Divalent Metal Ion Requirements of a Thermostable Multimetal β-Galactosidase from Saccharopolyspora rectivirgula

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To understand the roles of metal ions on the catalytic properties and thermostability of the thermostable β-galactosidase of Saccharopolyspora rectivirgula, a thermophilic actinomycete, we have investigated the binding kinetics and requirements of divalent metal ions by equilibrium dialysis, titration, and metal ion buffer techniques. We found that the monomeric multimetal enzyme (M, 136,977) had eight specific binding sites for divalent metal ions. These sites were classified as follows: a very tight (class I) site for Ca2+; three tight (class II) sites consisting of two Ca2+-specific sites (class IIa) and one Mn2+-specific site (class IIb; Kd for Mn2+, 2.0 mM), and four loose (class III) sites for Mn2+ (Kd, 1.2 μM) and Mg2+ (Kd, 2 μM). Removal of metal ions bound to class II and III sites of the holoenzyme (Ca2+Mn2+ species; relative Vmax (Vmax), 100%) by a chelating resin at 4 °C yielded a less thermostable Ca2+ species (Vmax, 1.7%) with a class I Ca2+ ion, removal of which by a chelating resin at 50 °C caused a complete irreversible inactivation of the enzyme. Titration studies revealed that stoichiometric binding of Mn2+ to a class IIa site of the Ca2+ species caused a 33-fold activation whereas binding of Ca2+ to class IIa sites had no effect on enzyme activity. Ca2+ species could be also activated 8-fold by heating at 60 °C for 20 min, suggesting that the catalytically important class II Mn2+ plays important roles in maintaining the native structure essential for activity. Occupation of class III sites by Mg2+ or Mn2+ was of physiological importance to attain sufficient thermostability by which this extracellular Mn2+-activated enzyme was secreted in medium with a molecular weight of 145,000 (11). It has been shown that the enzyme is strongly inhibited by EDTA or EGTA. The production of the enzyme is accomplished by addition of MnCl2 to the medium during actinomycetous cultivation. This divalent metal ion is suggested to be essential for both activity and thermostability in the S. rectivirgula enzyme (11). Because S. rectivirgula is a Gram-positive actinomycete, which is distant from Gram-negative E. coli and R. meliloti, it is of interest to compare the divalent metal ion requirements of the enzyme with those of the known β-galactosidases. In this study, we show that the S. rectivirgula β-galactosidase has multiple specific metal-binding sites for divalent metal ions that are essential for maximum activity and thermostability in the monomer. It was the first example of a heteronuclear multimetal glycosidase. We describe here the binding specificity, stoichiometry, dissociation constants, and possible roles of the bound metals in this unique multimetal β-galactosidase.

Many glycosidases require divalent metal ions for maximum activity and thermostability, but the roles and stoichiometry of such metal activators have been studied for only a few enzymes, including α-amylases (1, 2) and β-galactosidases (3-5). β-Galactosidase (EC 3.2.1.23) catalyzes the hydrolysis and transgalactosylation of β-D-galactopyranosides. The requirement for divalent metals to maintain the conformation and catalytic functioning of β-galactosidase has been intensively studied for the Escherichia coli enzyme. E. coli lacZ β-galacto-
sidase (M, 465,000) is composed of four identical subunits (6). The enzyme contains one tightly bound Mg2+/subunit (3, 5, 7), and the bound Mg2+ is required for maximum activity in the presence of an activator, Na+ or K+ (8). However, the role of the divalent metal ion in the enzyme mechanism is not yet known in detail (4, 5, 9).

