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EFFECTS OF VARIOUS CONCENTRATIONS OF Na^+ AND Mg^{2+} ON THE ACTIVITY OF β-GALACTOSIDASE

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SUMMARY

The interrelationships between the effects of Na^+ and Mg^{2+} on β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) were studied, together with the inhibition of the enzyme by high concentrations of the two ions and its reactivation on dilution. It was found that the optimum concentration of one ion depended on the concentration of the other and the effect was the opposite of that expected due to ionic strength. The time courses of inhibition and reactivation were found to be different for the two ions. Adjustments of rates on increasing the Mg^{2+} concentration to 1.0 M or on diluting from 1.0 M were found to be slow compared to the immediate changes observed with Na^+. Substrate was found to partially reverse the Mg^{2+} inhibition but had no effect on Na^+ inhibition or the reactivation with either ion. Increasing the Na^+ concentration slowed the Mg^{2+} inhibition.

INTRODUCTION

Cation activation of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) from *Escherichia coli* has been reviewed by WALLENFELS AND MALHOTRA[1] and summarised by REITHEL AND KIM[2]. Two later reports have also been published on the subject[3,4]. Activation by monovalent ion is generally greatest with Na^+ but with certain substrates and under certain conditions K^+ is better[1,4]. Broad peaks around the optimum are shown by the various workers (between 10^{-3} M and $5 \cdot 10^{-1}$ M). Evidence of slight inhibition at 1.0 M Na^+ has been shown[5,6] but, in contrast, it has also been stated that 1.0 M Na^+ strongly reduces the activity of the enzyme[7]. Even where the assay Na^+ concentrations have not reached 1.0 M, published curves suggest that there is inhibition[1,4,8]. The role of bivalent cation is somewhat more obscure. The apparent need for Mg^{2+} or Mn^{2+}, or lack of it, has been explained as being a requirement only in the hydrolysis of certain substrates. The degree of Mg^{2+} stimulation was shown to vary with the pH and this, together with the possibility that Mg^{2+} might not have been rigorously excluded in some cases, might also help to explain the confusion with regard to its need. Reasons given for variations in

ion activation and optimums include differences in substrate, pH, protein concentration and strain of *E. coli*[2].

Mg^{2+} and in particular Mn^{2+}, have been shown to protect the enzyme against denaturation by dilution[9]. Mg^{2+} and other bivalent cations (at 10^{-2} M) have also been shown to completely inhibit the renaturation of urea-denatured β-galactosidase. This was found not to be related to the enzyme's requirement for Mg^{2+}, but appears to be due to interaction between peptide chains. As the Mg^{2+} concentration was reduced the recovery increased, up to 100% in its absence[10].

A detailed study of interrelationships between monovalent and bivalent cation activation has not been carried out. For this reason the activity of the enzyme at a wide range of Na^+ and Mg^{2+} concentrations was determined. Inhibition at high concentrations of either ion was noted and the time course of this inhibition was followed.

MATERIALS AND METHODS

β-Galactosidase was purified as described previously[11]. The buffer used throughout this experimental work was 10^{-2} M Tris–HCl buffer (pH 7.6 at 30°) with 10^{-3} M 2-mercaptoethanol. Tris buffer was selected because a cationic buffer was required to enable low concentrations of Na^+ to be used in the absence of other monovalent cations. As some inhibition has been found with Tris (ref. 12), the buffer ion was kept constant at a low level. Na^+ was added as NaCl, but owing to the hygroscopic nature of $MgCl_2$, Mg^{2+} was added as $MgSO_4$. In this regard no significant difference was found between activation by Na_2SO_4 and NaCl. Before use, the stock enzyme was diluted in buffer containing 10^{-4} M EDTA (1:40) and then dialyzed extensively against the diluting buffer (500–1000 vol. for 48 h with one change). A similar dialysis was then carried out in the absence of EDTA. For the inhibition and reactivation studies a further dialysis was carried out to bring the enzyme to the required initial cation concentration. The enzyme was assayed in buffer with $1.33 \cdot 10^{-2}$ M *o*-nitrophenyl-β-D-galactoside or *p*-nitrophenyl-β-D-galactoside in the presence of the required concentrations of Na^+ and Mg^{2+}. The concentrations of Na^+ and Mg^{2+} at the lower ranges were verified by atomic absorption. The nitrophenol produced at 30° was measured at 420 nm in a Zeiss PMQII spectrophotometer and recorded with a Beckman linear-log recorder. Activity was expressed as $\Delta A_{420\,nm}$/min.

