Studies of the Mechanism of Activation of the Yeast Hypoxanthine–Guanine Phosphoribosyltransferase Catalyzed Reactions by Divalent Metal Ions†

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ABSTRACT: The yeast hypoxanthine–guanine phosphoribosyltransferase (HGPRTase) catalyzed formations of both GMP and IMP are activated by Mg^{2+}, Mn^{2+}, or Co^{2+}. These activations have been examined kinetically over wide concentration ranges of the metal ions with a high-pressure liquid chromatography assay procedure. Theoretical curve-fitting exercises were then employed to analyze these kinetic data in terms of specific models described by London and Steck [London, W. P., & Steck, T. L. (1969) *Biochemistry 8*, 1767–1779]. These results define a mechanism through which metal ions (M^{2+}) activate nucleotide formation. (1) A complex is formed between HGPRTase and M^{2+}. (2) This form of the enzyme then can bind the substrate phosphoribosyl pyrophosphate (PRibPP) and a second M^{2+} (in either order) or can bind a M^{2+}–PRibPP complex. (3) Thereafter, hypoxanthine or guanine binds, and products are formed. In addition, the relative values of the calculated dissociation constants, which characterize all of the binding events, and water proton relaxation rate measurements, which confirm the formation of Mn^{2+} complexes with PRibPP and the enzyme, suggest that the binding of a M^{2+}–PRibPP complex with M^{2+}–HGPRTase is the primary route for activation of these reactions.

Although it is relatively easy to determine that an enzyme-catalyzed reaction is activated by divalent metal ions, it is sometimes difficult to sort out which of the many possible metal ion complexations that occur in solution are required for activity. For example, all of the phosphoribosyltransferases that have been examined to date require Mg^{2+} for activity (Musick, 1981). Because phospho-α-ribosyl 1-pyrophosphate (PRibPP), the substrate for all of these enzymes, is known to form tight complexes with Mg^{2+} (Morton & Parsons, 1976; Thompson et al., 1978; Smithers & O'Sullivan, 1982) and Mn^{2+} (Victor et al., 1979), it is generally assumed that these complexes are the true substrates for the phosphoribosyltransferase-catalyzed reactions and that this explains why metal ions are required. However, other substrate and product ligations with metal ions are known to occur (Victor et al., 1979; Giacomello & Salerno, 1978; Dodin et al., 1982) as do formations of metal ion complexes with the enzymes themselves (Kosaka et al., 1977; Victor et al., 1979), and these complexes may also be required for the enzymes' activities.

London & Steck (1969) have devised three general models for enzyme activation in which both metal ion–enzyme and metal ion–substrate complexes may contribute to the catalytic activity, and these authors have described kinetic methods for distinguishing these models. In this paper, we report a kinetic analysis of the activation by Mg^{2+}, Mn^{2+}, and Co^{2+} of the yeast hypoxanthine–guanine phosphoribosyltransferase (HGPRTase) catalyzed formations of GMP and IMP. Theoretical curve-fitting exercises have been employed to define the sequence of metal ion binding events that activate HGPRTase. A preliminary account of this work has been presented (Ali & Sloan, 1983).

Materials and Methods

Materials. Bakers' yeast (Budweiser brand) was obtained from Valente Yeast Inc. (Flushing, NY). PRibPP (sodium salt), hypoxanthine, guanine, IMP, GMP, and triethanolamine were supplied by Sigma Chemical Co. Chelex-100 exchange resin was provided by Bio-Rad Laboratories. All other chemicals, including the chloride salts of manganese, magnesium, and cobalt, were analytical grade. Methanol (Mallencroft reagent grade) was distilled prior to use during high-pressure liquid chromatography (HPLC)[1] whereas HPLC-grade water was prepared in a Gelman Water I apparatus.

Enzyme Purification. HGPRTase was purified from bakers' yeast to apparent electrophoretic homogeneity by a published procedure (Ali & Sloan, 1982).

High-Pressure Liquid Chromatography. A Waters HPLC instrument equipped with a Model 6000A solvent delivery system, Model U6K sample injector, Model 440 absorbance detector, and a Houston Omniscribe chart recorder was used in the assay procedure. A single 4 mm × 40 cm Waters μBondapak C_{18} column (equilibrated with 10% methanol) was placed on-line with the solvent delivery system at a flow rate of 1 mL/min. Samples (10 μL) from solutions containing the enzyme, substrates, and the appropriate concentrations of divalent metal ions were injected onto the column with a Hamilton 801 microliter syringe and eluted under 700–1000 psi pressure. Nucleotides and bases in the eluent were detected at 254 nm with a 0.1 absorbance setting. All of the solvents used in the chromatographic procedures were eluted, by vacuum filtration, through a 0.45-mm HA Millipore filter.

