Interaction of Divalent Cations with β-Galactosidase (*Escherichia coli*)†

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ABSTRACT: Although the addition of various divalent metals to β-galactosidase resulted in apparent activation, only Mg^{2+} and Mn^{2+} actually did activate. The apparent activation by the other divalent metals was shown to be due to Mg^{2+} impurities. Calcium did not activate, but experiments suggested that it did bind. Other divalent metals which were studied failed to bind. The dissociation constants for Mg^{2+} and Mn^{2+} were 2.8×10^{-7} and 1.1×10^{-8} M, respectively, and in each case one ion bound per monomer. These constants corresponded very closely to apparent values which were obtained from activation studies. The apparent binding constant for Ca^{2+}, obtained from competition studies, was 1.5×10^{-5} M. Data were obtained which showed that Mg^{2+}, Mn^{2+}, and Ca^{2+} all compete for binding at a single site. Of interest and of possible molecular biological importance was the observation that, while Mg^{2+} bound noncooperatively ($n = 1.0$), Mn^{2+} did so in a highly cooperative manner ($n = 3.4$). The binding of Mn^{2+} (as compared to Mg^{2+}) resulted in a twofold drop in the V_{max} for the hydrolysis and transgalactosylis reactions of lactose but had little effect on the V_{max} of hydrolysis of allolactose, *p*-nitrophenyl β-D-galactopyranoside (PNPG), or *o*-nitrophenyl β-D-galactopyranoside (ONPG); K_m values were not affected differently for any of the substrates by Mn^{2+} as compared to Mg^{2+}. When very low levels of divalent metal ions were present (0.01 M EDTA added) or when Ca^{2+} was bound with lactose as the substrate, a greater decrease was observed in the rate of the transgalactosylic reaction than in the rate of the hydrolytic reaction, and the K_m values for lactose and ONPG were increased. Of the three divalent metal ions which bound to β-galactosidase, only Mn^{2+} had significant stabilizing effects toward denaturing urea and heat conditions.

Studies of the effects of divalent metal ions on the activity of β-galactosidase (EC 3.2.1.23) of *Escherichia coli* have been carried out numerous times and in various ways (Wallenfels & Weil, 1972). The literature regarding the divalent ion interactions with β-galactosidase is, however, somewhat confusing. β-Galactosidase from *Escherichia coli* is an enzyme which is coming under increasing scrutiny (Fowler & Zabin, 1978; Viratelle et al., 1977; Fowler et al., 1978; Brockhaus & Lehmann, 1978). Thus, it is important to obtain definitive information regarding divalent cation interactions with the enzyme.

Several divalent ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, and Ni^{2+}) have been reported to be activators of β-galactosidase (Wallenfels & Weil, 1972; Rickenberg, 1969), but this seemed rather unusual since most enzymes are fairly specific in their ion requirements. One of the goals of this study was to determine which metals actually bind to the enzyme and which of these activate.

Only one of the many reports of the effects of divalent metals on β-galactosidase has included a quantitation of the number of specifically bound divalent metal ions per monomer (Case et al., 1973). There is also only one account of a determination of a dissociation constant (Tenu et al., 1972), and the value reported was based only upon the activation effect. Both of those studies were done with Mg^{2+}. We set out to expand this research to other metals and to obtain actual binding constants.

The studies carried out with divalent metals in the past have been mainly with the synthetic substrates ONPG[1] and PNPG. In this study, the divalent cations which bound were also examined with respect to their effects on the action of β-galactosidase toward the physiological substrates lactose and allolactose.

Finally, the stabilizing effects (Wickson & Huber, 1969, 1970; Rickenberg, 1969) of divalent metal ions were studied and related to the activation effects of these metals.

Experimental Procedure

Reagents. Pure grades of all chemicals used were purchased. EDTA, EGTA, ATP, histidine, and PNPG were purchased from Sigma. NTA was from Eastman Organic, and urea was enzyme grade from Schwartz/Mann. $MgSO_4$, NaCl, $NiSO_4$, and $ZnSO_4$ were Fischer certified compounds, while $MnSO_4$, $FeCl_2 \cdot 4H_2O$ and lactose were Baker analyzed compounds. ONPG was from Terrochem and $CaCl_2 \cdot 2H_2O$ was from BDH (Analar). Attempts were made to purify EDTA, EGTA, and NTA by reprecipitations in acid solutions, but analysis showed that the Mg^{2+} content remained more or less constant (about 2×10^{-6} M in 0.01 M chelator). Possibly the acid used to precipitate the chelators and/or the base used to redissolve the chelators added back as much Mg^{2+} as was removed. Attempts were also made to extract the buffers with dithiazone in chloroform. This technique (Bosron et al., 1975) may work with metals which dithiazone chelates very tightly (e.g., Zn^{2+}), but no decrease in the Mg^{2+} content of the buffers was detected after the dithiazone treatments.