Cation concentration/activity profiles

About 5 μg (10 μl) of the dialyzed enzyme, which was essentially free of Na^+ and Mg^{2+}, was added to assay mixtures containing various concentrations of Na^+ and Mg^{2+} (total volume 2.6 ml). When adding enzyme from solutions essentially free of Na^+ and Mg^{2+} to assay mixtures with various concentrations of Na^+ and Mg^{2+}, the enzyme immediately attained an activity which did not change with incubation time. The initial enzyme activity was recorded at each separate condition and these activities were plotted.

Rates of inhibition and reactivation

For the inhibition studies of both ions the enzyme was dialyzed extensively against buffer containing 10^{-2} M Na^+ and 10^{-4} M Mg^{2+}. For Na^+ inhibition about 5 μg

(10 μl) of the dialyzed enzyme was added to 2.0 ml of assay solutions (without substrate) containing 1.0 M Na^+ (10^{-4} M Mg^{2+}). After incubation at 30° for various times, 0.6 ml of substrate (in buffer with assay salt concentrations) was added and the rate of hydrolysis followed. Similar studies of the inhibition of the enzyme at 1.0 M Mg^{2+} were carried out (at 10^{-1} M and 10^{-2} M Na^+). For the reactivation studies, the enzyme was dialyzed extensively against 1.0 M Na^+ (10^{-4} M Mg^{2+}) for Na^+ or against 1.0 M Mg^{2+} (10^{-2} M Na^+) for studies with Mg^{2+} and rates of hydrolysis after a series of preincubation times in 10^{-2} M Na^+, 10^{-4} M Mg^{2+} were obtained.

On dialysis against high salt concentrations some overall irreversible loss of activity was found, compared to a similar sample dialyzed against 10^{-2} M Na^+, 10^{-4} M Mg^{2+}. There was also some loss of volume due to the change in salt concentration. For these reasons the results were expressed as percentage changes. The final activity of the enzyme in 10^{-2} M Na^+, 10^{-4} M Mg^{2+} was taken as 100%. The addition of 10 μl of 1.0 M cation (from an enzyme sample) to 2.6 ml of a lower concentration, increases the cation concentration by $3.8 \cdot 10^{-3}$ M (10^{-2} M Na^+ to $1.38 \cdot 10^{-2}$ M and 10^{-4} M Mg^{2+} to $3.9 \cdot 10^{-3}$ M). This makes only a small difference in the expected activity at the original concentration as can be seen from Figs. 1 and 2.

Since dialysis was carried out in the cold room (2°) whereas assays for the rate of inhibition were done at 30°, a control study was carried out to see the rate of activity loss when β-galactosidase was transferred from 1.0 M Na^+ (10^{-4} M Mg^{2+}) at 2° to the same concentrations at 30°, in a series of timed incubations. A similar study was carried out on the enzyme at 1.0 M Mg^{2+} (10^{-2} M Na^+).