Enzymatic Assay Procedure. Measurements of the HGPRTase-catalyzed reactions and the activations of these reactions by divalent metal ions were accomplished by using a modification of the procedure described by Flaks (1963). The assay mixture consisted of 0.2 mL of a hypoxanthine or guanine solution (54 μM final concentration), 0.4 mL of a PRibPP solution (100 μM final concentration), 0.1 mL of one of the standard divalent metal ion solutions, 0.1 mL of 20 mM

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[1] Abbreviations: PRibPP, 5-phospho-α-ribosyl 1-pyrophosphate; HGPRTase, hypoxanthine–guanine phosphoribosyltransferase; HPLC, high-pressure liquid chromatography.

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triethanolamine (pH 7.8), and water to a final volume of 2.4 mL. This solution was placed in a 38 °C water bath, and the reaction was initiated with the addition of 1.6 mL of a dilute HGPRTase solution (approximately 1 milliunit of enzyme).[2] Thereafter, aliquots of the solution were removed at appropriate time intervals, and the reaction occurring in the aliquots was terminated by heating each sample in a boiling water bath (2 min). The samples were then clarified, first by centrifugation and then by passage through a 0.45-μm HA Millipore filter prior to HPLC injection. The HPLC elution profiles were employed to determine the time course of the reaction as described previously (Hanna & Sloan, 1980; Ali & Sloan, 1982). During the kinetic analysis, various concentrations of guanine or hypoxanthine, PRibPP, and either Co^{2+}, Mg^{2+}, or Mn^{2+} were employed. These concentrations are defined in the text and figure legends. To ensure that no metal ions were present in the assay solutions prior to the addition of the appropriate divalent metal ion, all of the substrate and enzyme solutions, as well as the triethanolamine buffer, were eluted through Chelex-100 minicolumns.

Water Proton Magnetic Relaxation Rate Measurements. Formation of the binary complex between Mn^{2+} and HGPRTase was detected by measuring the effect of addition of Mn^{2+} to a 40 μM solution of HGPRTase (0.1 mL) on the solution's water proton relaxation rate. These measurements were made at 25 °C and 30 MHz in a Spin-Lock Electronics Ltd. (Ontario, Canada) NMR spectrometer with a pulse sequence described previously (Carr & Purcell, 1954; Mildvan & Engle, 1972). The Mn^{2+} ligations with substrates and products of the HGPRTase-catalyzed reactions were monitored by published procedures (Victor et al., 1979). 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 and Discussion

Complete separations of guanine from GMP and hypoxanthine from IMP can be achieved by using reverse-phase HPLC and a 10% methanol elution buffer. The assay procedure described here, which takes advantage of this separation, is a modification of the methods described by Vasquez & Bieber (1978) and Hanna & Sloan (1980) and allows the HGPRTase-catalyzed reactions to be monitored quickly and with high sensitivity.

When divalent metal ions are removed completely from assay solutions containing all other reactants, the formation of IMP or GMP does not occur. With the addition of micromolar concentrations of Mg^{2+} to these solutions, nucleotide formations proceed over a period of 10–60 min. Activation with higher concentrations of Mg^{2+} and a range of concentration of Mn^{2+} or Co^{2+} require the removal of aliquots from the incubation mixture over a shorter time period (0.5–3 min) in order to evaluate initial rates. The concentrations of divalent ions that provide optimal activities varied with the concentration of bases and PRibPP in the incubation mixture and are within the ranges of 700–1100 μM for Mg^{2+} and 100–600 μM for Mn^{2+}. The highest concentration of Co^{2+} utilized in these studies was 100 μM because at higher concentrations an insoluble complex was formed between Co^{2+} and the base substrates (as determined by decreases in the hypoxanthine and guanine HPLC peak amplitudes). Thus a determination of the optimal concentration of this metal ion was not possible.