Labware. All glassware and cuvettes used in these experiments were soaked in 10^{-3} M EDTA for 0.5–1 h, then washed with concentrated nitric acid, and finally thoroughly rinsed with doubly deionized H_2O before use. Nalgene labware was treated as follows: (a) 1 h in 8 M urea (pH 2), (b) 1 h in 1 M KOH, and (c) 1 h in 1 mM EDTA, with copious rinses of doubly deionized H_2O between each treatment and after the last.

Enzyme. β-Galactosidase was isolated from a merodiploid strain of *E. coli* (A-324-5; a gift from Dr. A. Fowler, UCLA). The isolation procedure was essentially that reported by Fowler (1972).

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[1] Abbreviations used: ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; NTA, nitrilotriacetic acid; ONPG, *o*-nitrophenyl β-D-galactopyranoside; PNPG, *p*-nitrophenyl β-D-galactopyranoside.

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Protein concentrations were estimated by A_{280} by using an average of extinction coefficients reported in the literature (2.09 cm^2 mg^{-1}). The enzyme with a very low amount of divalent metal ion was prepared by dialysis, first against large volumes of a buffer (pH 7.6) containing 0.01 M histidine, 0.01 M NaCl, 0.001 M EDTA, and 0.001 M 2-mercaptoethanol for 2 days with several changes and then in the same buffer without the EDTA and mercaptoethanol for 2 days. After this treatment the enzyme was shown still to be in the tetrameric form by disc gel electrophoresis and had about 6–16% of the full hydrolytic activity, depending on the amount of free metal eliminated by the process.

Assay. The enzyme assays using ONPG and PNPG were carried out as described earlier (Hill & Huber, 1971). The assays of enzyme activity with lactose or allolactose as the substrates were carried out by gas–liquid chromatography or by linked enzyme assays as described earlier (Huber et al., 1976). In all cases where the term "unit" is used, it refers to 1 μmol of product per min. All assays were done at 30 °C.

Free Metal Ion Concentrations. Various concentrations of the metal in question and of NTA, EDTA, EGTA, or ATP were mixed to generate the particular free metal ion concentrations desired at the temperatures and ionic strengths of the studies. For the majority of metals, NTA was used since its instability constants (Sillen & Martell, 1964, 1971) for complexes with divalent ions were near the concentration at which each seemed to activate and thus would buffer the divalent metal ion concentration effectively. The chelating agent was always used at a high enough concentration relative to that of the enzyme to ensure that it was the sole determinant of the free metal ion concentrations. Total concentrations (free plus bound) of metals were determined by atomic absorption.

Binding. Binding was quantitated by equilibrium dialysis.

Stability. The stability of the enzyme (both of its enzymatic activity and of its tetrameric structure) to urea and heat was determined in the presence of various concentrations of divalent metal ions by the disc gel electrophoresis procedures previously described (Wickson & Huber, 1969, 1970).

Results

Effect of Chelating Agents on the Activity. In order to ensure that the chelating agents themselves did not have an effect on the results reported, controls in which the chelators were present along with adequate excess Mg^{2+} for activation were compared to the results with the same Mg^{2+} content but not including the chelators. This was studied under several different conditions, and there was no difference in the activities in any of the cases.

Activation of ONPG Hydrolysis by Various Divalent Metals. Activation curves for ONPG hydrolysis with respect to calculated free concentrations of several divalent metals (Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Ni^{2+}, and Co^{2+}) were obtained. All of the curves except that for Ca^{2+} are shown in Figure 1. The "apparent" dissociation constants (midpoints of the curves) increased in the following order: $Ni^{2+} < Co^{2+} < Zn^{2+} < Fe^{2+} \simeq Mn^{2+} < Mg^{2+}$. This order more or less follows the order of the instability constants for these metals ($Ni^{2+} = 3.4 \times 10^{-12}$ M; $Co^{2+} = 3.6 \times 10^{-11}$ M; $Zn^{2+} = 5.1 \times 10^{-11}$ M; $Fe^{2+} = 1.5 \times 10^{-9}$ M; $Mn^{2+} = 1.6 \times 10^{-8}$ M; $Mg^{2+} = 5.0 \times 10^{-6}$ M) with NTA. This suggested that the increase in activity may have been caused by some contaminant rather than by the divalent ions themselves. If the instability constant of a complexing agent (in this case, NTA) with the metal in question is smaller than the instability constant of the impurity by at least 2 orders of magnitude, the metal will bind to the chelator and occlude the impurity as the concentration of the metal is increased. Thus, increasing the concentration of free metal by increasing the metal concentration relative to the chelator concentration will cause the concentration of the free impurity to rise more rapidly than that of the free metal. This is because the poorly binding impurity cannot bind to a chelator which is binding the metal tightly already. The greater the affinity of a particular metal for a chelating agent, the greater will be this effect, and thus the instability constant of a metal with NTA should predict the apparent dissociation constant. Of course, the amount of impurity present is also important, and thus the order of the apparent dissociation constants may not always exactly match the order predicted on the basis of the instability constants. Since only a small amount of free Mg^{2+} ($<10^{-6}$ M) is required to activate β-galactosidase and since Fe^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and Mn^{2+} bind to NTA better than does Mg^{2+} by more than 2 orders of magnitude, it is possible that Mg^{2+} is an impurity in each of these metals and that it is the cause of the enzyme activations. Most of the compounds used in these studies were tested, and, indeed, Mg^{2+} was present at quite high levels in all of them (approximately 1×10^{-6} M Mg^{2+} in 1 mM metal solutions). Various approaches, as described in later sections, were used to determine whether activation by Mg^{2+} impurities was indeed responsible for the activity observed.