Leahy et al. (10) have studied whether Rhizobium meliloti β-galactosidase, a dimeric enzyme, has such metal ion requirements. The Rhizobium enzyme has an 16% sequence similarity to the E. coli enzyme, and it is the least closely related of those studied so far in terms of bacterial evolution (10). Unlike the E. coli enzyme, it does not require divalent metal ions for activity. Recently, we found that the Saccharopolyspora rectivirgula strain V2-2, a thermophilic actinomycete isolated from hay, secreted an extracellular thermostable β-galactosidase abundantly. We purified it and found it to be a monomeric enzyme with a molecular weight of 145,000 (11). It has been shown that the enzyme is strongly inhibited by EDTA or EGTA. The production of the enzyme is accomplished by addition of MnCl2 to the medium during actinomycetous cultivation. This divalent metal ion has been suggested to be essential for both activity and thermostability in the S. rectivirgula enzyme (11). Because S. rectivirgula is a Gram-positive actinomycete, which is distant from Gram-negative E. coli and R. meliloti, it is of interest to compare the divalent metal ion requirements of the enzyme with those of the known β-galactosidases. In this study, we show that the S. rectivirgula β-galactosidase has multiple specific metal-binding sites for divalent metal ions that are essential for maximum activity and thermostability in the monomer. It was the first example of a heteronuclear multimetal glycosidase. We describe here the binding specificity, stoichiometry, dissociation constants, and possible roles of the bound metals in this unique multimetal β-galactosidase.

EXPERIMENTAL PROCEDURES

Materials—p-Nitro-β-D-galactopyranoside, HEPES, EDTA, NTA, MnCl2-4H2O, MgCl2-6H2O, MgSO47H2O, CaCl2, HNO3, all of analytical grade, were purchased from Nakalai Tesque, Kyoto, Japan. Chelate Cellulofine was a product of Seikagaku Corp., Tokyo, Japan. β-Galactosidase was purified from the culture filtrate of S. rectivirgula strain V2-2, a stock culture of our laboratory, as previously described (11). The molecular weight of 136,977, which was calculated from the deduced amino acid sequence, and the absorption coefficient, A1%280 of 19.14 (11) were used for all calculations.

Metal Ion Studies—Distilled water used in the metal ion studies was of high performance liquid chromatography grade (Nakalai Tesque, }

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1 The abbreviations used are: NTA, nitrilotriacetic acid; ICPES, inductively coupled plasma emission spectrometry.

TABLE I

<table>
<thead>
<tr>
<th>Starting species</th>
<th>Treatment</th>
<th>Stoichiometry</th>
<th>Resulting species</th>
<th>Suggested sites occupied</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\text{Ca}^2+$</td>
<td>$\text{Mg}^{2+}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>atoms/enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Dialysis (10 µmol MnCl₂)</td>
<td>3.01</td>
<td>0.01</td>
<td>4.95</td>
</tr>
<tr>
<td>II</td>
<td>Ca₃Mn₄</td>
<td>3.20</td>
<td>0.20</td>
<td>0.87</td>
</tr>
<tr>
<td>III</td>
<td>Dialysis</td>
<td>3.13</td>
<td>0.05</td>
<td>0.95</td>
</tr>
<tr>
<td>IV</td>
<td>Chelate Cellulofine at 4 °C</td>
<td>0.50</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>V</td>
<td>Ca₃, Chelate Cellulofine at 50 °C</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>VI</td>
<td>MnCl₂/dialysis</td>
<td>3.13</td>
<td>ND</td>
<td>0.21</td>
</tr>
<tr>
<td>VII</td>
<td>Ca₃, CaCl₂/dialysis</td>
<td>2.26</td>
<td>ND</td>
<td>0.26</td>
</tr>
<tr>
<td>VIII</td>
<td>Ca₃Mn₄, CaCl₂/dialysis</td>
<td>2.92</td>
<td>ND</td>
<td>1.42</td>
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</tbody>
</table>

* Purified enzyme preparation obtained as described (11).

Kyoto, Japan). Buffers free of divalent metal ions were prepared by incubation with Chelate Cellulofine and stored in polystyrene bottles. The poly styrene were used throughout for the metal ion studies was washed with 1 N HNO₃ and then thoroughly rinsed with metal-free water. Metal-free dialysis tubing was prepared as described elsewhere (12).

Equilibrium Dialysis and Metal Analysis—Purified β-galactosidase (11), 38 µmol, was dialyzed in a polystyrene flask at 4 °C for 48 h against 0.01 M HEPES-NaOH buffer, pH 7.2 (buffer A), or buffer A supplemented with 0–10 µmol MnCl₂ or MgCl₂. For outer solutions for the dialysis were analyzed by ICPES for related metal elements, and the mixture was treated in the same way as above.