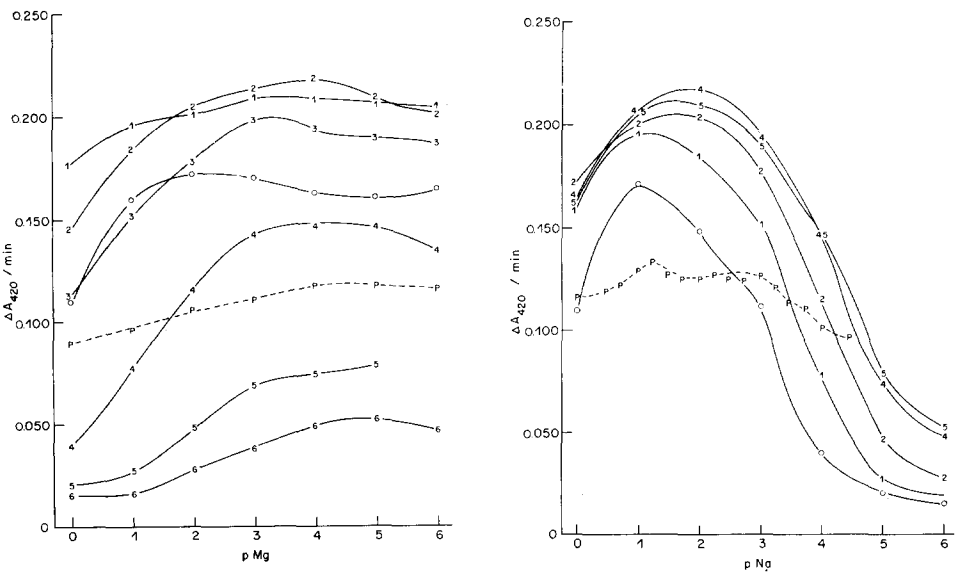

Fig. 1. Effect of Mg^{2+} concentration on activity ($\Delta A_{420\ nm}$/min) (pMg = $-\log_{10}Mg^{2+}$), at Na^+ concentrations: 1.0 M (o—o), 10^{-1} M (1—1), 10^{-2} M (2—2), 10^{-3} M (3—3), 10^{-4} M (4—4), 10^{-5} M (5—5), 10^{-6} M (6—6). At 10^{-2} M Na^+ with *p*-nitrophenyl-β-D-galactopyranoside as substrate P- - -P).

Fig. 2. Effect of Na^+ concentration on activity ($\Delta A_{420\ nm}$/min) (pNa = $-\log_{10}Na^+$), at Mg^{2+} concentrations: 1.0 M (o—o), 10^{-1} M (1—1), 10^{-2} M (2—2), 10^{-4} M (4—4), 10^{-5} M (5—5). At 10^{-4} M Mg^{2+} with *p*-nitrophenyl-β-D-galactopyranoside as substrate (P- - -P).

RESULTS

Cation concentration/activity profiles

Fig. 1 shows the effect of varying the Mg^{2+} concentration at various fixed Na^+ concentrations. With *o*-nitrophenyl-β-D-galactoside as substrate the highest activity was obtained at 10^{-2} M Na^+, 10^{-4} M Mg^{2+}. However, it can be noted that the optimum Mg^{2+} concentration varies between 10^{-2} M at 1.0 M Na^+ and 10^{-5} M at 10^{-6} M Na^+. There was inhibition at Mg^{2+} concentrations above the optimum. This inhibition was more marked at the lower Na^+ concentrations, with the exception of that at 1.0 M Na^+, 1.0 M Mg^{2+} where the overriding effect was probably due to the high ionic strength.

There were only small differences between the activities of the enzyme at the optimum and sub-optimum Mg^{2+} concentrations, giving the broad flat peaks of Fig. 1. Owing to difficulties of demonstrating a requirement for Mg^{2+} by completely excluding the ion as an impurity, the enzyme was assayed in the presence of 10^{-4} M EDTA. Under these conditions the activity was very low (only about 5% of that at 1 μM Mg^{2+}) suggesting a possible requirement for a bivalent ion. The ability of a variety of bivalent cations to reverse this EDTA inhibition was investigated (Mg^{2+}, Mn^{2+}, Co^{2+}, Sn^{2+}, Ni^{2+}, Fe^{2+}, Sr^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Cr^{2+}; all at 10^{-4} M). Both Mg^{2+} and Mn^{2+} activated to a level expected from Fig. 1 while none of the other ions did more than partially reverse the EDTA inhibition. Since EDTA is needed to show this requirement, it would appear that a small amount of Mg^{2+} or Mn^{2+} is bound tightly and required for activity. Alternatively, EDTA may have other effects on β-galactosidase which are reversed on addition of Mg^{2+} and Mn^{2+}.

In Fig. 2 results are plotted to show the effect of varying the Na^+ concentration at several fixed Mg^{2+} concentrations. The optimum Na^+ concentration varied with the Mg^{2+} concentration and concentrations of Na^+ above optimum were inhibitory. The effects of high Mg^{2+} concentrations on the Na^+ inhibition can be seen by the shift of the Na^+ optimum from 10^{-2} M at 10^{-4} M Mg^{2+} to 10^{-1} M at 1.0 M Mg^{2+}. The Na^+ optimum and inhibition were affected by variations in the Mg^{2+} concentration in a similar way to those of Mg^{2+} by Na^+, although the effects were not as marked.

The results with *p*-nitrophenyl-β-D-galactoside (dashed lines in Figs. 1 and 2) gave curves of basically the same shape as those with *o*-nitrophenyl-β-D-galactoside. The curves were much flatter, however, indicating that the activation and inhibition observed was not as great. It should be noted that these results have not been corrected for the differences in extinction coefficient of the two nitrophenol products.