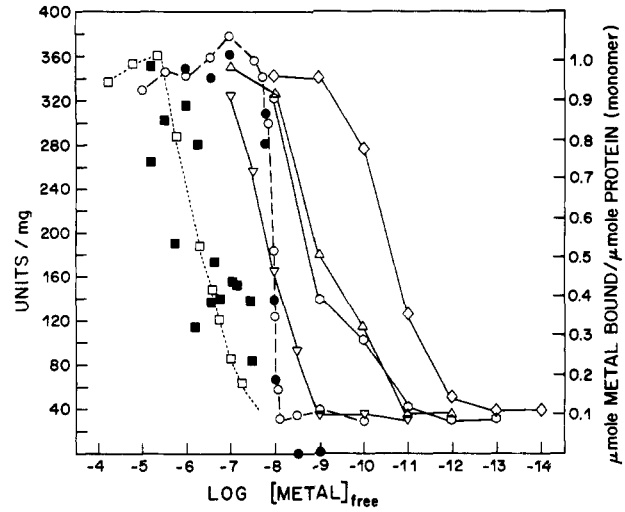

FIGURE 1: The activities of β-galactosidase (ONPG hydrolysis assays) and actual binding (in the case of Mg^{2+} and Mn^{2+}) at various free concentrations of several divalent metal ions. Fisher seamless cellulose dialysis tubing (2.50 cm wide for a large surface area) was boiled in 1 mM EDTA (pH 8.0) for 15 min and then 3 times in fresh doubly deionized H_2O for 5 min before being thoroughly rinsed with doubly deionized H_2O. Aliquots (3 mL) of "metal-free" β-galactosidase (about 1 mg/mL) in 10 mM histidine buffer (containing 0.1 mM mercaptoethanol and sufficient NaCl to give a final conductivity of 18 ± 3 mS) at pH 7.6 ± 0.05 were added to the dialysis bags. These were placed into 2-L beakers containing histidine buffer with various free ion concentrations. After stirring for 48 h at 4 ± 1 °C, we determined the protein concentrations within the dialysis bags. Metal ion concentrations of solution inside and outside the bags were measured by atomic absorption, and the amount of metal per monomer was calculated. The figure is a representative sample of three similar experiments. The open symbols represent activity observed (ONPG hydrolysis) while the closed symbols show the fraction bound as studied by equilibrium dialysis. (●, ○) Mn^{2+}; (■, □) Mg^{2+}; (◇) Ni^{2+}; (△) Co^{2+}; (○) Zn^{2+}; (▽) Fe^{2+}.

The ONPG hydrolytic activity of the enzyme at various free Ca^{2+} concentrations is represented in Figure 2. The data can best be explained by assuming that Ca^{2+} causes inhibition and that most of the activation seen arises from Mg^{2+} impurities. The curve on Figure 2 for increasing concentrations of Ca^{2+} in the absence of chelators but with constant Mg^{2+} concentration (2×10^{-5} M) shows that the enzyme has high activity

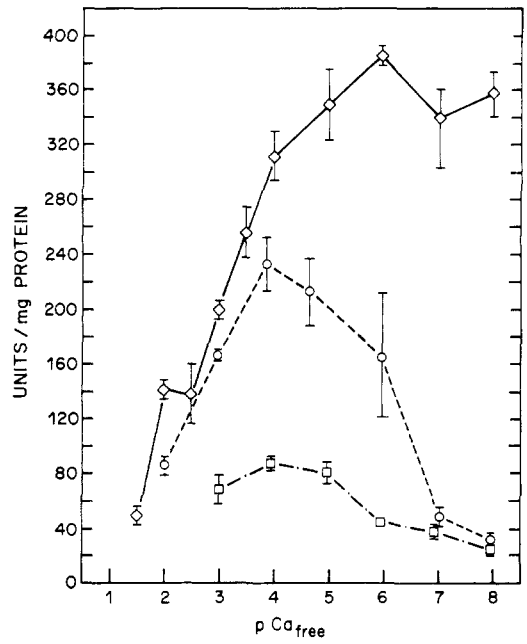

FIGURE 2: The activities of β-galactosidase (ONPG hydrolysis at various concentrations of free Ca^{2+}. The free [Ca^{2+}] was generated by the use of various chelating agents. (◇) 2×10^{-5} M Mg^{2+}; (○) 0.01 M EDTA; (□) 0.1 M ATP.