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

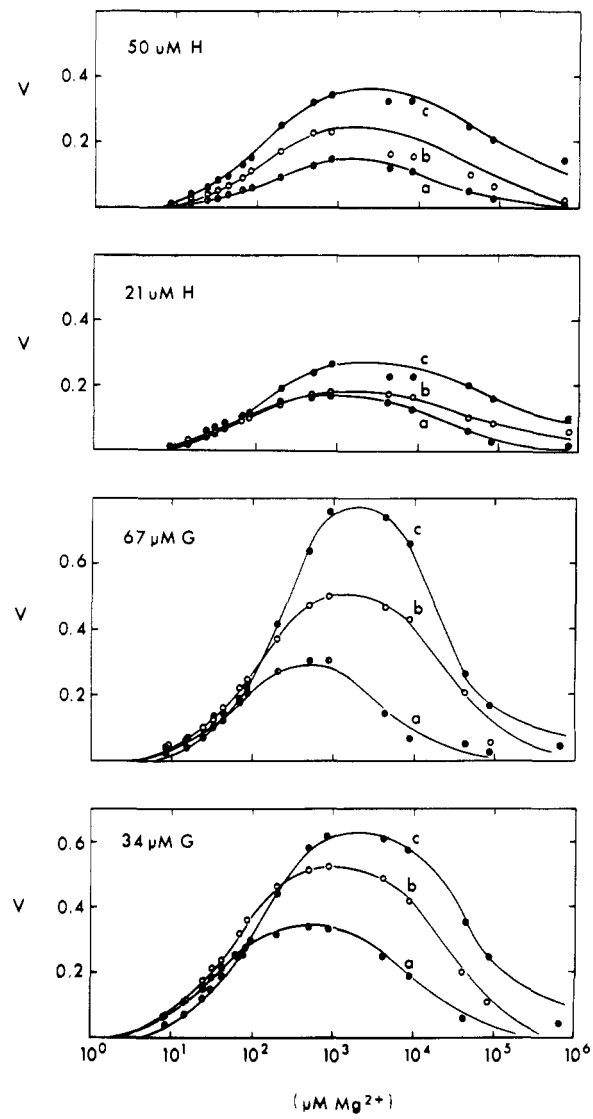


FIGURE 1: Initial velocity (V) measurements of the yeast HGPRTase-catalyzed formations of IMP and GMP, using the HPLC assay procedure, over a range (8 μM–0.8 M) of Mg^{2+} concentrations. The concentrations of bases present in the incubation solutions are defined in the graphs. Lines a, b, and c of each graph illustrate incubations during which 33, 66, and 167 μM PRibPP, respectively, were present in the incubation solutions. Theoretical curves were generated by using eq 4 as described in the text.

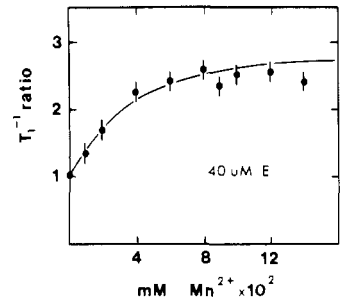


FIGURE 2: Effects of addition of Mn^{2+} on water proton longitudinal relaxation rates (T_1) of 40 μM HGPRTase solutions (pH 7.4). The T_1 values are presented in the form of a ratio in which the effect of paramagnetic Mn^{2+} is divided by the effect of addition of an equivalent concentration of diamagnetic Mg^{2+}.

At the higher concentrations of Mg^{2+} (1–160 mM) and Mn^{2+} (500 μM–160 mM), decreased rates for the formations of IMP and GMP were observed. Hence, for these metal ions three ranges of concentrations are observed: an activation

Table I: Calculated Kinetic Constants for Metal Ion Activation of Hypoxanthine-Guanine Phosphoribosyltransferase from Yeast[a]

