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Divalent Metal Ion Activation of the Yeast Orotate Phosphoribosyltransferase Catalyzed Reaction†

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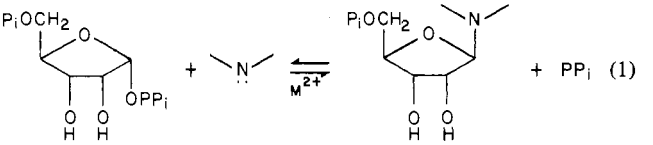
ABSTRACT: An analysis of the divalent metal ion activation of the orotate phosphoribosyltransferase catalyzed reaction has been performed using homogeneous preparations of this enzyme from yeast. Electron paramagnetic resonance (EPR), UV spectroscopy, and water proton magnetic relaxation rate (PRR) measurements have been utilized to characterize dissociation constants ($\bar{K}_0$) for the formation of binary complexes between Mn^{2+} and orotate ($\bar{K}_0$ = 500 μM), phosphoribosyl pyrophosphate (P-Rib-PP; $\bar{K}_0$ = 30 μM), PP_i ($\bar{K}_0$ = 12 μM), and OMP ($\bar{K}_0$ = 200 μM). EPR and PRR evidence has been collected for the formation of a binary Mn^{2+}–enzyme complex (maximal stoichiometry 4:1). The shape of the binding isotherm which defines this complex formation is sigmoidal, suggesting cooperation between Mn^{2+} binding sites. Kinetic analysis of the activation of the phosphoribosyl transfer reaction revealed a biphasic nature for Mg^{2+} and Mn^{2+} activation. These results are interpreted in terms of an enzyme–metal ion complex and a metal-free enzyme, both of which catalyze this reaction but at different rates. Although Co^{2+} and Ca^{2+} do not activate the phosphoribosyl transfer, Co^{2+} inhibits Mg^{2+} activation (perhaps as a result of the formation of a Co^{2+}–orotate complex, K_0 = 40 μM). Previous kinetic studies [Victor, J., Greenberg, L. B., & Sloan, D. L. (1979) *J. Biol. Chem. 254*, 2647–2655] revealed that this phosphoribosyl transfer proceeds through the use of a ping-pong kinetic mechanism in the presence of optimal Mg^{2+} concentrations and that both half reactions, as defined by radioactive exchange between substrate/product pairs, require Mg^{2+}. The present results suggest that the M^{2+}–orotate phosphoribosyltransferase complex may be the enzyme form which catalyzes the reaction via this mechanism.

Divalent metal ion activators such as Mg^{2+} or Mn^{2+} are required for all phosphoribosyltransferase-catalyzed reactions in which β-glycosidic linkages are formed between the C-1 position of phosphoribosyl α-1-pyrophosphate[1] (P-Rib-PP) and the appropriate nitrogenous second substrates (eq 1).



(1)

The nature of this activation, however, is not clearly understood. It has been suggested or assumed that the monomagnesium or dimagnesium salts of P-Rib-PP are the true substrates for reactions catalyzed by adenine phosphoribosyltransferase from *Escherichia coli* (Berlin, 1969; Hochstadt-Ozer & Stadtman, 1971), by hypoxanthine–guanine

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[1] Abbreviations used: P-Rib-PP, 5-phosphoribosyl α-1-pyrophosphate; OMP, orotidine 5′-phosphate; PP_i, inorganic pyrophosphate; PRR, water proton magnetic relaxation rate; EPR, electron paramagnetic resonance.

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phosphoribosyltransferase from rabbit brain (Krenitsky et al., 1969) and human erythrocytes (Giacomello & Salerno, 1978), by orotate phosphoribosyltransferase from ascites cells (Kavipurapu & Jones, 1976), by ATP phosphoribosyltransferase from *Salmonella typhimurium* (Morton & Parsons, 1976), and by the anthranilate synthase–anthranilate phosphoribosyltransferase from this same bacterium (Henderson et al., 1970). Whereas certain of these investigators have considered other substrate–metal ion interactions (Giacomello & Salerno 1978; Morton & Parsons, 1976), the possible existence of binary metal ion–phosphoribosyltransferase complexes was not considered in these investigations. In contrast, studies of the nicotinate phosphoribosyltransferase catalyzed reaction in baker's yeast (Kosaka et al., 1977) suggest that nicotinate mononucleotide is formed from either Mg^{2+}–P-Rib-PP or P-Rib-PP and that a Mg^{2+}–enzyme complex might be required to bring about the phosphoribosyl transfer.

The reaction catalyzed by orotate phosphoribosyltransferase from yeast has also been observed to require Mg^{2+} or Mn^{2+} (Lieberman et al., 1955; Umezu et al., 1971). This enzyme-catalyzed reaction proceeds through the use of a bibi ping-pong kinetic mechanism under conditions of optimal Mg^{2+} concentrations (Victor et al., 1979). Moreover, both half reactions which describe this mechanism (eq 2 and 3)

$$E + \text{P-Rib-PP} \rightleftharpoons E\text{–P-Rib} + PP_i \quad (2)$$

$$E\text{–P-Rib} + \text{orotate} \rightleftharpoons E + \text{OMP} \quad (3)$$

require Mg^{2+}, as determined by the effect of Mg^{2+} concentrations on the exchange of label between [^{14}C]orotate and orotidine 5′-monophosphate (OMP) in the absence of P-Rib-PP and PP_i and the exchange of label between [^{32}P]PP_i and P-Rib-PP in the absence of orotate and OMP (Victor et al., 1979). In order to characterize the nature of the metal ion activation of these exchange reactions as well as the overall orotate phosphoribosyl transfer reaction, we have elected to characterize the possible binary metal ion–substrate and –product complexations and to search for possible metal ion–orotate phosphoribosyltransferase interactions. These results along with kinetic analysis of metal ion activation of this catalysis by Mg^{2+} and Mn^{2+} provide evidence for a multifunctional role for metal ions in this reaction.

Materials and Methods

Pressed baker's yeast (Budweiser brand) was purchased from Valenti Yeast, Inc., Flushing, NY. The reagents supplied by Sigma Chemical were P-Rib-PP (sodium salt) and orotidine 5′-monophosphate (sodium salt). Orotic acid was purchased from Calbiochem. Bio-Rad Laboratories supplied Chelex-100 cation exchange resin. The chloride salts of Mn^{2+}, Mg^{2+}, and Co^{2+} in addition to all other chemicals used in this investigation were analytical grade.

