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

Mohit B. Bhatia and Charles Grumbmeyer

Department of Biology, New York University, New York, New York 10003

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Orotate phosphoribosyltransferase (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 Mg²⁺ is necessary for catalysis, the mechanism of activation of the phosphoribosyl transfer by Mg²⁺ remains unclear. The divalent cation may activate the phosphoribosyl transfer by binding to either or both substrates PRPP and orotate or 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 Mg²⁺ 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 MgCl₂. Mn²⁺ could replace Mg²⁺ 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 $\times$ 10⁶ and 6 $\times$ 10⁴ 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 $\times$ 10⁸ M⁻¹ s⁻¹ for orotate. Spectroscopic studies failed to reveal the existence of Mg²⁺-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),¹ one of the family of 10 PRTases, catalyzes the Mg²⁺-dependent formation of orotidine 5'-monophosphate (OMP) from the

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nitrogenous base orotate and α-D-5-phosphoribosyl-1-pyrophosphate (PRPP). Direct studies on a number of PRTases have shown that Mg²⁺ is necessary for catalysis. The Mg²⁺ requirement was demonstrated in the case of APRTase from monkey liver (1), yeast APRTase and HGPTase (2), Salmonella typhimurium anthranilate PRTase (3) and S. typhimurium ATP–PRTase (4). Ali and Sloan (5) showed that the Mg²⁺ requirement in yeast HGPTase could be substituted by Mn²⁺, Co²⁺, or Zn²⁺. Although all PRTases that have been studied require Mg²⁺, the actual mechanism of Mg²⁺ activation of the phosphoribosyl transfer is not known.

Activation of the phosphoribosyl transfer could result from Mg²⁺ 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 Mg²⁺ in solution. Gadd and Henderson (6) suggested three possible complexes of PRPP with Mg²⁺ and proposed that the monomagnesium complex of PRPP (Scheme I) dominated at concentrations of Mg²⁺ below 10 mM. Thompson et al. (7) employed pH titration methods to determine association constants for Mg²⁺–PRPP complexes and concluded that the monomagnesium complex of PRPP with Mg²⁺ bound at the pyrophosphate moiety of PRPP

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¹Current address: Department of Biochemistry, The Rockefeller University, 1230 York Avenue, New York, NY 10021.
²To whom correspondence should be addressed at Department of Biochemistry, Temple University Medical School, 3400 North Broad Street, Philadelphia, PA 19140.
³Abbreviations used: APRTase, adenine PRTase; HGPTase, 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.
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 HGPTase. 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 HGPTase to define a kinetic mechanism in which a Mg$^{2+}$-HGPTase 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 HGPTase are the only two cases in which E·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. typhimurium 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 sub-

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**Scheme I.** Mg$^{2+}$-PRPP complex with metal bound at the pyrophosphate group.

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**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)−HCO₃⁻, 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, in 75 mM Tris−HCl, 6 mM MgCl₂, pH 8.0. The reaction was initiated by the addition of 40 µg of ORPTase 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 MgCl₂, pH 8.0, was incubated at 30°C. To three such reaction mixtures 0, 15, and 30 µg of ORPTase 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 Rs 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 containing 1 mM PRPP, 1 mM uracil (100,000 cpm), in 75 mM Tris−HCl, 6 mM MgCl₂, pH 8.0. Data were analyzed by the program HYPER (13).

To investigate the effect of OMP upon the ORPTase-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 Bhata et al. (12).

**Effect of divalent metal upon the orotate spectrum.** Solutions (1.0 ml) containing 60 µM orotate, MnCl₂ (0−2 mM) or MgCl₂ (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 Mg²⁺ upon the Mn²⁺−orotate spectrum, a 1.0-ml solution contained 0.5 mM MnCl₂, 60 µM orotate, and 0−5 mM MgCl₂ in 75 mM Tris−HCl, pH 8.0.

**Enzyme−metal binding studies.** To investigate the binding of Mn²⁺ to ORPTase, a 100-µl reaction mixture containing 40 µM ORPTase (subunit concentration based on M, 23,000), 40 µM MnCl₂ (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 600g. From the top and bottom chambers of the filter units, 30-µl samples were removed for determination of Mn²⁺ by liquid scintillation counting.