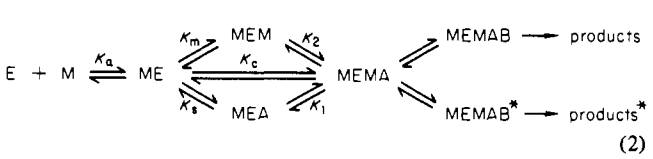
metal ion	[PRibPP] (μM)	[H] (μM)	[G] (μM)	K_s (μM)	K_m (mM)	K_a (μM)	$\bar{K}_c$ (μM)	μmol of IMP or GMP/min V_H	V_G
Mn^{2+}	33	21		45	2	7	15	0.4	
	33	50		45	2	12	6	0.5	
	33		34	9	0.5	5	3		0.6
	33		67	8	0.3	5	3		0.6
	83	21		20	0.8	10	8	0.4	
	83	50		23	1	5	8	0.5	
	83		34	10	0.5	5	5		0.7
	83		67	12	0.5	5	6.4		0.9
	167	21		23	1	4	11.4	0.5	
	167	50		20	1	4	11.3	0.7	
	167		34	30	2	4	5.4		0.8
	167		67	23	2	4	10		1.0
Co^{2+}	33	21		31	2	5	10	0.4	
	33	50		47	2	7	9	0.5	
	33		34	30	2	5	11		0.5
	33		67	18	2	4	8		0.5
	83	21		22	0.3	5	4.8	0.4	
	83	50		17	0.3	4	5.4	0.5	
	83		34	12	0.3	5	4.8		0.5
	83		67	24	0.3	5	4		0.5
	167	21		15	1	4	7.3	0.4	
	167	50		13	1	4	12	0.7	
	167		34	16	1	4	5.9		0.8
	167		67	19	1	4	11.5		0.9
Mg^{2+}	33	21		11	0.3	20	10	0.2	
	33	50		39	0.3	25	7	0.2	
	33		34	7	0.5	25	10		0.4
	33		67	9	2	27	20		0.4
	83	21		15	1	20	8	0.2	
	83	50		24	1	20	8	0.3	
	83		34	9	0.5	15	5		0.6
	83		67	19	0.5	20	5		0.6
	167	21		15	1	20	10	0.3	
	167	50		17	1	20	12	0.4	
	167		34	23	0.5	20	13		0.7
	167		67	40	2	20	25		0.9

[a] Calculations were made by using eq 4 and 5.

range, an optimal range, and an inhibition range. These effects of Mg^{2+} concentration on the observed rates of formation of IMP and GMP are illustrated in Figure 1. As described below, the general shape of the curves shown in Figure 1 and the analogous curves for Mn^{2+} provide clues to the mechanism by which metal ion activations of these reactions proceed.

London and Steck have described three limiting models for the activation of an enzymatic reaction in which the enzyme (E) and substrate (A) form complexes with an activator (M). Four enzymic structures are defined in each model (E, EA, EM, and EMA for model I; ME, MEM, MEA, and MEMA for model II; E, ME, EMA, and MEMA for model III). Model II (eq 1–3) predicts that the inhibitory concentration

$$M + A \overset{K_0}{\rightleftharpoons} MA \quad (1)$$



(2)

$$\frac{v}{V_{max}} = \frac{K_c K_a [M]_f [MA]}{K_a [M]_f (K_s [A] + K_m [M]_f + K_c [MA] + 1) + 1} \quad (3)$$

range of activator will occur at concentrations far greater than that of the substrate and that the enzyme will form a metal ion complex. Since we have observed that the inhibitory concentrations of Mn^{2+} and Mg^{2+} greatly exceed that of PRibPP and that HGPRTase forms a complex with Mn^{2+} ($\bar{K}_a$ = 20 μM, Figure 2),[3] we elected to generate theoretical curves to define the data for Mg^{2+} activation shown in Figure 1 (and for the analogous data for Mn^{2+} activation) on the basis of this model.

Values for $\bar{K}_0$, the dissociation constant defining the PRibPP–metal ion interaction (eq 1), equal to 260 μM and 17 μM have been evaluated previously for Mg^{2+} (Thompson et al., 1978; Morton & Parsons, 1976) and Mn^{2+} (Victor et al., 1979), respectively, and were employed to generate these theoretical curves on the basis of eq 4. It was observed that

$$\frac{v}{V_{max}} = \frac{K_c K_a ([M]_t - [MA])[MA]}{\{K_a([M]_t - [MA]) \times [K_s[A]_t + K_m[M]_t + (K_c - K_s - K_m)[MA] + 1] + 1\}} \quad (4)$$

the values of $\bar{K}_s$ and $\bar{K}_m$ define the inhibitory portion of the kinetic curves. These values, which provided the best fit of the experimental results for the Mg^{2+} and Mn^{2+} activations at each concentration of PRibPP and base, are listed in Table

[3] The nomenclature for association constants and dissociation constants of London & Steck (1969) has been adapted for use in this paper. For example, K_0 is an association constant for the PRibPP (A)–metal ion (M) interaction and is equal to $[MA]/([M]_f[S]_f)$. $\bar{K}_0$ is a dissociation constant for this same interaction and is equal to the reciprocal of K_0. The binding events defined by association constants K_0, K_a, K_m, K_s, K_1, and K_2 are shown in eq 1 and 2.