The purification of orotate phosphoribosyltransferase from baker's yeast was accomplished as described previously (Victor et al., 1979). Each of the three enzyme preparations used in these investigations was homogeneous by the criteria of polyacrylamide gel electrophoresis and was characterized by a specific activity[2] (in the absence of any added OMP decarboxylase) of 40 units/mg of protein. These preparations can be stored at −76 °C in the presence of orotate for 6 months with no appreciable loss of activity.

Measurements of the enzymic activity and metal ion activation of the phosphoribosyl transfer reaction were carried out spectrophotometrically at 25 °C and 290 nm by methods previously described (Umezu et al., 1971) using a Cary-15 recording spectrophotometer. The final concentrations of reactants in 1 mL of assay solution under conditions of substrate and activator saturation were 300 μM P-Rib-PP, 300 μM orotate, 1 mM $MgCl_2$ or $MnCl_2$, 50 mM Tris-HCl buffer (pH 8), and 2×10^{-4} mg of enzyme. The concentrations of the substrates and metal ion activators were varied as described in the text and figure legends. All of the substrate and enzyme solutions as well as the Tris-HCl buffer were eluted through Chelex-100 minicolumns to remove metal contaminants. No change in absorbance at 290 nm was observed until the appropriate aliquot of metal ion was added to the enzyme–substrate solution.

The UV spectra characterizing the formation of the Co^{2+}–orotate and Mn^{2+}–orotate complexes were obtained by adding appropriate concentrations of orotate and metal ion to solutions containing 50 mM Tris-HCl (pH 8). The final volume of all of these solutions was 1 mL, and the final concentrations of buffer and orotate were held constant over the entire metal ion concentration range. The UV absorbance of each solution was monitored on a Cary-15 recording spectrophotometer using reference samples which contained analogous Tris-HCl and metal ion concentrations.

Samples to be studied by electron paramagnetic resonance (EPR) were prepared as follows. Solutions of OMP, P-Rib-PP, PP_i, and orotate phosphoribosyltransferase (concentrations are listed in the Figure 3 legend) in Tris-HCl (50 mM; pH 8) were eluted through Chelex-100 minicolumns to remove metal contaminants. An appropriate aliquot of 100 mM $MnCl_2$ was added to each sample in order to achieve the required concentration of Mn^{2+}. The EPR spectrum of each of these samples was recorded on a Varian V-4500 X-band EPR spectrometer at 9.295 GHz over a sweep-field range of 3835–2835 G at room temperature. We are grateful to Dr. W. Sweeney of Hunter College for affording us the opportunity to use this instrumentation. The concentration of $Mn^{2+}(H_2O)_6$ was assumed to be proportional to the peak amplitudes of the EPR signal (Cohn & Townsend, 1954), and standard curves were constructed for each experiment by using aqueous Mn^{2+} solutions of known concentration.

The various binary and ternary complexes of substrates, products, and enzyme with Mn^{2+} were observed by measuring the water proton magnetic relaxation rates (PRR) of these solutions at 25 °C and 30 MHz by using a Spin-Lock Electronic Ltd. (Ontario, Canada) NMR spectrometer and the Carr–Purcell procedure (Carr & Purcell, 1954), the technical details of which have been described previously (Mildvan & Engle, 1972). Separate solutions (0.1-mL final volume) of $MnCl_2$ in Tris-HCl (50 mM; pH 8) containing appropriate concentrations of orotate, OMP, P-Rib-PP, or PP_i were prepared in 5-mm cellulose nitrate tubes for PRR determinations. A single enzyme solution (100 μM in Tris-HCl, pH 8) was titrated with appropriate μL aliquots of 0.1 M Mn^{2+}, and these results were compared to an analogous titration of buffer alone in order to discern dilution effects on the observed relaxation rates. The formation of the binary Mn^{2+}–P-Rib-PP complex was also monitored at 24.3 MHz by similar procedures using the laboratory-built instrument in the laboratory of Dr. A. S. Mildvan at the Institute for Cancer Research. We are grateful to Dr. Mildvan for this instrument time. Samples containing fixed concentrations of $MnCl_2$ (250 μM) and orotate phosphoribosyltransferase (75 μM) and various fixed concentrations of OMP or P-Rib-PP were employed to study the formation of ternary metal

[2] One unit of activity for the phosphoribosyl transfer reaction is defined as 1 μmol of OMP formed per min.

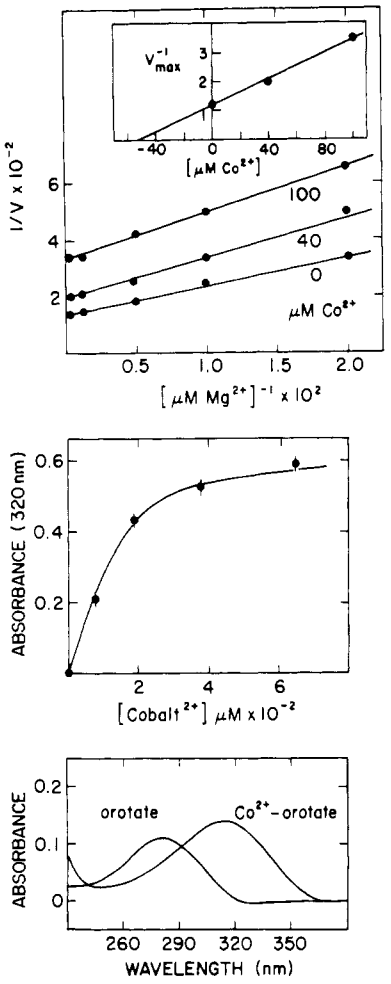


FIGURE 1: Kinetic analysis of Co^{2+} inhibition of the Mg^{2+} activation of the reaction catalyzed by orotate phosphoribosyltransferase from yeast (top figure) and analysis of Co^{2+} ligation of orotate (middle and bottom figures) in 50 mM Tris-HCl buffer, pH 8.0 at 25 °C. Final concentrations of orotate, P-Rib-PP, and enzyme utilized in the kinetic analysis over the divalent metal ion concentration ranges were 300 μM, 100 μM, and 2×10^{-4} mg/mL, respectively. The UV spectrum (bottom figure) of the Co^{2+}–orotate complex with an absorbance maximum at 315 nm was recorded by using 30 μM orotate whereas the increase in absorbance at 320 nm with addition of concentrations of Co^{2+} (middle figure) was recorded by using 140 μM orotate. Theoretical curve fitting relating this absorbance increase to Co^{2+}–orotate formation was accomplished as described under Results.

ion–substrate–enzyme complexes. Diamagnetic control experiments for all of the above-described investigations were carried out at 30 MHz employing equivalent concentrations of Mg^{2+} in place of Mn^{2+}.