Enzyme Assay—Various enzyme species were defined and prepared as described under “Results” and Table I except that Ca₃, and Ca₃Mn₄, species were prepared by the addition of 2 and 1 equiv of CaCl₂ and MnCl₂, respectively, to Ca₃, species (49 µmol) followed by incubation for 25 °C for 1 h. For kinetic studies for Ca₃, heat-activated Ca₃, Ca₃Mn₄, and Ca₃Mn₄, species, the metal-free standard assay mixture contained 2–10 mmol of p-nitrophenyl β-D-galactopyranoside, 20 µmol of HEPES-NaOH buffer, pH 7.2, and the enzyme in a final volume of 2.0 ml. For the assay of the Ca₃, and heat-activated Ca₃, species, the assay mixture also contained 2 µmol of EDTA. Ca₃Mn₄, and Ca₃Mn₄, species were assayed in the standard assay mixture supplemented with 40 mmol of MnCl₂ and MgCl₂, respectively. The reaction was started by the addition of enzyme (2–10 µl), and changes in absorbance at 405 nm were recorded at 25 °C in a polystyrene cuvette (1937 PMMA, Kartell) using a Shimadzu UV-160 spectrophotometer (kinetic mode; gain, 10) equipped with a temperature-controlled cell holder TCC-240A. The extinction coefficient for p-nitrophenol under these conditions was 15,400 cm⁻¹ M⁻¹ (11).

RESULTS

Preparation of Various Enzyme Species and Site Nomenclature—We previously observed that S. rectirigula β-galactosidase was activated and stabilized in the presence of 10 µmol Mn²⁺ or Mg²⁺ (11). Analysis of the metal content of the holoenzyme, which was dialyzed at 4 °C for 48 h against buffer A containing 10 µmol MnCl₂ revealed that it contained 3 mol of Ca²⁺ and 5 mol of Mn²⁺/mol of enzyme, which was the Ca₃Mn₄ species (Table I relative $V_{\text{max}}$ (V₉₀), 100% (Fig. 1)). Exhaustive dialysis of the Ca₃Mn₄ species against metal-free buffer A yielded the Ca₃Mn₄ species (V₉₀, 56%). When the Ca₃Mn₄ species was dialyzed against 20 µmol MgCl₂, 4 mol of Mn²⁺/mol of enzyme were displaced by Mg²⁺ to yield the Ca₃Mn₄Mg₄ species (V₉₀, 92%). Intercconversion among Ca₃Mn₄, Ca₃Mn₄, and Ca₃Mn₄Mg₄ species by dialysis was fully reversible.

When the Ca₃Mn₄ or Ca₃Mn₄ species (90 µmol) was treated repeatedly with Chelate Cellulofine at 4 °C, ICPES analysis of the enzyme showed formation of a Ca₃ species (V₉₀, 1.7%), which contained 1 mol of Ca²⁺/mol of enzyme. To the Ca₃ species (50 µmol in 2.0 ml of buffer A) was added Chelate Cellulofine (1.0 ml), and the mixture was stirred at 50 °C for 1 h. ICPES analysis of the supernatant showed that the enzyme contained 0.09 mol of Ca²⁺/mol of enzyme, indicating formation of an apoenzyme. Loss of this very tightly bound Ca²⁺ ion was accompanied by a complete irreversible loss of enzyme activity (V₉₀, 0%); Ca²⁺, Mn²⁺, or Mg²⁺, or all together failed to reanimate the apoenzyme. On the other hand, the Ca₃ species could be reactivated fully by incubation with Mn²⁺ (see below).

The results showed that the monomeric β-galactosidase had three kinds of binding sites for divalent metals with different
metal affinities: one very tight binding site for Ca\(^{2+}\) ion (class I site), three tight binding sites that could bind two Ca\(^{2+}\) ions and one Mn\(^{2+}\) ion (class II sites), and four loose binding sites for Mn\(^{2+}\) or Mg\(^{2+}\) ions (class III sites). The divalent metal ions bound to class I and II sites could be removed by incubation with Chelex Cellulose at 50 and 4 °C, respectively, and those bound to the class III sites could be easily removed by dialysis against metal-free buffer.