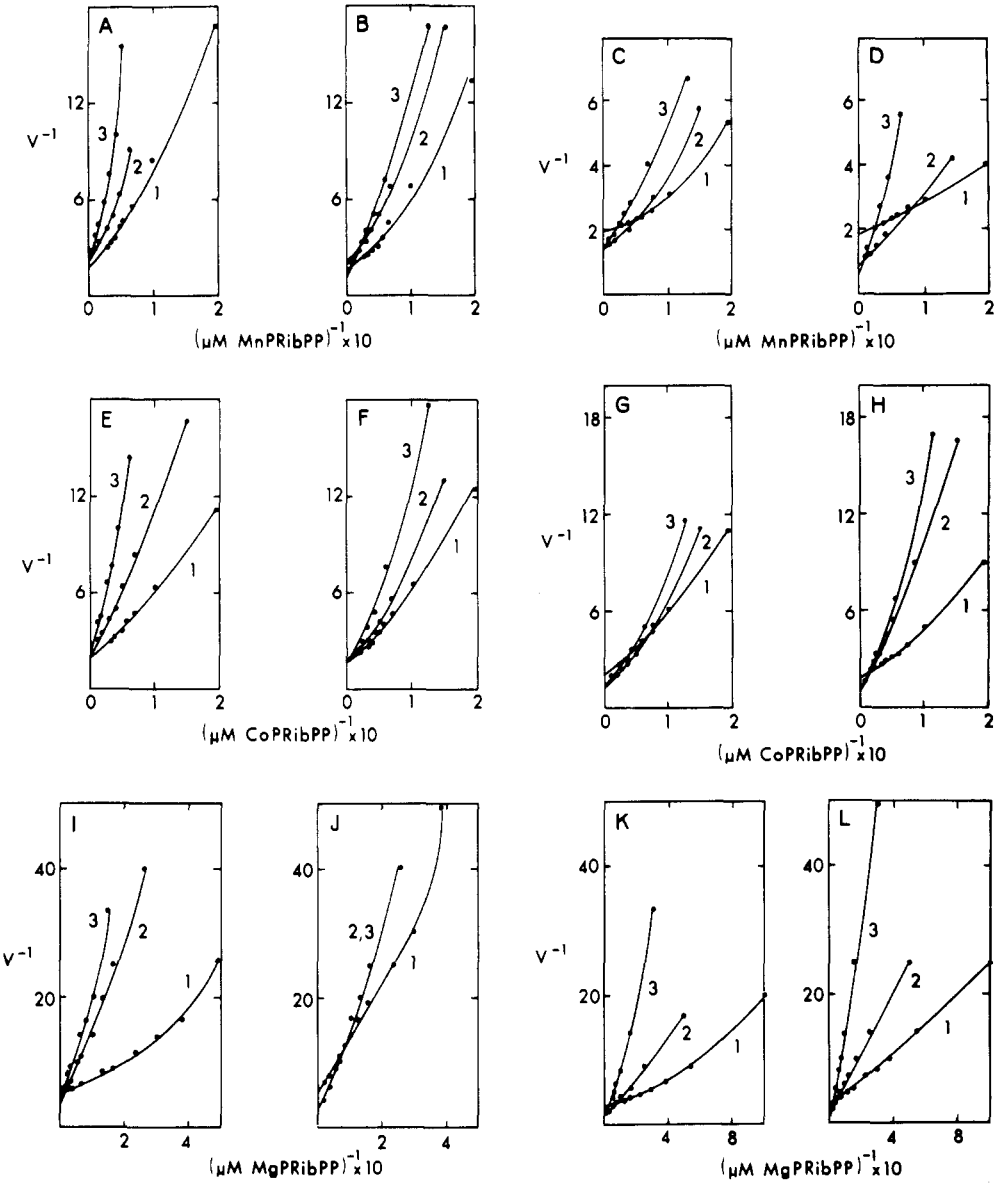


FIGURE 3: Double-reciprocal plots of initial velocity measurements (V^{-1}) of yeast HGPRTase-catalyzed formations of IMP and GMP vs. calculated reciprocal concentrations of metal ion–PRibPP complexes. Plots A, E, and I illustrate experiments where 21 μM hypoxanthine was present in the incubation solution. Plots B, F, and J illustrate experiments where 50 μM hypoxanthine was present. Plots C, G, and K illustrate experiments where 34 μM guanine was present. Plots D, H, and L illustrate experiments where 67 μM gaunine was present. Lines 1, 2, and 3 in each of the graphs illustrate incubations during which 33, 66, and 167 μM PRibPP, respectively, were present in the incubation solutions. Theoretical curves were generated by using eq 5 as described in the text.

