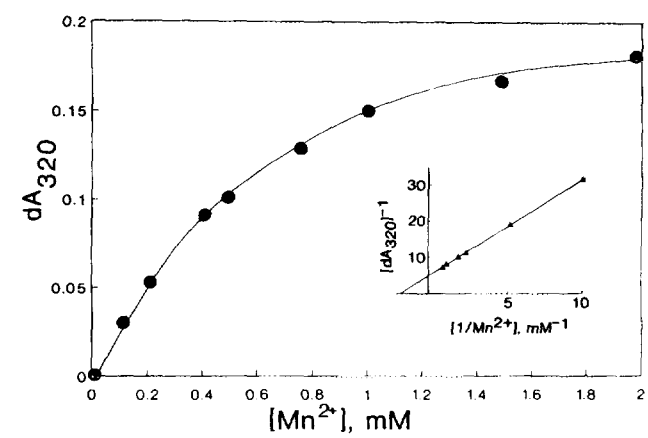


FIG. 3. Effect of increasing $MnCl_2$ concentration upon the absorbance difference spectrum of [Mn^{2+}-orotate] in 75 mM Tris-HCl, pH 8.0, measured at 320 nm. (Inset) Data are replotted as a double-reciprocal plot. The horizontal axis represents the inverse of [Mn^{2+}] and the vertical axis represents the inverse of the difference spectrum at 320 nm.

μM) did not result in any change in the absorbance (320 nm) of the Mn–orotate complex, indicating that Mg^{2+} does not bind to orotate under the present assay conditions (K_D > 5 mM). These studies, together with the alternative substrate studies, confirm that Mg^{2+} does not activate the OPRTase-catalyzed reaction by binding to orotate.

Metal–enzyme binding studies. Since divalent metal is not involved in binding orotate, another possibility is that it may bind to the enzyme as suggested for yeast OPRTase (8). As earlier mentioned, Sloan and co-workers had observed a K_D of 50 μM for the binding of Mn^{2+} to yeast OPRTase (8). In *S. typhimurium* OPRTase, the Mg^{2+} function can be replaced by Mn^{2+} and thus $^{54}Mn^{2+}$ binding provided a convenient probe for the investigation of metal binding in OPRTase. The conditions we employed in the metal–enzyme binding assays (see Materials and Methods) would have allowed us to conveniently detect binding with a K_D in the range of 50 μM [as proposed by Sloan and co-workers (8)]. However, our results indicated that any binding was very weak, with minimal K_D of 1 mM. Because of technical problems in quantitation, resulting from the weakness of the interaction, no additional studies of this potential interaction were undertaken.

DISCUSSION

The results presented in this work indicate that Mg^{2+} does not activate the reaction catalyzed by OPRTase by binding orotate or the enzyme. Since Mg^{2+}–PRPP complexes are well documented in the literature (6, 7), it is logical to assume that the only role of Mg^{2+} in the OPRTase-catalyzed reaction is to form a monomagnesium complex with PRPP.

Divalent Mg^{2+} could activate the OPRTase-catalyzed reaction by binding to orotate, as proposed by Dodin *et al.* (11). Binding of Mn^{2+} between the carboxyl group and the N1 nitrogen of orotate is well documented (10). In this work we found that Mn^{2+}–orotate complexes are formed in solution (K_D = 510 μM) as previously reported (8). However, no binding of Mg^{2+} to orotate was observed either by perturbation of orotate spectrum or by competition (K_D > 5 mM). Uracil and OAME are two orotate analogs that are unable to bind divalent metal in a manner similar to orotate. Both were found to be substrates for the OPRTase-catalyzed reaction. These results indicate that a metal complex of orotate is not the required substrate for the OPRTase-catalyzed reaction.

Since it was determined that a Mg^{2+}–orotate complex does not activate the phosphoribosyl transfer reaction, the alternative explanation that metal could activate the OPRTase-catalyzed reaction by binding to enzyme was investigated by enzyme–$^{54}Mn^{2+}$ binding studies. Since the Mg^{2+} requirement in *S. typhimurium* OPRTase could be substituted by Mn^{2+}, it is reasonable to assume that Mn^{2+} would bind to similar sites on the enzyme and would follow the same mechanism of activation as Mg^{2+}. Although Sloan and co-workers had proposed a K_D = 50 μM for the binding of metal to yeast OPRTase (8), we determined that Mn^{2+} bound poorly to *S. typhimurium* OPRTase with a K_D at least as high as 1 mM.

The effects of Mg^{2+} and Mn^{2+} on the OPRTase-catalyzed reaction were investigated. In this work, the rates of the forward OPRTase-catalyzed reaction were measured at varying Mg^{2+} and Mn^{2+} concentrations. The plots in Fig. 1 indicate that bacterial OPRTase requires Mg^{2+} and that this requirement could be substituted by Mn^{2+}. The maximal rate obtained with Mn^{2+} is, however, slower than that with Mg^{2+}. These plots, when compared with the computed rate at varying Mg^{2+} concentrations (Fig. 1), indicate that in *S. typhimurium* OPRTase the substrate for the OPRTase-catalyzed reaction is the monomagnesium complex of PRPP (Scheme I). This conclusion agrees with that proposed for APRTase by Gadd and Henderson (6) and yeast HGPRTase (9).

From these studies a simple model for the role of divalent metal has emerged. Although studies on metal–orotate complexes have suggested that Mg^{2+}–orotate may be the substrate for OPRTase, it has been clearly shown that the proposed complex does not form. Metal–enzyme complexes formed in bacterial OPRTase are at best weak. Instead, it appears that a MgPRPP complex forms the substrate for OPRTase. Whether the metal ion then assists in catalysis directly or serves to position the substrate or transition state is the subject of current experimentation.

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