at low Ca^{2+} concentration and that the activity decreases as the concentration of Ca^{2+} increases. The enzyme is probably active at low Ca^{2+} concentration because Mg^{2+} is bound to it, but then at high Ca^{2+} concentration the Mg^{2+} is displaced from the enzyme by the Ca^{2+}, causing a loss of activity. A study was also done with EGTA. EGTA binds Ca^{2+} well but Mg^{2+} only poorly (instability constants: $Ca^{2+} = 1.0 \times 10^{-11}$ M; $Mg^{2+} = 6.3 \times 10^{-6}$ M), and thus one would expect a curve very similar to that without a chelator since the Mg^{2+} concentration would not be greatly affected by a chelator (even at low Ca^{2+} concentration) which binds Ca^{2+} very tightly in comparison. The activity curve (not shown) was very similar to that obtained without a chelator, as expected. When NTA, which binds both divalent ions moderately well (instability constants: $Ca^{2+} = 3.2 \times 10^{-7}$ M; $Mg^{2+} = 5.0 \times 10^{-6}$ M) was the chelator, a similar curve was again obtained (not shown), but the activity at low Ca^{2+} concentration was lower. The lower activity was, presumably, due to the fact that NTA binds Mg^{2+} better (in relation to Ca^{2+}) than does EGTA, and thus it lowers the Mg^{2+} as well as the Ca^{2+} concentration, and the low Mg^{2+} concentration causes the lowering of the activity. When EDTA was the chelator, the activity at low free Ca^{2+} concentration (Figure 2) was low since this reagent binds Mg^{2+} effectively even in the presence of Ca^{2+} (instability constants: $Ca^{2+} = 2.5 \times 10^{-11}$ M; $Mg^{2+} = 3.2 \times 10^{-9}$ M), and so, since it is Mg^{2+} which activates, the enzyme has low activity. In the case of EDTA, however, as the Ca^{2+} concentration was increased, the Ca^{2+} did displace enough Mg^{2+} from the EDTA to activate the enzyme. Then again, as the Ca^{2+} concentration became high again the activity was inhibited. The presence of ATP (Figure 2), which binds Mg^{2+} better than it binds Ca^{2+} (instability constants: $Ca^{2+} = 1.6 \times 10^{-4}$ M; $Mg^{2+} = 4.0 \times 10^{-5}$ M), gave low activity over the entire range of Ca^{2+} concentrations. This is expected since it would keep Mg^{2+} concentrations low even when a large amount of Ca^{2+} is present. Calculations of actual free Mg^{2+} levels, using known chelator and total Ca^{2+} and Mg^{2+} concentrations, were performed, and there was excellent correlation between the calculated free Mg^{2+} and the observed enzyme activity. Thus, a large amount of the activity noted was due to the level of Mg^{2+} present, and Ca^{2+} competes for binding at this Mg^{2+} site and inhibits the activity when it is bound.

Table I: Amount of Metal Bound per Monomer of β-Galactosidase as Measured by Equilibrium Dialysis[a]

metal	free concn (M)	amount bound per monomer	Mg^{2+} per monomer
Ni^{2+}	10^{-9}	<0.05	0.96
Zn^{2+}	10^{-7}	0.25	1.05
Mn^{2+}	10^{-6}	1.06	[b]
Mg^{2+}	$10^{-6.5}$	0.90	0.90

[a] Free concentrations of metals used for these studies were near to the levels where the maximum activation was achieved for that metal (see Figure 1). The amount of Mg^{2+} bound with each metal is also shown. The results shown are those of one representative experiment of three which were carried out. [b] Not determined; see Table II for the amount bound.

The concentration of Ca^{2+} needed to obtain inhibition, especially in the presence of Mg^{2+}, made binding studies very difficult, and thus quantitation of Ca^{2+} binding could not be carried out directly. Since Ca^{2+} competes with Mg^{2+} and is inhibitory, one can, however, obtain an apparent binding constant for Ca^{2+} from the inhibition caused by Ca^{2+} in the presence of a fixed amount of Mg^{2+}. This curve is shown in Figure 2 (Mg^{2+} at 2.0×10^{-5} M). The dissociation constant calculated was 1.5×10^{-5} M, assuming a dissociation constant for Mg^{2+} of 2.8×10^{-7} M (see Binding Studies) and a residual enzyme activity in the presence of Ca^{2+} of 15% (chosen so that it gave approximately equal dissociation constants at each point of the curve at which it was calculated).

Binding Studies. The actual binding of some divalent metal ions (Ni^{2+}, Zn^{2+}, Mn^{2+}, and Mg^{2+}) to β-galactosidase was measured by equilibrium dialysis (Table I) at the free concentrations at which they appeared to cause full activation of the enzyme (Fe^{2+} and Co^{2+} were not done because of problems involved in keeping Fe^{2+} reduced and because of color interference by Co^{2+}). The proposal that the apparent activations observed are really due to Mg^{2+} binding was true for Ni^{2+} and Zn^{2+} (it was found that 0.25 Zn^{2+} was bound per monomer, but this was probably nonspecific since it is unlikely that 0.25 Zn^{2+} per monomer would result in full activation). The results indicate that Mn^{2+} also binds to the enzyme in a specific fashion since one Mn^{2+} was found bound per monomer.