Results

Binary Orotate–Metal Ion Complexes. Umezu et al. (1971) first observed the UV spectroscopic shift associated with the binding of metal ions to orotate. As shown in Figures 1 and 2, these shifts can be used to characterize the formation of Co^{2+}–orotate and Mn^{2+}–orotate complexes, respectively. Stability constants[3] [K_0 as defined by London & Steck (1969)] were calculated for these two complexes by generating theoretical curves which best fit the data by using the general equation (eq 4) shown below. As shown in Figure 1, a theoretical isotherm which fits the experimental points for the appearance of absorption at 320 nm vs. Co^{2+} concentration can be generated by using a K_0(orotate) value of 2.5×10^4 M^{-1} and a value of 1 for both p and q. This K_0 value corresponds to a dissociation constant ($\bar{K}_0$) equal to 40 μM. None of the theoretical curves, which were generated over a wide range of K_0 values by using eq 4 and values of p and q other than 1, provided a good fit to the experimental data. These results suggest that Co^{2+} and orotate form primarily a 1:1 complex in solution. A similar conclusion was reached for the Mn^{2+}–orotate ligation (Figure 2).

$$K_0(\text{ligand}) = \frac{[M_p\text{–ligand}_q]}{[M]^p[\text{ligand}]^q} \quad (4)$$

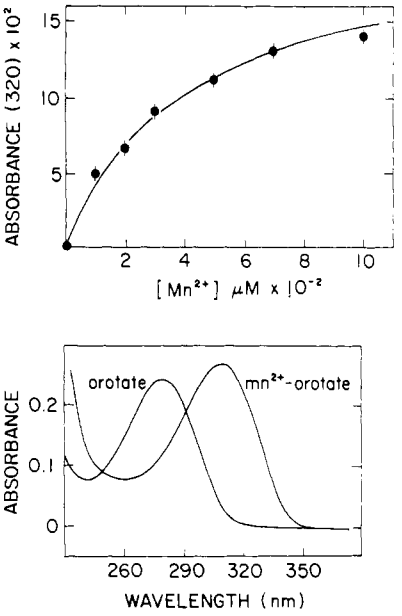


FIGURE 2: Analysis of Mn^{2+} ligation of orotate in 50 mM Tris-HCl buffer, pH 8.0 at 25 °C. The UV spectrum (bottom figure) of the Mn^{2+}–orotate complex with an absorbance maximum at 310 nm, as well as the 320-nm absorbance increase (top figure), was recorded by using 60 μM orotate. Theoretical curve fitting relating this absorbance increase to Mn^{2+}–orotate formation was accomplished as described under Results.

As shown in Figure 2, an isotherm generated by assuming a K_0(orotate) of 2×10^3 M^{-1} fits the experimental points for the appearance of 320-nm absorbance vs. Mn^{2+} concentration. This value corresponds to a $\bar{K}_0$ value of 500 μM. Spectral shifts were not observed over a concentration range of 0.1–10 mM Mg^{2+} in the presence of 100 μM orotate, which suggests but does not prove that Mg^{2+} has a low affinity for an orotate ligand. By the criteria of UV spectral shifts, then, the order of affinity for orotate by metal ions is $Co^{2+} > Mn^{2+} > Mg^{2+}$. Whereas both Mn^{2+} and Mg^{2+} are essential activators of orotate phosphoribosyltransferase activity (vide infra), Co^{2+} is not an activator of this enzymatic reaction. However, Co^{2+} serves as an effective noncompetitive inhibitor of activation by Mg^{2+} (Figure 1).

Electron Paramagnetic Resonance Studies. As shown in Figure 3, the concentration of Mn^{2+} in solution is proportional to the observed EPR spectral amplitude of this ion. In the presence of OMP, P-Rib-PP, and PP_i, this spectral amplitude is decreased over the concentration range of Mn^{2+}, indicating ligation between the paramagnetic ion and each of these

[3] The nomenclature for association constants and dissociation constants of London & Steck (1969) has been adapted for use in this manuscript. K_0(S) is an association constant for a substrate (S)–metal ion (M) interaction and is equal to $[MS]/[M]_f[S]_f$. $\bar{K}_0$(S) is a dissociation constant for this same interaction and is equal to the reciprocal of K_0(S). K_A is an association constant for the enzyme (E)–metal ion interaction and is equal to $[EM]/([M]_f[E]_f)$. $\bar{K}_A$ is a dissociation constant for this same interaction and is equal to the reciprocal of K_A.

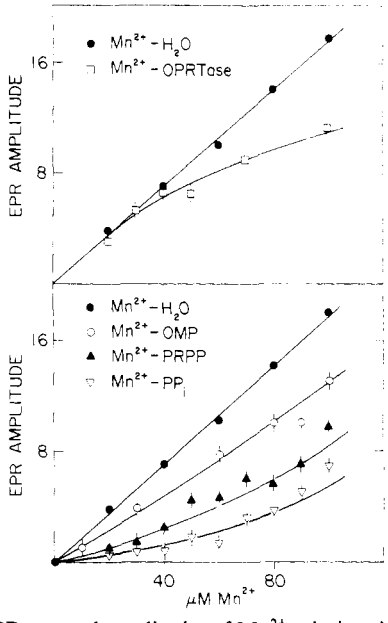


FIGURE 3: EPR spectral amplitudes of Mn^{2+} solutions in the absence of orotate phosphoribosyltransferase (closed circles) and presence of orotate phosphoribosyltransferase (open squares), OMP (open circles), P-Rib-PP (closed triangles), or PP_i (open triangles). The concentrations of enzyme, OMP, P-Rib-PP, and PP_i solutions used in these studies were all 100 μM, and all of the solutions were made up in 50 mM Tris-HCl buffer, pH 8.0. Theoretical curve fitting relating the observed EPR amplitude to the concentration of each of the Mn^{2+} complexes with substrates was accomplished as described under Results.