When a 5-fold excess amount of Mn\(^{2+}\) or Ca\(^{2+}\), or both, was added to the Ca\(_{i}\), species (110 μL, 1.0 mM) followed by dialysis against metal-free buffer A, the enzyme species obtained was the Ca\(_{i}\)Mn\(_{i}\), Ca\(_{i}\), or Ca\(_{i}\)Mn\(_{i}\), species, respectively, as found by ICPES. These results indicate that class II sites are further resolved into two Ca\(^{2+}\)-specific sites (termed class II\(_{a}\)) and one Mn\(^{2+}\)-specific site (class II\(_{b}\)). In addition, analysis of equilibrium binding of 1-100 μM Ca\(^{2+}\) to the Ca\(_{i}\)Mn\(_{i}\), species showed that 12 additional Ca\(^{2+}\) ions per enzyme could also bind to this enzyme species with a significantly larger \(K_d\) (130 μM) than those observed for Mn\(^{2+}\) and Mg\(^{2+}\) binding to class III sites (see below). However, the binding of Ca\(^{2+}\) to the Ca\(_{i}\)Mn\(_{i}\), species caused no effect on enzyme activity and stability. Taken together that activation and stabilization of the enzyme by Mn\(^{2+}\) binding to class II\(_{a}\) sites (see below) were not inhibited by excess Ca\(^{2+}\) (300 μM), Ca\(^{2+}\) should not bind to the functional class III sites but should bind to the other weak sites with no biological significance. Thus, Mn\(^{2+}\) and Mg\(^{2+}\) should be specific functional metal ions for class III sites. Therefore, the Ca\(_{i}\)Mn\(_{i}\), species, for example, can be described in more detail as Ca\(_{i}\)/Ca\(_{i}\)Mn\(_{i}\)/Ca\(_{i}\)Mn\(_{i}\)/Mn\(_{i}\) according to what metals occupied the sites, labeled here by the Roman numerals (Table I).

**Catalytic Properties of Various Enzyme Species**—Enzyme species are plotted as functions of steady-state kinetic parameters for hydrolysis of p-nitrophenyl \(\beta\)-galactopyranoside as shown in Fig. 1. Binding of Mn\(^{2+}\) to a class II\(_{a}\) site of Ca\(_{i}\) or Ca\(_{i}\)

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**Fig. 1.** Enzyme species are plotted as functions of \(K_a\) and relative \(V_{\text{max}}\) (V\(_{\text{max}}\)). Enzyme species were assayed for hydrolysis of p-nitrophenyl \(\beta\)-galactopyranoside at 25 °C and pH 7.2 as described under "Experimental Procedures." \(K_a\) and \(V_{\text{max}}\) were calculated from double-reciprocal plots. \(V_{\text{max}}\) of 4.48 μmol·min\(^{-1}\)·mg\(^{-1}\) was taken to be 100%.

**Fig. 2.** Titration of class II site with divalent metal ions. To 49 μL Ca\(_{i}\), species in buffer A (200 μL) were added 2-20 μL of 1 mM MnCl\(_2\), MgCl\(_2\), or CaCl\(_2\), and incubation was at 25 °C for 1 h. A 2-μL portion of the mixtures was then diluted with 2 μL of metal-free assay mixture (substrate concentration, 1 mM) to be assayed for enzyme activity as described under "Experimental Procedures." dA405/min, increase in absorbance at 405 nm/min (see "Enzyme Assay").

**Stability Studies**—S. rectirigula \(\beta\)-galactosidase requires 10-20 μM Mn\(^{2+}\) or Mg\(^{2+}\) for the thermostability that permits the enzyme to retain activity under optimum growth conditions of the thermophile (11). To elucidate the effect of metal binding on enzyme stabilization, inactivation studies of the various enzyme species were conducted at 60 °C and pH 7.2 (Fig. 3). The Ca\(_{i}\)Mn\(_{i}\), species was the most thermostable, followed by Ca\(_{i}\)Mn\(_{i}\)Mg\(_{i}\), and Ca\(_{i}\)Mn\(_{i}\), under the same conditions. Other species, whose thermostabilities were essentially the same, were less stable than the above three species.