Binding of Mn^{2+} and Mg^{2+} was followed as a function of the free concentrations of these metals (Figure 1, closed symbols). The binding of Mn^{2+} followed the activity curve closely except that the amount of Mn^{2+} bound dropped to zero while the activity did not. This was probably because of the ubiquitous Mg^{2+} contamination of compounds used. The free concentration of Mg^{2+} at pH 7.6 could not be reduced to less than about $10^{-7.7}$ M with any of the common chelating agents (Sillen & Martell, 1964, 1971) despite efforts to remove the Mg^{2+} as outlined under Experimental Procedure. Thus, when Mn^{2+} was reduced to very low free concentrations, free Mg^{2+} was still present at about $10^{-7.7}$ M, and at this point there is still enough Mg^{2+} present for partial activity. The Mg^{2+} binding values are somewhat more scattered, but they still follow the activity curve closely. Dissociation constants of 2.8×10^{-7} M for Mg^{2+} and 1.1×10^{-8} M for Mn^{2+} were calculated from the curves, and in each case at high concentrations of metal, 1:1 binding to monomer was observed. It should be stated that the dissociation constants for Mg^{2+} and Mn^{2+} did not seem to be greatly dependent on temperature as often is the case (Sillen & Martell, 1964, 1971). The activity studies were done at 30 °C and the equilibrium dialysis

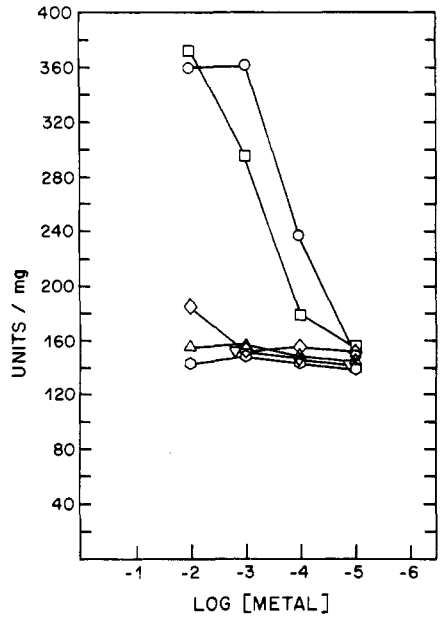

FIGURE 3: The activities (ONPG hydrolysis) of β-galactosidase in the presence of 10^{-3} M Ca^{2+} and various concentrations of several divalent metals. Each assay solution contained 10^{-3} M Ca^{2+} as well as a concentration of another metal. The results shown are those of a representative of three similar experiments which were carried out. (○) Mn^{2+}; (□) Mg^{2+}; (◇) Ni^{2+}; (△) Co^{2+}; (○) Zn^{2+}; (▽) Fe^{2+}.

experiments were done at 4 °C and yet the curves compared well. (The free concentrations of Mg^{2+} and Mn^{2+} were calculated for the temperature at which the studies were carried out.)

At values of free Mg^{2+} concentrations which were greater than 2×10^{-6} M, especially when only small amounts of the chelating agent were present to buffer the Mg^{2+}, there was evidence of nonspecific binding of Mg^{2+}, and this nonspecific binding seemed to vary unpredictably.

Activation of the Enzyme in the Presence of High Calcium Concentrations. Assuming that Ca^{2+} binds to the same site as do Mg^{2+} and Mn^{2+} but that it does not activate, excess concentrations of Mg^{2+} or Mn^{2+} should be able to activate the enzyme by displacing the Ca^{2+}. Other metals should not be able to do this unless they contain very high levels of Mg^{2+} or Mn^{2+} impurities. The results of a study of this type are shown in Figure 3. It is noted that, indeed, at high levels of Mg^{2+} or Mn^{2+} there is activation. This is good evidence for the fact that Ca^{2+}, Mg^{2+}, and Mn^{2+} compete for the same site and that Ca^{2+} binds but does not activate significantly. A further study was carried out which showed that the level of Mg^{2+} or Mn^{2+} needed to activate the enzyme was dependent on the Ca^{2+} concentrations. At higher [Ca^{2+}] more Mg^{2+} or Mn^{2+} was needed to activate. When other divalent metals were added to the Ca^{2+}-inhibited enzyme, they did not cause activation because they do not bind. The Mg^{2+} and Mn^{2+} impurities they contained were obviously insufficient to overcome the Ca^{2+} inhibition (concentrations of Mg^{2+} and Mn^{2+} in excess of 10^{-4} M are necessary to do this).

Number of Divalent Metal Sites. Although it has generally been assumed that β-galactosidase has only one divalent metal ion binding site per monomer, this has never been confirmed. There is a small possibility that since the enzyme binds three different divalent metals it has a specific site for each. This is highly unlikely for Ca^{2+} since both Mg^{2+} and Mn^{2+} were able to overcome the inhibition of the enzyme even in the presence of high Ca^{2+}, and this was shown to be competitively dependent on the Ca^{2+} concentrations (see Activation of the Enzyme in the Presence of High Calcium Concentrations).