I. Interestingly, only values of $\bar{K}_s$, $\bar{K}_m$, and $\bar{K}_0$ were required to fit the V vs. $[M^{2+}]_T$ plots. In order to evaluate the remaining constants, a second set of theoretical curves and a new plotting device were employed.

As shown by a rearrangement of eq 3 (eq 5), plots of V^{-1}

$$V^{-1} = V_{max}^{-1}\bar{K}_c(1 + K_s[A]_f + K_m[M]_f + \bar{K}_a[M]_f^{-1}) \times [MA]^{-1} + V_{max}^{-1} \quad (5)$$

vs. $[M^{2+}\text{–PRibPP}]^{-1}$ should be nonlinear with the slope of the curve dependent upon five constants and the concentrations of free PRibPP and free metal ion. The observed nonlinearity of these slopes essentially eliminates one model (model III) described by London & Steck (1969) since these plots are predicted to be linear. Curve-fitting exercises defined the nonlinear slope of each kinetic experiment (Figure 3) where different concentrations of PRibPP, hypoxanthine, and guanine were employed. The only assumptions that were made in generating these curves was that the $\bar{K}_0$ values for the Mg^{2+}–PRibPP and Mn^{2+}–PRibPP complexations were equal to 260 μM and 17 μM, respectively, and that the $\bar{K}_0$ value for the Co^{2+}–PRibPP interaction was equal to that of Mn^{2+} (J. Victor and D. L. Sloan, unpublished results). From this analysis, values for $\bar{K}_s$, $\bar{K}_m$, $\bar{K}_a$, and $\bar{K}_c$ were determined for each experiment (Table I). The calculated values for all constants were averaged to define the activation of the HGPRTase-catalyzed reactions (Table II). No distinction has been made between the values determined for the enzymatic formation of IMP and that of GMP because all of the metal ion complexes should form prior to the binding of either base (defined as substrate B in eq 2). Since insoluble Co^{2+} complexes and to a lesser extent Mn^{2+} complexes form with hypoxanthine and guanine only at relatively high ion concentrations, these complexes, or possibly more soluble ones, should not contribute to the activation processes. We have been unable to detect any base–metal or nucleotide–metal interactions using UV spectroscopy, but we have in fact observed the formation of relatively low-affinity soluble Mn^{2+} complexes with hypoxanthine, IMP, and GMP, using water

Table II: Dissociation Constants for Metal Ion Activation of Hypoxanthine-Guanine Phosphoribosyltransferase from Yeast

metal ion	$\bar{K}_0$[a] (μM)	$\bar{K}_s$ (μM)	$\bar{K}_m$ (mM)	$\bar{K}_a$ (μM)	$\bar{K}_c$ (μM)	$\bar{K}_2$[b] (μM)	$\bar{K}_1$[c] (μM)
Mn^{2+}	17 ± 8	22 ± 9	1.1 ± 0.6	6 ± 2	8 ± 4	0.1	6.2
Mg^{2+}	260	19 ± 8	0.9 ± 0.5	21 ± 2	11 ± 4	3.2	150
Co^{2+}	17	22 ± 7	1.1 ± 0.6	5 ± 1	8 ± 2	0.1	6.2

[a] The $\bar{K}_0$ values for Mn^{2+} and Mg^{2+} were obtained from Victor et al. (1979) and Morton & Parsons (1976). The value of $\bar{K}_0$ for Co^{2+} was assumed to be equivalent to that of Mn^{2+}. [b] The values of $\bar{K}_2$ were calculated by using the average values of $\bar{K}_0$, $\bar{K}_c$, and $\bar{K}_m$ for each metal ion and the relationship $\bar{K}_0\bar{K}_c = \bar{K}_m\bar{K}_2$. [c] The values of $\bar{K}_1$ were calculated by using the average values of $\bar{K}_0$, $\bar{K}_c$, and $\bar{K}_s$ for each metal ion and the relationship $\bar{K}_0\bar{K}_c = \bar{K}_s\bar{K}_1$.

proton relaxation rate measurements. Listed in Table II are the average calculated values for $\bar{K}_s$, $\bar{K}_a$, and $\bar{K}_c$ for each divalent metal ion activation. In addition to these values, calculated values for $\bar{K}_2$ and $\bar{K}_1$, which were determined as described in Table II, are presented.