reactants. Since the signal amplitude represents a measurement of free Mn^{2+} in solution (Cohn & Townsend, 1954), theoretical curves have been generated (Figure 3) characterizing $\bar{K}_0$ values for P-Rib-PP, OMP, and PP_i equal to 40 ± 10, 200 ± 50, and 12 ± 5 μM, respectively. The uncertainty of these measurements impedes an evaluation of multiple binding sites for Mn^{2+} or evaluation of metal ion–ligand stoichiometries other than 1:1. However, theoretical plots of the EPR amplitude vs. Mn^{2+} concentrations generated over a wide range of K_0 values by using eq 4 and values for p and q of 1 and 2, respectively, all showed an abrupt upward curvature at a Mn^{2+} concentration of 50 μM. Moreover, analogous theoretical plots, generated by using p and q values of 2 and 1, respectively, were essentially linear over the experimental range of Mn^{2+} concentrations (0–100 μM). Because the experimental points do not fit either of these two criteria, and because a 1:1 stoichiometry (p and q both equal to 1) can be utilized to fit the data (Figure 3), it can be assumed that PP_i, P-Rib-PP, and OMP primarily form 1:1 complexes with Mn^{2+} in solution. Further proof of a Mn^{2+}–P-Rib-PP complex is provided by our water proton magnetic relaxation measurements (vide infra). Recently, $\bar{K}_0(PP_i)$ and $\bar{K}_0$(P-Rib-PP) values equal to 8 and 17 μM, respectively, were determined by using higher substrate and Mn^{2+} concentration ranges and this EPR technique (Tebar et al., 1977).

Effects of orotate phosphoribosyltransferase on the Mn^{2+} EPR signal are observed only at Mn^{2+}/enzyme ratios greater than 1:2 (Figure 3). This observation can be interpreted qualitatively in terms of complexation between Mn^{2+} and the phosphoribosyltransferase with the metal ion occupying more than a single enzyme site. The unexpected nature of this curve also suggests the occurrence of allosteric interaction between metal binding sites. This interpretation is supported by water proton magnetic relaxation rate measurements (vide infra) made over a more extended range of Mn^{2+} concentrations.

Water Proton Relaxation Rate Measurements. Complexation between Mn^{2+} and P-Rib-PP has been monitored by measuring the effect of Mn^{2+} on the water proton relaxation rates (PRR) of the P-Rib-PP solution [$T_{1(\text{P-Rib-PP})}^{-1}$] and comparing these values to the effect of Mn^{2+} on the PRR of the water solvent [$T_{1(H_2O)}^{-1}$] (Figure 4A). Both of these observed paramagnetic contributions to the PRR [defined as $T_{1\rho}^{-1}$ by Mildvan & Cohn (1970)] are related to a theoretical value for this effect (T_{1m}^{-1}) by eq 5 (Luz & Meiboom, 1964), where f is the fraction of the total concentration of ligand (in this case P-Rib-PP) bound to the metal ion, q is the number of water ligands, τ_m is the residence time of the ligand in the metal coordination sphere, and T_{os}^{-1} is the contribution to $T_{1\rho}^{-1}$ made by ligand molecules outside the metal ion coordination sphere. For the Mn^{2+} aquo ion, under conditions where T_{os}^{-1}

$$T_{1\rho}^{-1} = \frac{fq}{T_{1m} + \tau_m} + \frac{1}{T_{os}} \tag{5}$$

is neglible and $\tau_m \ll T_{1m}$, the observed paramagnetic effect ($T_{1\rho}^{-1}$) is described by the following equation (Solomon, 1955; Bloembergen, 1957)

$$T_{1\rho}^{-1} = \left[\frac{qB}{r^6} f(\tau_c)\right] \frac{[M]}{55.6} \tag{6}$$

where B is a collection of constants characteristic of Mn^{2+}, r is the distance between the ligand and Mn^{2+}, $f(\tau_c)$ is a correlation function for the dipolar Mn^{2+}–ligand interaction, and [M] is the molarity of bound Mn^{2+}. An observed $T_{1\rho}^{-1}$ change can be interpreted theoretically as indicating a change in q, r, or $f(\tau_c)$, and a successful interpretation requires extensive measurements of the temperature dependence and frequency dependence of the $T_{1\rho}^{-1}$ value (Mildvan & Gupta, 1978; Koenig, 1978). However, observed $T_{1\rho}^{-1}$ values can be related to the concentration of the Mn^{2+}–ligand complex prior to a full working knowledge of the nature of the effect.

As shown in Figure 4A, the complexation of P-Rib-PP with Mn^{2+}, as determined by changes in $T_{1(\text{P-Rib-PP})}^{-1} - T_{1(H_2O)}^{-1}$ with P-Rib-PP concentration at 30 and 24.3 MHz, is characterized by a theoretical curve generated by using a 1:1 metal ion–ligand stoichiometry and $\bar{K}_0$(P-Rib-PP) values of 30 (30 MHz) and 20 μM (24.3 MHz). The existence of significant concentrations of $(Mn^{2+})_2$–P-Rib-PP or Mn^{2+}–$(\text{P-Rib-PP})_2$ under these experimental conditions has been ruled out by curve-fitting exercises similar to those described for the Co^{2+}–orotate complex.

As shown in Figure 4B, a unique observation was made when Mn^{2+} was added to orotate phosphoribosyltransferase solutions. Although the shape of the resultant isotherm was sigmoidal as suggested by our EPR results, the value of $T_{1(E)}^{-1} - T_{1(H_2O)}^{-1}$ decreased [as shown by an increase in $T_{1(H_2O)}^{-1} - T_{1(E)}^{-1}$ in Figure 4B] with increased Mn^{2+} concentration. This effect, which is equal to an enhancement value (ϵ^*) as defined by Mildvan & Cohn (1970) of 0.1, is the opposite of that observed for the P-Rib-PP additions (Figure 4A).