Table II: Amounts of Mg^{2+} and Mn^{2+} Bound to β-Galactosidase Monomers at Various Mg^{2+} and Mn^{2+} Concentration Combinations[a]

[Mg^{2+}] (M)	[Mn^{2+}] (M)	Mg^{2+} per monomer	Mn^{2+} per monomer
2.2×10^{-6}	1.0×10^{-6}	*b*	0.87 (0.91)
2.2×10^{-6}	1.0×10^{-7}	*b*	0.54 (0.56)
2.2×10^{-6}	1.0×10^{-8}	*b*	undetectable (0.00)
2.2×10^{-6}	0	*b*	undetectable (0.00)
1.0×10^{-5}	1.0×10^{-5}	*b*	0.90 (0.96)
2.0×10^{-5}	2.0×10^{-5}	*b*	0.99 (0.96)
2.0×10^{-5}	2.0×10^{-6}	*b*	0.74 (0.72)
2.0×10^{-5}	2.0×10^{-7}	*b*	0.33 (0.21)
2.0×10^{-5}	2.0×10^{-8}	*b*	undetectable (0.04)
2.0×10^{-5}	0	*b*	undetectable (0.00)
6.5×10^{-7}	3.8×10^{-10}	0.93	undetectable
4.7×10^{-7}	3.8×10^{-10}	0.86	0.11
4.2×10^{-7}	3.8×10^{-10}	0.81	0.06
4.0×10^{-7}	3.8×10^{-10}	1.18	0.03
3.9×10^{-7}	3.8×10^{-10}	0.38	0.10
2.0×10^{-5}	1.0×10^{-7}	0.59 ± 0.08	0.54 ± 0.01
2.0×10^{-5}	3.4×10^{-8}	0.89 ± 0.42	0.24 ± 0.09
3.0×10^{-7}	3.0×10^{-8}	0.07 (0.22)	*c*
1.0×10^{-7}	3.0×10^{-8}	0.09 (0.09)	*c*
3.0×10^{-8}	3.0×10^{-8}	0.17 (0.03)	*c*

[a] For the cases where determinations of Mg^{2+} and Mn^{2+} bound could not both be made, there is listed in parentheses beside each number the calculated amount that should be bound, assuming that binding for the metals is competitive and using the values of the dissociation constants determined. In the two cases where the studies were done in replicate (three or more), standard deviations are given. [b] At Mg^{2+} levels higher than 10^{-6} M, when chelating agent was not used, there was nonspecific binding of Mg^{2+}, and the values of Mg^{2+} bound were high and variable. These are not reported. The reason for the variable nonspecific binding is not known. [c] In order to obtain these combinations of free [Mg^{2+}] and [Mn^{2+}] in the presence of NTA, we had to use large concentrations of NTA and of total [Mn^{2+}]. The high total [Mn^{2+}] made accurate estimates of the amount of Mn^{2+} bound impossible since the enzyme concentrations could not be made larger than about 10^{-4} M.

To determine whether Mn^{2+} and Mg^{2+} compete for the same site, we varied their free concentrations in relation to each other and measured their binding by equilibrium dialysis. This experiment was complicated by significant and highly variable nonspecific binding of Mg^{2+} at high Mg^{2+} concentrations when a chelator was absent and by the fact that, in order to buffer Mg^{2+} and Mn^{2+} with NTA at levels where one would expect good competition between Mg^{2+} and Mn^{2+}, we had to add very high levels of total Mn^{2+}, making differences between Mn^{2+} inside and outside of the dialysis bag less than experimental error. Many different combinations were used to give a wide spectrum of determinations and thus compensate for the problems. The results are shown in Table II. In the cases where accurate measurements of both Mn^{2+} and Mg^{2+} could be made, the combined levels of Mn^{2+} bound and Mg^{2+} bound were never significantly greater than one cation per monomer. In the cases where the binding of one or the other of the metals could not be measured, the expected binding per monomer was calculated and is listed in parentheses. The observed values corresponded quite well to the expected ones. The results of this section, taken together with those of the last, show that there is really only one specific cation binding site per monomer and that Mg^{2+}, Mn^{2+}, and Ca^{2+} compete for it.

Cooperativity of Mn^{2+} Binding. Analysis of Figure 1 shows that the Mn^{2+} curve is much steeper than the Mg^{2+} curve, suggesting cooperativity. When the activity results for Mn^{2+} and Mg^{2+} were plotted in the Hill form, the cooperativity for