It has been suggested[13] that the action of Na^+ on β-galactosidase can be explained by considering two binding sites for Na^+ on the enzyme. At one of these sites Na^+ activates, at the other Na^+ inhibits. This effect is described by the equation:

$$\frac{v_{max}}{v} = 1 + k\left(\frac{M_0}{M} + \frac{M}{M_0}\right)$$

where v is the observed velocity at an activator concentration M, v_{max} is the maximum velocity at the optimum activator concentration M_0, and k is a constant. The plot $1/v$ against $(M_0/M + M/M_0)$ should then be a straight line. Fig. 3 shows the results plotted according to this treatment (10^{-4} M Mg^{2+}). The results do not fit the straight line obtained by the previous workers[13], either above the optimum or below it. It, therefore, appears that this explanation of the action of Na^+ does not hold.

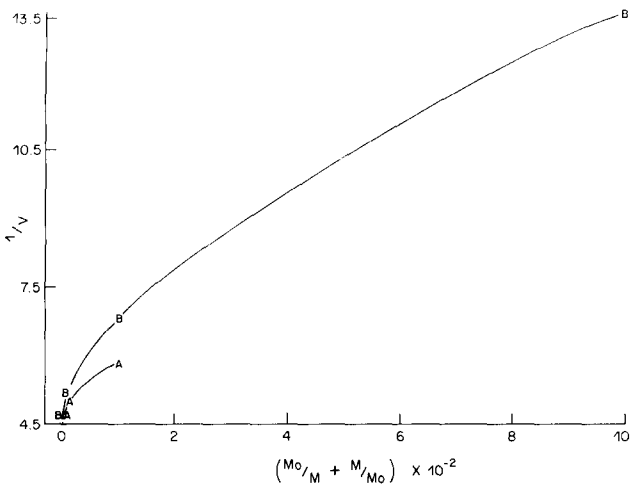

Fig. 3. Dependence of $1/v$ on $(M_0/M + M/M_0)$. A—A, $1/v$ at Na^+ concentrations above optimum; B—B, $1/v$ at Na^+ concentrations below optimum. M_0 = optimum Na^+ concentration, M = Na^+ concentration, $v = \Delta A_{420\ nm}$/min.

Inhibition and reactivation studies

Na^+ inhibition and reactivation (Fig. 4). There was an immediate loss of approx. 20% in the activity of the enzyme when the Na^+ concentration was increased from 10^{-2} M to 1.0 M. This corresponds to the change shown in the activity profile (Fig. 2). Over the next 240 min there was a further loss of activity of about 20%. When enzyme at 1.0 M Na^+ (10^{-4} M Mg^{2+}) at 2° was incubated in the same Na^+ and Mg^{2+} concentrations there was loss of activity at 30° which very closely followed the second stage (Fig. 4). This suggests that the latter loss is due to heat denaturation of the

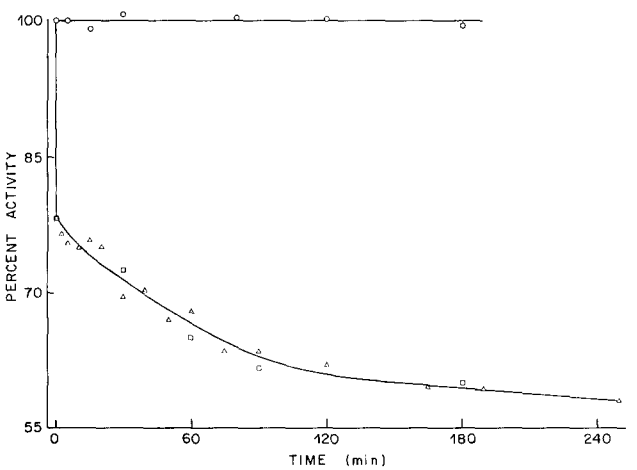

Fig. 4. Na^+ inhibition and reactivation (100% activity is the activity in 10^{-2} M Na^+). △--△, activity remaining after various times in 1.0 M Na^+ (10^{-2} M Na^+ to 1.0 M Na^+); ○ —○, activity after various times in 10^{-2} M Na^+, (1.0 M Na^+ to 10^{-2} M Na^+); □—□, activity with a temperature change, (at 1.0 M Na^+) from 2 to 30°. In all cases the Mg^{2+} concentration was 10^{-4} M.