The relative value of $\bar{K}_m$ for each of the metal ion activations suggests that the formations of $Co^{2+}{}_2E$, $Mn^{2+}{}_2E$, and $Mg^{2+}{}_2E$ are unlikely except at high metal ion concentrations, but the relative values of $\bar{K}_2$ suggest that PRibPP easily binds to these complexes once formed. The relatively small values for $\bar{K}_s$ and $\bar{K}_1$ suggest that PRibPP and a second metal ion may indeed bind in that order to the M^{2+}–E complex to form the active M^{2+}–HGPRTase–M^{2+}–PRibPP complex, but the even smaller values for $\bar{K}_c$ suggest that the binding of the M^{2+}–PRibPP complex to M^{2+}–HGPRTase is the preferred route for formation of this active complex. Moreover, the calculated values of $\bar{K}_a$ suggest that, at catalytic concentrations of HGPRTase and optimal M^{2+} concentrations, complete ligation between the metal ion and the enzyme will have occurred (in which case the equations and theory described as model I also describe these activations).

The absolute values of the constants listed in Table II allow a comparison of HGPRTase activation by the different metal ions. The activations by Co^{2+} and Mn^{2+} are essentially equal, although this may be the result of our assumption of equivalent $\bar{K}_0$ values. However, plots of the observed inital velocity vs. total metal ion concentration were equivalent for these two metal ions as well. In contrast, Mg^{2+} appears to form enzyme and substrate complexes with a lower affinity than those of Mn^{2+} or Co^{2+}, although the $\bar{K}_s$ and $\bar{K}_c$ values are similar for all metal ions, confirming that these binding events are independent of the metal ion used. All of these results suggest that the role of the metal ion in these reactions is to form active enzyme and substrate structures (the metal ion complexes) and that these structures form a substrate-bridge complex (Mildvan & Cohn, 1970).

Previous studies have established the requirement for metal ions during the course of the HGPRTase-catalyzed reactions (Krenitsky et al., 1969; Nakagawa et al., 1976; Nagy & Ribet, 1977; Giacomello & Salerno, 1978; Salerno & Giacomello, 1981). The most detailed of these studies has been carried out by Giacomello & Salerno (1978) and Salerno & Giacomello (1981) in their examination of the activation of HGPRTase from human erythrocytes. They have defined the activation by Mg^{2+} of the enzymatic formation of IMP exclusively in terms of formation of a Mg^{2+}–PRibPP–Mg^{2+} complex. Using the two previously determined constants that define the formation of this complex (Salerno & Giacomello, 1981), we have been unable to generate theroretical curves that would fit the plots shown in Figures 1 and 3 for the activation of the yeast HGPRTase by Mg^{2+}. Indeed, our calculations suggest that for the yeast enzyme the optimal concentration of Mg^{2+} is reached prior to the complete formation of the Mg^{2+}–PRibPP–Mg^{2+} complex. However, the model that provides the best fit for our results for all of the divalent metal ion activations (eq 2) includes a structure (MEMA) that may be an enzyme-bound M^{2+}–PRibPP–M^{2+} complex. Moreover, the studies of the Mg^{2+} activation of HGPRTase from another strain of yeast (Nagy & Ribet, 1977) suggest that the mechanism for activation of yeast enzymes may differ from this mechanism for HGPRTase from human erythrocytes. Nagy & Ribet (1977) determined that high concentrations of Mg^{2+} inhibit noncompetitively the binding of PRibPP to yeast HGPRTase, and this result contrasts with the observation (Salerno & Giacomello, 1981) that this inhibition is competitive for the human HGPRTase. Our results and theoretical considerations are fully consistent with the results of Nagy & Ribet (1977), which suggest the occurrence of independent binding of Mg^{2+} to the yeast enzyme. We conclude that this binding event is but one of a series of binding events that define the mechanism for the metal ion activation of the yeast HGPRTase.

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Registry No. Mn, 7439-96-5; Mg, 7439-95-4; Co, 7440-48-4; hypoxanthine phosphoribosyltransferase, 9016-12-0; 5-phospho-α-ribosyl 1-pyrophosphate, 7540-64-9.