Table III: V_{max} and K_m Values for the Action of β-Galactosidase on Various Substrates in the Presence of Mg^{2+}, Mn^{2+}, and Ca^{2+} and at Very Low Divalent Metal Concentrations (0.01 M EDTA)[a]

substrate	Mg^{2+}	Mn^{2+}	Ca^{2+}	no metal (0.01 M EDTA)
PNPG: V_{max}	59.7 ± 0.5	50.5 ± 0.6		8.1 (±0.2)
K_m	3.4 (±0.2) × 10^{-5}	3.8 (±0.2) × 10^{-5}		1.2 (±0.1) × 10^{-4}
ONPG: V_{max}	360.0 ± 15.2	358.2 ± 14.2		38.5 (±1.6)
K_m	1.4 (±0.1) × 10^{-4}	2.0 (±0.2) × 10^{-4}		6.2 (±0.6) × 10^{-4}
lactose				
(a) hydrolysis V_{max}	30.9 ± 1.07	14.28 ± 0.57	1.77 ± 0.2	0.95 ± 0.35
K_m	1.35 (±0.12) × 10^{-3}	1.14 (±0.13) × 10^{-3}	13.3 (±3.35) × 10^{-3}	6.81 (±6.48) × 10^{-3}
(b) transgalactosylis (calcd): V_{max}	31.6	14.4	0.6	0.3
(c) ratio allo/mono[b] (V_{max})	1.05	1.01	0.35	0.30
allolactose: V_{max}	47.9 ± 4.76	48.21 ± 3.58		
K_m	0.94 (±0.22) × 10^{-3}	0.92 (±0.16) × 10^{-3}		

[a] The data all represent experiments with at least 14 points. The results were weighted, and the error was estimated by the method recommended by Cornish-Bowden (1976). Minimal amounts of enzyme were used as recommended by Segel (1975). In each V_{max} is in terms of micromoles of product per milligram per minute and K_m is in molarity. The V_{max} for lactose transgalactosylic action is the calculated theoretical V_{max}, obtained by extrapolating data back to zero time and assuming the same K_m as for hydrolysis (Huber et al., 1976). [b] Ratio of the allolactose V_{max} to the hydrolysis (monosaccharide) V_{max}.

Mn^{2+} was confirmed (the least-squares slope of the Mn^{2+} line was 3.4 while that of the Mg^{2+} line was 1.0). These results have been reported previously in a preliminary form (Woulfe-Flanagan & Huber, 1978).

Effect of Divalent Metals on Reactions with Various Substrates. Table III shows how the three divalent metals which bind affect the activity of β-galactosidase with a series of different substrates. Also shown are results with very low metal concentrations (high EDTA). In both the low metal concentration and the Ca^{2+} cases, the results reflect whatever residual activity remains. It is seen that when Mg^{2+} and Mn^{2+} are compared, only the action of the enzyme with lactose was affected differently by the two metals. The two synthetic substrates and allolactose were hydrolyzed at essentially the same rates with either of these metals. In the case of lactose the V_{max} of both the hydrolytic reaction and of the transgalactosylic reaction was about half as large in the presence of Mn^{2+} as in the presence of Mg^{2+}. The presence of $10^{-1.5}$ M free Ca^{2+} resulted in a large reduction in the V_{max} and a several-fold increase in the K_m for lactose hydrolysis. The transgalactosylic reaction in this case, however, occurred at a slow rate relative to the hydrolytic reaction. Similar effects were seen when high EDTA concentrations were added to reduce the free divalent metal concentrations. It seems that elimination of most metal binding or, alternatively, saturation of most sites of β-galactosidase with Ca^{2+} had similar effects.

Stabilization Effects. With 5.5 M urea at 30 °C, only Mg^{2+} and Mn^{2+} had any significant stabilizing effects on the activity; of the two, Mn^{2+} was quite a lot more effective than Mg^{2+}. The loss of activity occurred at a faster rate than the loss of tetrameric structure in the presence of each metal. Figure 4 shows that the urea stabilization curve as a function of $[Mn^{2+}]$ is not simple, and this suggests that there may be other temporary sites for divalent metals formed on the enzyme during the denaturation. It was seen that for stabilization a concentration of Mn^{2+} greater than 10^{-4} M was needed.

The effect of divalent metals on the stabilization of β-galactosidase under heat-denaturing conditions (55 °C) was very similar to the effect in urea. Only Mn^{2+} stabilized significantly, and the activity was again lost more rapidly than the tetrameric structure. The insert of Figure 4 shows the concentration vs. stabilization curve. The curve, which is much simpler than that for urea, has a midpoint of about 10^{-5} M Mn^{2+}.

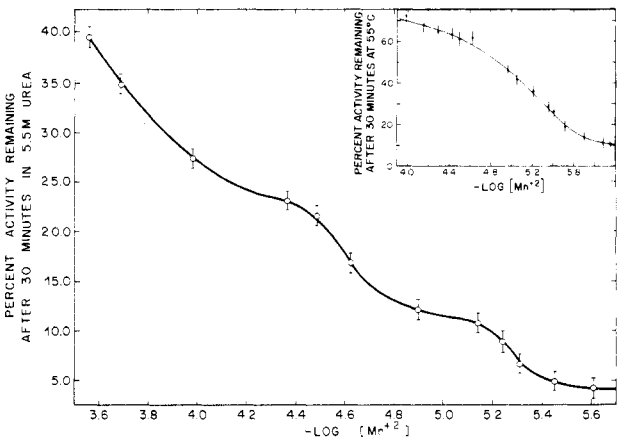

FIGURE 4: Activity (ONPG hydrolysis) of β-galactosidase remaining 30 min after incubation of the enzyme in 5.5 M urea at 25 °C with the listed concentrations of Mn^{2+} added. The enzyme was incubated in 5.5 M urea at each of the Mn^{2+} concentrations listed for 30 min, and then its activity was determined. The insert shows the activity remaining 30 min after incubation of the enzyme at 55 °C at various Mn^{2+} concentrations. The enzyme was incubated at 55 °C at each of the Mn^{2+} concentrations and then assayed at 30 °C.