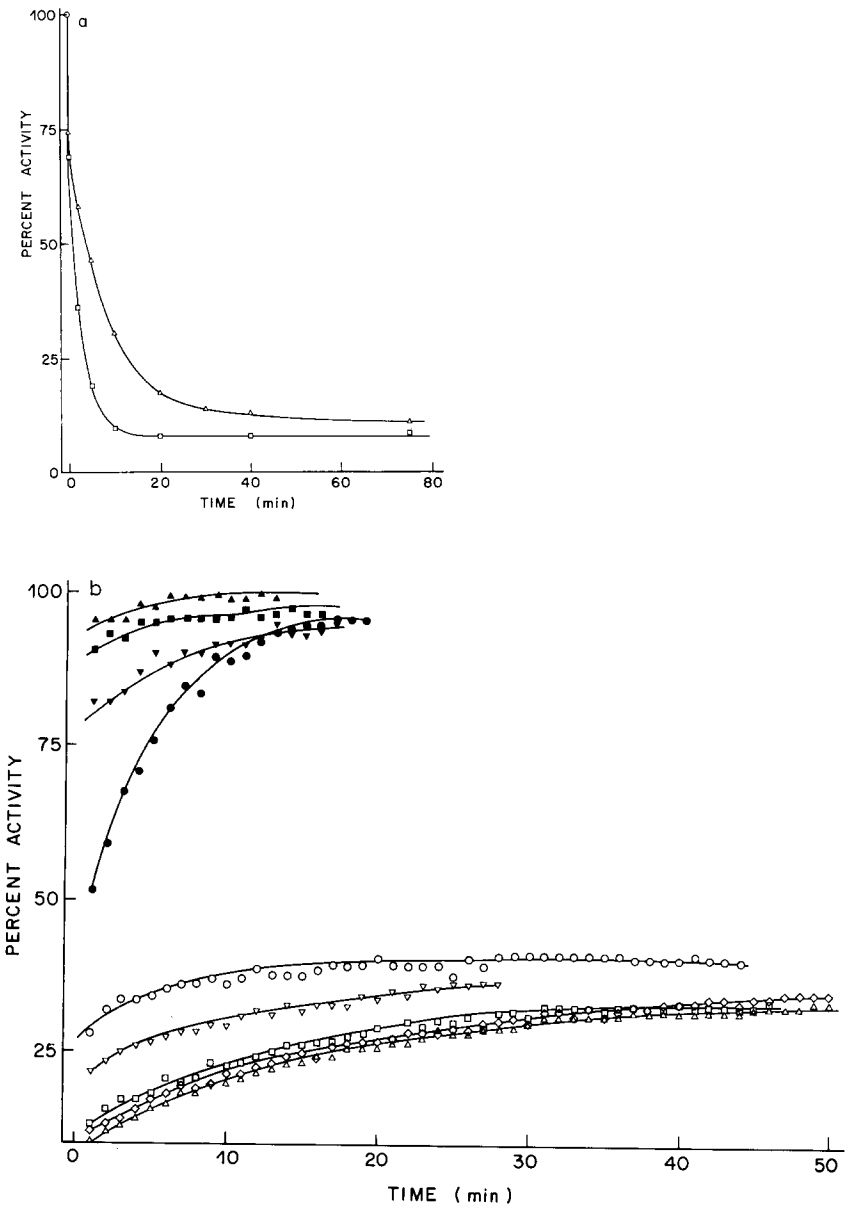

Fig. 5.a. Mg^{2+} inhibition (100% activity is the activity in 10^{-4} M Mg^{2+}, 10^{-2} M Na^+). Activity remaining after various times in 1.0 M Mg^{2+}, (10^{-4} M Mg^{2+}, 10^{-2} M Na^+ to 1.0 M Mg^{2+}) at 10^{-1} M Na^+ (△—△) and at 10^{-2} M Na^+ (□—□). b. Mg^{2+} reactivation (100% activity is the final activity in 10^{-4} M Mg^{2+}, 10^{-2} M Na^+). Percent activity per min (enzyme dialyzed in 1.0 M Mg^{2+}, 10^{-2} M Na^+ and transferred to 10^{-4} M Mg^{2+}, 10^{-2} M Na^+) after preincubation times (without substrate) of: 0 min (●—●), 5 min (▼—▼), 15 min (■—■), and 35 min (▲—▲). Percent activity per min after a temperature change from 2 to 30° (at 1.0 M Mg^{2+}, 10^{-2} M Na^+) for preincubation times (30° without substrate) of: 0 min (○—○), 2 min (▽—▽), 10 min (◇—◇), 20 min (△—△) and 60 min (□—□).

enzyme at the high salt concentration. The rate of reactivation of the enzyme on diluting the Na^+ is also shown in Fig. 4. The regain of activity was of the magnitude predicted from Fig. 2 and was immediate.