An attempt has been made to characterize the sigmoidal shape of the binding isotherm shown in Figure 4B by fitting this data with a theoretical curve generated using eq 7 (Monod

$$Y_f = \frac{Lc\alpha(1 + c\alpha)^{n-1} + \alpha(1 + \alpha)^{n-1}}{L(1 + c\alpha)^n + (1 + \alpha)^n} \tag{7}$$

et al., 1965). Although eq 7 is a more general equation for cooperative binding events, we have assumed a two-state conformational equilibrium between two enzyme forms (R and

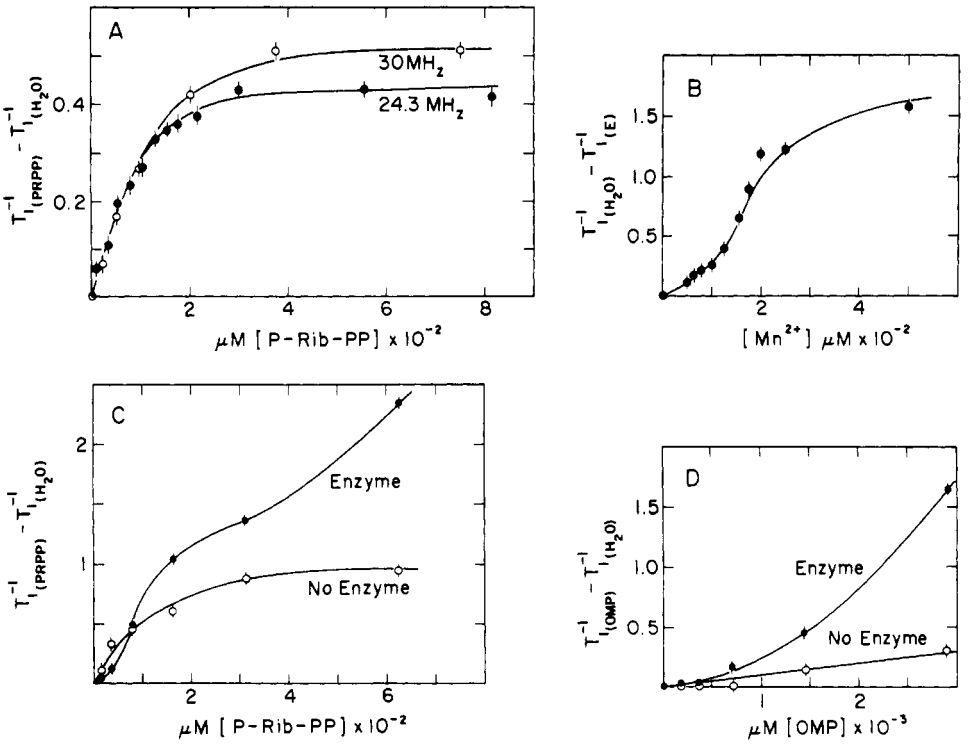


FIGURE 4: Effects of orotate phosphoribosyltransferase from yeast $[T_{1(H_2O)}^{-1} - T_{1(E)}^{-1}]$ and/or substrates of this reversible phosphoribosyl transfer reaction $[T_{1(P\text{-}Rib\text{-}PP)}^{-1} - T_{1(H_2O)}^{-1}$ or $T_{1(OMP)}^{-1} - T_{1(H_2O)}^{-1}]$on the paramagnetic effect of Mn^{2+} on the water proton magnetic relaxation rate $[T_{1(H_2O)}^{-1}]$. (A) The change in the observed relaxation rates with the addition of P-Rib-PP to 100 μM Mn^{2+} solutions (50 mM Tris-HCl, pH 8.0 and 25 °C) at 30 (open circles) and 24.3 MHz (closed circles). (B) The change in the observed relaxation rates with the addition of Mn^{2+} to a 100 μM phosphoribosyltransferase solution (50 mM Tris-HCl, pH 8.0) subtracted from this change observed with the addition of Mn^{2+} to buffer solutions at 30 MHz. (C) The change in the observed relaxation rates with the addition of P-Rib-PP to 250 μM Mn^{2+} solutions (open circles) and this change with the addition of P-Rib-PP to a solution of 75 μM phosphoribosyltransferase and 250 μM Mn^{2+} (closed circles) in 50 mM Tris-HCl, pH 8 at 25 °C, and at 30 MHz. (D) The change in the observed relaxation rates with the addition of OMP to 250 μM Mn^{2+} solutions (open circles) and this change with the addition of OMP to a solution of 75 μM phosphoribosyltransferase and 250 μM Mn^{2+} (closed circles) in 50 mM Tris-HCl, pH 8 at 25 °C, and at 30 MHz.

T) where $\bar{K}_A(R)$ and $\bar{K}_A(T)$ are dissociation constants[3] defined by London & Steck (1969) for the Mn^{2+}–R and Mn^{2+}–T complexes, respectively. In eq 7 L is a constant, α is equal to $[Mn^{2+}]/\bar{K}_A(R)$, c is equal to $\bar{K}_A(T)/\bar{K}_A(R)$, and n is equal to the number of metal ion binding sites on the enzyme. Because we had determined previously that orotate phosphoribosyltransferase from yeast was composed of two subunits of molecular weight 20000 (Victor et al., 1979), curve fitting was initiated by assuming a value of n equal to 2. However, varying values of c, L, and α over several orders of magnitude did not lead to an effective fit of the experimental points. For a value of n equal to 4, sufficiently sigmoidal curvatures were generated, and the best fit was achieved by using values of L = 250, c = 0.01, and $\bar{K}_A(R)$ = 50 μM (Figure 4B). We conclude therefore that a $(Mn^{2+})_4$–phosphoribosyltransferase complex forms (two metal ions per subunit) and that certain of these enzyme sites for Mn^{2+}, perhaps two sites, are characterized by a dissociation constant for Mn^{2+} equivalent to the K_m for P-Rib-PP in the phosphoribosyl transfer reaction (Victor et al., 1979).