Discussion

The studies showed that only Mn^{2+}, Mg^{2+}, and Ca^{2+} bound and affected the activity of β-galactosidase. The apparent activation seen in the presence of the other metals was due to the Mg^{2+} impurities found in these metals and the concentrating effects which chelating agents had on the free Mg^{2+} concentrations. It was found that Mg^{2+} and Mn^{2+} bound to the enzyme and caused activation while the evidence strongly suggested that Ca^{2+} bound but did not activate significantly. The binding curves of Mg^{2+} and Mn^{2+} closely followed the activating effects of these two metals. There is only one binding site per monomer for divalent cations on β-galactosidase, and the three metals compete for this site. Binding dissociation constants for Mg^{2+} and Mn^{2+} are reported, and an apparent constant for Ca^{2+} was determined. The dissociation constant for Mg^{2+} (2.8×10^{-7} M) is similar to that reported by other workers (Tenu et al., 1972). The one reported here, however, was derived from an actual binding curve, not just inferred from an activation curve.

It is interesting that Mn^{2+} bound and activated cooperatively. Since Mn^{2+} slowed the rate of utilization of lactose by a factor of greater than 2 compared to the rate with Mg^{2+}, these results could be of physiological significance. Stadtman & Ginsburg (1974) have suggested that these two metals might be of regulatory importance in another enzyme system in *E. coli* (glutamine synthetase). It is also interesting that, in a recent study with β-galactosidase from a mutant strain of *E. coli* (Deschavanne et al., 1978), Mn^{2+} was able to

activate the enzyme but Mg^{2+} was not.

The only divalent cation which significantly stabilized the enzyme to denaturing forces was Mn^{2+}. The large effect demonstrated by Mn^{2+} may be related to its cooperative binding with the enzyme, but it could also simply be related to the greater binding ability of Mn^{2+} as compared to the binding of Mg^{2+} to the enzyme.

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Cadmium-109 as a Probe of the Metal Binding Sites in Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The noncatalytic and catalytic zinc atoms of horse liver alcohol dehydrogenase, $[(LADH)Zn_2Zn_2]$ or LADH, have been replaced differentially with ^{109}Cd by equilibrium dialysis, resulting in two new enzymatically active species, $[(LADH)^{109}Cd_2Zn_2]$ and $[(LADH)^{109}Cd_2{}^{109}Cd_2]$. The UV difference spectra of the cadmium enzymes vs. native $[(LADH)Zn_2Zn_2]$ reveal maxima at 240 nm with molar absorptivities, $\Delta\epsilon_{240}$, of 1.6×10^4 M^{-1} cm^{-1} per noncatalytic ^{109}Cd atom and 0.9×10^4 M^{-1} cm^{-1} per catalytic ^{109}Cd atom, consistent with coordination of the metals by four and two thiolate ligands, respectively, strikingly similar to the 250-nm charge-transfer absorbance in metallothionein. Carboxymethylation of the Cys-46 ligand to the catalytic metal in LADH presumably lowers the overall stability constant of the coordination complex and results in loss of catalytic ^{109}Cd or catalytic cobalt but not catalytic zinc from the enzyme.

Twenty years ago horse liver alcohol dehydrogenase was found to be a zinc metalloenzyme (Vallee & Hoch, 1957). Some 10 years later its four zinc atoms were first shown to encompass one set each of two functional and two structural atoms as established in solution (Drum et al., 1967) and confirmed by the elegant X-ray crystallographic studies of Brändén et al. (1975).

We have previously replaced the four zinc atoms of the enzyme with both cobalt and cadmium to facilitate spectroscopic and/or kinetic exchange experiments and ultimately to lead to mechanistic insights. More recently, it has proven possible to delineate conditions critical to the specific and selective replacement of zinc by cobalt and these have now led to an extension of earlier investigations with cadmium (Druyan & Vallee, 1962; Drum & Vallee, 1970b).

Comparison of the exchange properties, spectral characteristics, and inhibition kinetics of these cadmium derivatives with those of cobalt and zinc provides further insight regarding the metal coordination chemistry of LADH.[1]

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[1] Abbreviations used: $[(LADH)Zn_2Zn_2]$ or LADH, native horse liver alcohol dehydrogenase; CD, circular dichroism; OP, 1,10-phenanthroline. In order to differentiate and clarify presentation, we designated the first pair of exchangeable metal (Me) atoms in the standard formulation the "N" (noncatalytic) pair and the second the "C" (catalytic) pair, i.e., $[(LADH)N_2C_2]$. Hence, Zn and Co represent the N pair in $[(LADH)Zn_2Me_2]$ and $[(LADH)[Co_2Me_2]$, while they are the C pair in $[(LADH)Me_2Zn_2]$ and $[(LADH)Me_2Co_2]$.

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