Mg^{2+} inhibition and reactivation. Fig. 5a shows the rate of Mg^{2+} inhibition at two different Na^+ concentrations. The initial loss in activity is that predicted from Fig. 1, however, the activity continues to drop rapidly with time. The higher Na^+ concentration appears to protect the enzyme activity as the loss is slower. This agrees with the evidence shown in Fig. 1 for the protection, by Na^+, of activity when the Mg^{2+} concentration is above optimum. The lower curves of Fig. 5b show how enzyme, dialyzed finally against 1.0 M Mg^{2+} (10^{-2} M Na^+) at 2°, and then preincubated without substrate at 30° for various times also in 1.0 M Mg^{2+} (10^{-2} M Na^+), increases in activity with time after the addition of substrate. The activity immediately after substrate addition in each case shows that activity is lost rapidly for up to the 10-min preincubation time even though there is no change in the concentration of Mg^{2+}. The only difference is the temperature (2–30°). This suggests that part of the loss of activity in 1.0 M Mg^{2+} is due to the increase in temperature. After addition of substrate the rate increases stopping any further loss in activity. With no preincubation a maximum value of about 40% was reached. This value was not reached when the enzyme was first preincubated at 30° but may have been if longer assay times were used.

The upper curves of Fig. 5b show the rate of reactivation with time after various preincubation times on dilution of the Mg^{2+} concentration from 1.0 M to 10^{-4} M. It can be seen that, unlike inhibition, the rate increases with incubation time are independent of substrate. The initial rate after a period of preincubation is approximately the same as that after a similar period of assay in the presence of substrate (no preincubation). Regardless of the time of preincubation, the activity tended towards a common final rate.

DISCUSSION

The cation activity profiles for Na^+ show a broad peak similar to those curves published by other workers[5,6,8]. The shape and extent of the peak, however, depends on the Mg^{2+} concentration, with a corresponding variation in the optimum. An activation was observed with Mg^{2+} after EDTA treatment (*o*-nitrophenyl-β-D-galactoside as substrate), agreeing with some previous results[2,8,10]. The activation was not as great as that found by REITHEL AND KIM[2] nor was the optimum concentration as high. Variations of cation activity with pH shown by these authors suggested that differences between the results might be due to the different pH's used. A preliminary experiment showed that decreasing the pH increased the optimum concentration and the degree of activation by Mg^{2+} (J. A. HILL AND R. E. HUBER, unpublished results).

The interrelationships between Na^+ and Mg^{2+} can be explained by a more general expansion of the treatment used by NEVILLE AND LING[3]. Amino acid analysis of β-galactosidase shows a large excess of free glutamic and aspartic carboxyl groups over positively charged side chains[14]. This could lead to a variety of sites being available for cation binding. With several of these sites in a position to affect the activity, the particular ion bound at any one of these might alter the rate of hydrolysis of

substrate. Depending on their concentration, one particular distribution of ions at effecting sites could lead to an optimum activity.

Inhibition and reactivation rate curves for Mg^{2+} indicate that a period of time is necessary for the equilibration of the enzyme, in contrast to the immediate activity obtained with Na^+. The difference might be explained by the bivalent nature of Mg^{2+} requiring two bonds to be formed or broken. The ability of Mg^{2+} to protect the conformation of β-galactosidase has been demonstrated when the enzyme is both active[9] and denatured[10]. The ionic inhibition could involve a change in the secondary or tertiary structure, or an alteration in an equilibrium between an active and an inactive structure. The presence of Mg^{2+} although causing the change could also act in a secondary manner to oppose it, by its ability to hold together two polypeptide chains with a salt linkage or complex formation between them. This would not be expected with the monovalent ion, Na^+. Thus Mg^{2+} inhibition and reactivation would be slower than that with Na^+.

The substrate protection of inhibitions by the ions could be the result of the substrate position keeping the ions from reaching an inhibiting area. On the other hand, the lack of any effect of substrate on reactivations can be explained by substrate not being in a position to affect loss of ions on dilution.

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