Also illustrated in Figure 4 are the effects of addition of P-Rib-PP (Figure 4C) or OMP (Figure 4D) on the PRR of solutions containing Mn^{2+} and orotate phosphoribosyltransferase. We have elected to illustrate these effects to show that a reversal of the above-described decrease in the value of $T_{1(E)}^{-1} - T_{1(H_2O)}^{-1}$ occurs with subsequent additions of substrates and products. The observation of these reversals may indicate either formation of ternary Mn^{2+}–P-Rib-PP–enzyme and Mn^{2+}–OMP–enzyme complexes or competition between reactants and the enzyme for the metal ion. Moreover, these two enzyme complexes have the potential to form the products PP_i and orotate, respectively, via a ping-pong kinetic mechanism (Victor et al., 1979), and their formation would perturb the PRR of these solutions. Definitive interpretation of the PRR of solutions containing Mn^{2+}, enzyme, and substrate (as well as interpretation of the ϵ^* decrease associated with the enzyme additions) must await further studies employing higher concentrations of the phosphoribosyltransferase and new studies of the frequency and temperature dependences of these effects.

Kinetic Analysis of Metal Ion Activation. Previous studies of the effects of metal ions on the orotate phosphoribosyltransferase catalyzed reaction established Mg^{2+} as an effective activator of the reaction (Lieberman et al., 1955) and Hg^{2+} as an inhibitor (Umezu et al., 1971). We have determined that Co^{2+} is an effective noncompetitive inhibitor of Mg^{2+} activation (Figure 1) and that Ca^{2+} neither activates this reaction nor inhibits activation by Mg^{2+}. In spite of the fact that shifts in the UV spectrum of orotate occur with addition of Mn^{2+} to the assay mixture, the disappearance of orotate during the catalytic reaction can be monitored at 290 nm because both orotate and the Mn^{2+}–orotate complex have equivalent absorbances at that wavelength (Figure 2). Thus, both Mg^{2+} and Mn^{2+} activation studies can be utilized to delineate the role of metal ions in the orotate phosphoribosyltransferase reaction.

We have examined the effect of additions of both Mg^{2+} and Mn^{2+} on the initial velocity for OMP formation. The concentrations of free Mg^{2+} or Mn^{2+} in each of the assay mixtures were calculated by using the $\bar{K}_0$ and $\bar{K}_A$ values described in the text and by assuming that (1) the concentrations of metal–enzyme complexes are negligible compared to the free

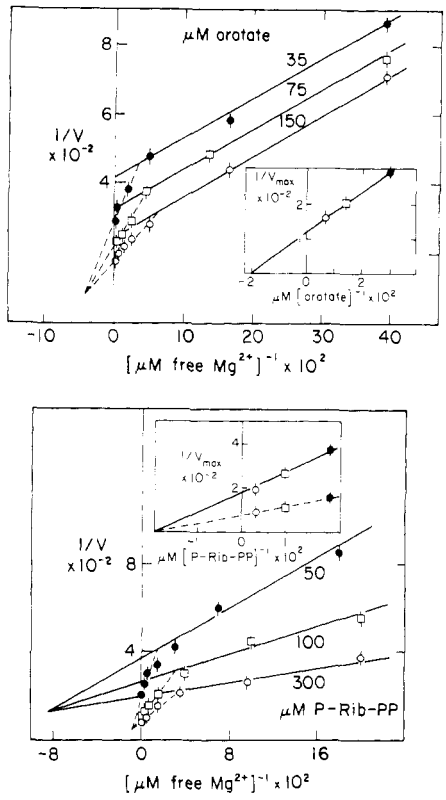


FIGURE 5: Double-reciprocal plots of free $[Mg^{2+}]^{-1}$ vs. v_0^{-1} in the presence of 300 μM P-Rib-PP and various fixed concentrations of orotate (top figure) and in the presence of 300 μM orotate and various fixed concentrations of P-Rib-PP (bottom figure). All v_0 measurements (in units of micromoles of OMP formed per minute) were taken under conditions of 50 mM Tris-HCl buffer, pH 8.0 at 25 °C, using 2×10^{-4} mg of enzyme. Each point in this figure corresponds to an average of nine v_0 measurements.

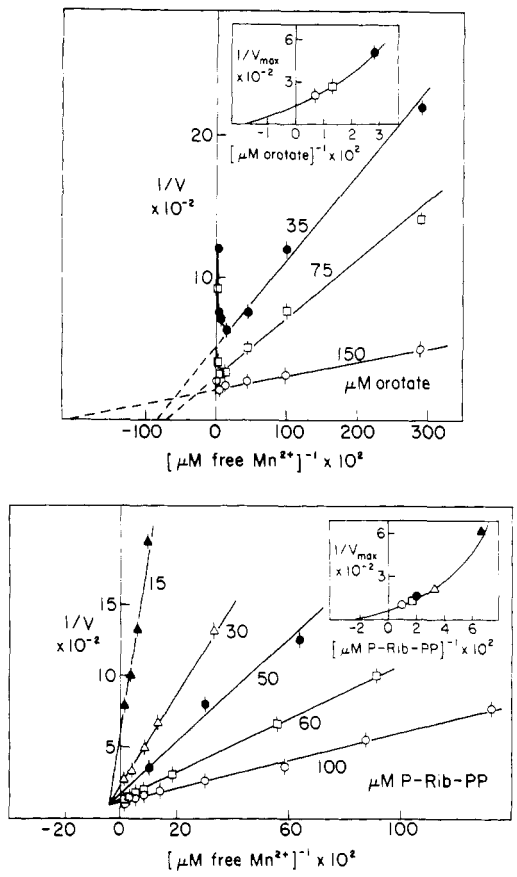


FIGURE 6: Double-reciprocal plots of free $[Mn^{2+}]^{-1}$ vs. v_0^{-1} in the presence of 300 μM P-Rib-PP and various fixed concentrations of orotate (top figure) and in the presence of 300 μM orotate and various fixed concentrations of P-Rib-PP (bottom figure). The conditions are as described in Figure 5.

metal ion concentration (5 nM enzyme was utilized in each assay solution), (2) Mg^{2+} does not complex with orotate but forms a 1:1 complex with P-Rib-PP primarily characterized by a $\bar{K}_0$(P-Rib-PP) value of 260 μM (Morton & Parsons, 1976), and (3) Mn^{2+} forms 1:1 complexes with P-Rib-PP and orotate. The possibility that $(M^{2+})_2$–P-Rib-PP and M^{2+}–$(P\text{-Rib-PP})_2$ complexes form was not considered because neither complex was detected in our binding studies. However, these complexes may indeed form at high relative concentrations of metal ion and P-Rib-PP, respectively, and increase the error in the calculation of free metal ion under these conditions.