**RESULTS**

**Effect of Mg²⁺ and Mn²⁺ on the ORPTase-catalyzed reaction.** The metal requirement for the reaction catalyzed by bacterial ORPTase was investigated. In the absence of added Mg²⁺, a residual ORPTase activity of 1 unit/mg was measured. Upon addition of 1 mM EDTA, pH 8.0, to the assay mixture (no Mg²⁺), residual ORPTase activity was reduced to 0.01 units/mg. In a second experiment, [Mg²⁺] was varied at 1 mM PRPP (Fig. 1). ORPTase activity increased with increasing [Mg²⁺]. A maximal rate of 70 units/mg was achieved at 2 mM MgCl₂. A theoretical curve was drawn in which the known K₅ for the MgPRPP complex (14) was used to predict the concentration of MgPRP at each Mg²⁺ concentration. The previously determined K₅ for PRPP, 44.1 µM (12), was then used to predict the reaction velocity at each Mg²⁺ concentration. The line provides a reasonable fit to the experimental data. In another experiment, [Mn²⁺] was varied at 1 mM PRPP. A maximal rate of 48 units/mg was measured at 10 mM MnCl₂. These experiments indicated that ORPTase required Mg²⁺ for catalysis and that the Mn²⁺ requirement could be replaced by Mn²⁺, as previously shown for yeast ORPTase (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 exhibited spectral properties similar to orotate (λₘₐₓ at 278 nm). Addition of 6 µg of ORPTase to a 1.0-ml solution containing 100 µM OAME, 1 mM PRPP in 75 mM Tris−HCl, 6 mM MgCl₂, 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 ORPTase. 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

**FIG. 1.** Effect of Mn²⁺ and Mg²⁺ on ORPTase activity. Reactions were carried out in 75 mM Tris−HCl, pH 8.0, 300 µM orotate, and 1 mM PRPP. (A) Effect of increasing MgCl₂ concentration upon the rate of the forward ORPTase-catalyzed reaction. Reactions were carried out at 1 mM PRPP and 0−10 mM MgCl₂. (B) Effect of increasing MnCl₂ concentration upon the forward ORPTase-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 Mg²⁺ on activity was computed from the Michaelis−Menten equation employing a K₅ of 44.1 µM for PRPP (12) and Vₘₐₓ of 70 units/mg. The concentration of MgPRP was computed as described by Salerno and Giacomelli (14) employing a K₅ of 1700 M⁻¹ for the binding of Mg²⁺ to PRPP.
TABLE I
Determination of Kinetic Constants for Orotate and Its Substrate Analogues

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orotate</td>
<td>0.028</td>
<td>27.5</td>
<td>$9.8 \times 10^3$</td>
</tr>
<tr>
<td>Orotic acid methyl ester</td>
<td>0.19</td>
<td>17.2</td>
<td>$9.1 \times 10^2$</td>
</tr>
<tr>
<td>Uracil</td>
<td>2.63</td>
<td>$1.6 \times 10^{-3}$</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* 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 pyrophosphorylase reaction in a large scale preparation. When this reaction was carried out, a single product with a $\lambda_{max}$ 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 $\mu$g 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 ($K_i = 8.1$ $\mu$M, 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 $\mu$M 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, 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 $\mu$M 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 $\mu$M 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 Mn$^{2+}$ 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

![Graph](image-url)
\( \mu M \) 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 \( (K_D > 5 \mu M) \). 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 \( \mu M \) 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 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 \( \mu M \) [as proposed by Sloan and co-workers (8)]. However, our results indicated that any binding was very weak, with minimal \( K_D \) of 1 \( \mu M \). 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 \( (K_D = 510 \mu M) \) as previously reported (8). However, no binding of Mg\(^{2+} \) to orotate was observed either by perturbation of orotate spectrum or by competition \( (K_D > 5 \mu M) \). 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-\(^{58} \)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 \( K_D = 50 \mu M \) 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.

**REFERENCES**