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Active Site Cobalt(II)-Substituted Liver Alcohol Dehydrogenase: Characterization of Intermediates in the Reduction of *p*-Nitrobenzaldehyde by Rapid-Scanning Ultraviolet–Visible Spectroscopy†

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ABSTRACT: A detailed mechanistic investigation of the effects of Co(II) substitution for Zn(II) at the catalytic site of horse liver alcohol dehydrogenase (LADH) during the reduction of *p*-nitrobenzaldehyde (NBZA) by NADH using UV–visible rapid-scanning spectroscopy and stopped-flow rapid-mixing techniques is presented. Reaction was limited to a single turnover of sites by inclusion of pyrazole (pyr) to trap and inactivate the enzyme via formation of the E(NAD–pyr) adduct. The changes in the d–d and charge-transfer bands of the active site cobalt and the changes in the spectral bands of NADH and the E(NAD–pyr) adduct were studied via rapid scanning. The reaction to form the Co(II)E(NAD–pyr) complex and *p*-nitrobenzyl alcohol (NBZOH) was observed to occur in two distinct kinetic processes with apparent rate constants of 150–200 s^{-1} and 0.08 s^{-1} at pH 8.38. The time-resolved spectra and difference spectra established that NADH oxidation occurs in the first phase. Kinetic studies showed that in the first phase the oxidation of enzyme-bound NADH is preceded by at least two, rapid, preequilibrium processes: the first is presumed to be the binding of NBZA; the second is proposed to be a relaxation of the ternary Co(II)E(NADH, NBZA) complex involving NADH activation and/or an obligatory enzyme conformation change. Deuterium kinetic isotope experiments establish that hydride transfer does not limit the oxidation of NADH. On the basis of spectral comparisons, the product formed in the second phase is identified as the Co(II)E(NAD–pyr) complex. Analyses of the time-resolved spectra and difference spectra and comparisons with stable ternary complexes provide strong evidence indicating that the rate of formation of the Co(II)E(NAD–pyr) complex is limited by the dissociation of NBZOH from the Co(II)E(NAD^+, NBZOH) complex. The overall reaction time course was found to be significantly different from the time course observed for the native Zn(II) enzyme [Koerber, S. C., & Dunn, M. F. (1981) *Biochimie 63*, 97–102]. Although the microscopic origins for the different behaviors of the Co(II) and Zn(II) enzymes have not been determined, it is clear that substitution of Co(II) for Zn(II) substantially alters the mechanism of NBZA reduction by LADH.

The specific replacement of the active site zinc ion in horse liver alcohol dehydrogenase (LADH)[1] by other divalent metal ions is of considerable theoretical and experimental interest. The difficult task of replacement has been accomplished by several groups with varying degrees of success (Sloan et al., 1975; Shore & Santiago, 1975; Sytkowski & Vallee, 1978; Maret et al., 1979). However, the demonstration by Zeppezauer and co-workers (Maret et al., 1979) that the catalytic zinc ion can be specifically removed from the crystalline enzyme (Tilander et al., 1965) without changing the content of noncatalytic zinc and that the catalytic activity can be reconstituted in the crystalline state with other divalent metal ions resolves much ambiguity arising from previous reports.

This report is one of a series from our laboratories detailing mechanistic investigations of the reactions of specifically substituted Co(II)E(NADH) with aromatic aldehyde substrates. Cobalt substitution for zinc at the active site gives a chromophoric probe (Dietrich et al., 1979; Dunn et al., 1982) of the state of the metal ion along the reaction pathway. Since only the active site metal ion has been replaced, changes in observed kinetics with Co(II)E vis-à-vis native enzyme are presumed to reflect subtle changes in the chemistry and structure of the active site. Further, it is presumed that the

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[1] Abbreviations: E, LADH, horse liver alcohol dehydrogenase; Co(II)E, Zn(II)E, Cd(II)E, and Ni(II)E, specific active site substituted alcohol dehydrogenase species; NAD^+ and NADH, respectively oxidized and reduced nicotinamide adenine dinucleotides; DACA, *trans*-4-(dimethylamino)cinnamaldehyde; NBZA and NBZOH, respectively *p*-nitrobenzaldehyde and *p*-nitrobenzyl alcohol; TFE, 2,2,2-trifluoroethanol; pyr, pyrazole; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.

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