As shown in Figure 5, the double-reciprocal plots of free $[Mg^{2+}]^{-1}$ vs. v_0^{-1} at various fixed levels of P-Rib-PP are composed of two sets of intersecting lines. This type of curved pattern of lines has been considered by Frieden (1964) and may be described in terms of two Mg^{2+} binding sites on the enzyme, one of which is allosteric in character. The intersection of lines of each pattern to the left of the y axis also supports the conclusion that a catalytically active Mg^{2+}–phosphoribosyltransferase complex as well as an active metal-free enzyme is involved in this reaction. The utilization of two enzyme forms to produce products has been described as model III by London & Steck (1969) and case III by Adolfsen & Moudrianakis (1978a,b) and will be described under Discussion. The two points of intersection of the patterns of lines should also provide an estimation of two K_A values for the metal interaction with enzyme (Mildvan & Cohn, 1965). These two values are determined to be 100 and 12 μM from the plot of free $[Mg^{2+}]^{-1}$ vs. v_0^{-1} at various fixed levels of P-Rib-PP (Figure 5). A single value of $\bar{K}_A$ equal to 25 μM can be determined from this plot at various fixed levels of orotate (Figure 5), and a second $\bar{K}_A$ value can be established from the seemingly parallel-line pattern by extrapolation of the data taken at the two lowest metal ion concentrations to a common point of intersection. This $\bar{K}_A$ value is 3 μM. Although these values calculated from the orotate data do not agree numerically with those calculated for the P-Rib-PP data, the numerical ratio of both sets of $\bar{K}_A$ values is 8:1. Secondary plots of the y intercepts vs. the reciprocals of varied fixed concentrations of P-Rib-PP and orotate (Figure 5) intersect the x axis at points corresponding to reciprocal values of the K_m values (Victor et al., 1979) of these two substrates.

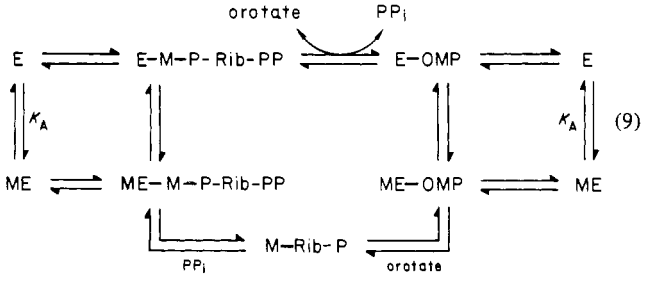
Although the nonlinear character of the double-reciprocal plots of free $[Mn^{2+}]^{-1}$ vs. v_0^{-1} at varied fixed levels of orotate or P-Rib-PP is not readily apparent (Figure 6), we have observed that at high concentrations of Mn^{2+} and relatively low concentrations of orotate or P-Rib-PP, the reaction is inhibited. This effect is seen most definitively at low concentrations of orotate (Figure 6) under conditions in which orotate is known to ligand with Mn^{2+}. However, we have also denoted that secondary plots of the y intercepts, of both of the above-described reciprocal plots vs. the varied fixed substrate concentrations, are nonlinear, which is consistent with the hypothesis that a Mn^{2+}–phosphoribosyltransferase complex as well as the metal-free phosphoribosyltransferase may be catalytically active (Aldolfsen & Moudrianakis, 1978b). A single $\bar{K}_A$ value equal to 20 μM can be evaluated from the double-reciprocal plot involving fixed P-Rib-PP concentrations (Figure 6). This $\bar{K}_A$ value is the same order of magnitude as the theoretical $\bar{K}_A(R)$ value utilized to fit the Mn^{2+}–enzyme binding isotherm (Figure 4B) but is also similar to the $\bar{K}_0$

values characterizing the Mn^{2+}–P-Rib-PP interaction as determined by the EPR (Figure 3) and PRR (Figure 4) techniques. A single point of interaction of lines was not obtained for the double-reciprocal plot of free $[Mn^{2+}]^{-1}$ vs. v_0^{-1} at fixed levels of orotate. Thus, a $\bar{K}_A$ value was not determined from these data.

Discussion

Several lines of evidence suggest that more than a single role is played by divalent metal ions in activating the enzyme-catalyzed orotate phosphoribosyltransfer reaction. (1) The high affinity for ligation of P-Rib-PP by Mn^{2+} and by Mg^{2+} (Morton & Parsons, 1976), which we believe occurs primarily through interaction with the pyrophosphate group, indicates that a metal ion complex with P-Rib-PP is the predominant species of this substrate under conditions of optimum catalytic activity and is the true substrate for this reaction. (2) Binary Mn^{2+}–orotate phosphoribosyltransferase complexes have been detected, initial formations of which may induce conformational changes in the enzyme which allows additional Mn^{2+} interactions. A $(Mn^{2+})_4$–phosphoribosyltransferase complex may be the predominant enzyme species under optimal reaction conditions with this metal ion. (3) Although Mn^{2+}, and presumably Mg^{2+}, has a relatively low affinity for OMP, the exchange of label between [^{14}C]orotate and OMP requires the presence of a divalent metal ion. (4) The biphasic nature of the double-reciprocal plots of free $[Mg^{2+}]^{-1}$ vs. v_0^{-1} suggests two reaction pathways for this catalysis governed by two different V_{max} values (V_{max} and V_{max}'). Our indirect evidence (nonlinear plots, Figure 6) suggests but does not prove two reaction pathways for Mn^{2+} activation as well. Thus, if it is assumed that the collective results for both Mg^{2+} and Mn^{2+} characterize a common pathway, a scheme can be devised which delineates the role divalent metal ions (abbreviated as M) play in this catalytic reaction (eq 8 and 9).

$$M + \text{P-Rib-PP} \overset{K_0}{\rightleftharpoons} \text{M-P-Rib-PP} \qquad (8)$$



As shown in eq 8 and 9, both E and ME represent catalytically active orotate phosphoribosyltransferases which combine with P-Rib-PP–M (the true substrate) and orotate to form OMP and PP_i. Theoretical aspects of this pathway have been considered previously by London & Steck (1969) and more recently by Adolfsen & Moudrianakis (1978b). We propose that the order of product release is different, however, for each reaction pathway, with the reaction catalyzed by the ME complex proceeding through the use of the ping-pong kinetic mechanism, and that different V_{max} values govern each pathway. An analogous mechanism has been proposed for the hypoxanthine–guanine phosphoribosyltransferase catalyzed reaction (Krenitsky et al., 1969). These proposals are fully consistent with the kinetic data presented previously (Victor et al., 1979) and in this paper.

Our characterization of more than a single role for divalent metal ions in a catalytic reaction involving a phosphorylated substrate has several precedents. Mildvan et al. (1976) through a series of NMR investigations have proposed a mechanism for the phosphotransfer reaction catalyzed by pyruvate kinase which makes use of two divalent metal ions and a monovalent ion. Additionally, Aldolfsen & Moudrianakis (1978b), Butler & Sperow (1977), and Switzer (1971) have utilized kinetic analysis to propose that multiple metal ion binding sites are involved in the reactions catalyzed by adenosine triphosphatase, inorganic pyrophosphatase, and P-Rib-PP synthetase, respectively. All of these enzyme-catalyzed reactions, as well as the reaction catalyzed by orotate phosphoribosyltransferase, involve the breaking of phosphate linkages. More recent studies of the role of metal ions in the P-Rib-PP synthetase catalyzed reaction (Li et al., 1978) have provided evidence for formation of a catalytically active M^{2+}–enzyme complex. Thus, P-Rib-PP synthesis, as well as its utilization, may require such ligation.

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Role of Metal Cofactors in Enzyme Regulation. Differences in the Regulatory Properties of the *Escherichia coli* Nicotinamide Adenine Dinucleotide Specific Malic Enzyme Depending on Whether Mg^{2+} or Mn^{2+} Serves as Divalent Cation†

Judi A. Milne and Robert A. Cook*

ABSTRACT: A number of differences in the kinetic properties of the *Escherichia coli* nicotinamide adenine dinucleotide (NAD^+) dependent malic enzyme have been found depending upon whether Mg^{2+} or Mn^{2+} served to fulfill the divalent cation requirement. With Mg^{2+} as cation, the velocity–malate saturation curve in the absence of effectors is complex at pH 7.4, indicating a combination of apparent positive and negative cooperativity, while the velocity-free Mg^{2+} saturation curve exhibits positive cooperativity. If Mn^{2+} served as cation, however, the velocity–malate and velocity-free Mn^{2+} saturation curves exhibit a simple hyperbolic response. The velocity–NAD^+ saturation curves, in contrast, exhibit a simple hyperbolic response in the presence of either metal cofactor, but the affinity for NAD^+ and the V_{max} are increased in the presence of Mn^{2+}. When Mg^{2+} serves as cation, the enzyme activity is much more sensitive to regulation by the allosteric inhibitor CoA and the allosteric activator aspartate. If Mn^{2+} replaces Mg^{2+}, the enzyme activity is more sensitive to inhibition by ATP. This inhibition is shown to be due to chelation but may be of physiological importance. The inhibitor, CoA, increases the interaction between malate-binding sites in the presence of Mn^{2+} but has little effect on subunit interaction in the presence of Mg^{2+}. The kinetic data can be explained by a model involving sequential ligand-induced conformational changes of the enzyme, resulting in a mixture of apparent positive and negative cooperative behavior. Alternate explanations involving different classes of noninteracting binding sites or different enzyme forms are also considered. The metal cofactors Mg^{2+} and Mn^{2+} appear to stabilize two distinct forms of the enzyme which differ in response to varying substrate and effector concentrations. The results are strikingly similar to previous results reported on the NAD^+-dependent isocitrate dehydrogenase, supporting the suggestion that metal cofactors function as regulatory entities.

The NAD^+-specific[1] malic enzyme (EC 1.1.1.38) of *Escherichia coli* has been reported to exhibit characteristics of a modulator dependent cooperative (MDC) system (Sanwal, 1970a); i.e., the substrates, malate and NAD^+, exhibit normal hyperbolic behavior in the absence of effectors, but cooperative behavior in the presence of allosteric inhibitors (Sanwal, 1970b; Yamaguchi et al., 1974). The enzyme has been shown to be specifically inhibited by CoA, acetyl-CoA, and ATP and activated by aspartate (Takeo et al., 1967; Sanwal, 1970b; Yamaguchi et al., 1974). The catalytic and regulatory properties of the enzyme have been determined almost exclusively in the presence of Mn^{2+} as the required cofactor, although the divalent cation requirement can be fulfilled by Mg^{2+} (Takeo et al., 1967; Takeo, 1969; Sanwal, 1970b; Murai et al., 1972; Yamaguchi et al., 1974). In view of the recent results reported with the NAD^+-specific isocitrate dehydrogenase suggesting that the metal cofactors, Mg^{2+} and Mn^{2+}, may possibly function as allosteric effectors (Barratt & Cook, 1978), a detailed examination of the function of metal cofactors in the NAD^+-specific malic enzyme system as well as other enzyme systems capable of utilizing Mg^{2+} and Mn^{2+} is warranted. Distinct effects of Mg^{2+} and Mn^{2+} on the NAD^+-specific malic enzyme have, in fact, been reported by Yamaguchi et al. (1974), primarily in regard to aspartate activation. In their study, it was reported that (1) the enzyme was more sensitive to inhibition by CoA in the presence of Mg^{2+}; (2) the interaction between malate-binding sites was weakly cooperative in the presence of Mg^{2+}; (3) high concentrations of aspartate were inhibitory only in the presence of Mn^{2+}; and (4) Mg^{2+} and Mn^{2+} exhibited sigmoidal behavior in the absence of effectors. A definitive role for the metal cofactor in the enzyme mechanism was not suggested, however, from these preliminary results.

Since the substrates and effectors utilized in the present study are capable of chelating Mg^{2+} and Mn^{2+} to different extents, the experimental data was routinely subjected to computer analysis to determine the concentration of the various free and chelated forms of all ligands. Such analyses have allowed us to distinguish true allosteric effects from simple

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[1] Abbreviations used: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; $NADP^+$, NAD^+ phosphate; CoA, coenzyme A; n_H, Hill coefficient or interaction coefficient; V_{max}, maximum velocity; $S_{0.5}$, half-saturating concentration of substrate; $I_{0.5}$, concentration of inhibitor giving 50% inhibition; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid.

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