**Dissociation Constant of Mn\(^{2+}\) at Class II\(_{a}\) Sites**—The metal ion buffer technique (15) was used to determine the metal dissociation constant for class II\(_{a}\) site in the S. rectirigula \(\beta\)-galactosidase. Fig. 4 shows the relationship between restored \(\beta\)-galactosidase activity and the calculated concentration of free Mn\(^{2+}\) in the metal-buffered system (15), indicating a hyperbolic binding of Mn\(^{2+}\) to class II\(_{a}\) site. The dissociation constant was calculated to be 2.0 nm.

**Characterization of Class III Sites**—We characterized the binding of Mn\(^{2+}\) to the class III sites by equilibrium dialysis
against buffer A containing the 0–20 μM metal ion (Fig. 5). The Mn²⁺ content of the enzyme increased as the equilibrium Mn²⁺ concentration increased, as shown by the rectangular hyperbola of Fig. 5A, whereas the Ca²⁺ content was constant at 3 mol/mol of the enzyme. Scatchard plots of the equilibrium binding data were linear (Fig. 5A, inset), so the four class III sites were kinetically indistinguishable in their metal affinities. A linear relationship was also obtained for Scatchard plots of the equilibrium binding of Mn²⁺ to class III sites. The apparent dissociation constants of a class III site for Mn²⁺ and Mg²⁺ were estimated from the slope to be 1.2 and 2 μM, respectively. The horizontal axis intercept of the Scatchard plots yielded the number of class III sites for Mn²⁺ and Mg²⁺ of 4.

When the enzyme was dialyzed against buffer A containing 10 μM MnCl₂ at different pH values, the Mn²⁺ content of the enzyme decreased with decreasing pH below 7 (Fig. 5B). We previously reported that the β-galactosidase could be irreversibly denatured under acidic conditions (below pH 5.0 at 37 °C for 30 min) (11). Although this provides a possibility that the loss of Mn²⁺ is due to an irreversible denaturation of the enzyme, this is not the case because the enzyme was fully active during pH titration studies, which were executed at 4 °C. When the enzyme was dialyzed against buffer free of divalent metal ions at pH 6.2, it contained 3 mol of Ca²⁺ and 1 mol of Mn²⁺/mol of enzyme, as found by ICPES. Thus, the decrease in the Mn²⁺ content under acidic conditions could arise from loss of the metal from class III sites. The apparent pK for Mn²⁺ binding to class III sites was 6.1.

**DISCUSSION**

Our results demonstrate that *S. rectirugula* β-galactosidase has eight binding sites for divalent metal ions in the monomeric protein. The binding data presented here indicate that these consisted of three classes based on metal affinities (Scheme I): a very tight Ca²⁺-specific site (class I site), three less tight sites (class II sites) consisting of one Mn²⁺-specific (Kᵢ = 2 mM) and two Ca²⁺-specific sites, and four weak Mn²⁺- and Mg²⁺-specific sites (class III sites, Kᵢ = 1–2 μM).

*Class I Site—Removal of Ca²⁺ at this site required heating (at 50 °C for 1 h) of the enzyme with a chelating resin and resulted in an irreversible loss of activity. In spite of many attempts, we failed to obtain an apoenzyme that could be reversibly reconstituted. These results suggest that the apoenzyme should be more unstable than the heat-activated Ca₃ species, which retained significant activities after incubation at 60 °C for 1 h. Thus, class I Ca²⁺ ion may be essential in maintaining the enzyme structure that confers "basal" thermostability and ensures reversible activation or further stabilization by subsequent binding of divalent metal ions to class II and III sites (see below).**
Class II Sites—Removal of the bound metals from these sites of the Ca,Mn species yield a Ca species with significant loss of enzyme activity, which could be restored to the original by stoichiometric binding of Mn$^{2+}$ to the class II$_{M}$ site, suggesting an important role of the Mn$^{2+}$ in catalytic functioning of the enzyme. This catalytically important Mn$^{2+}$ ion could not be fully replaced by Mg$^{2+}$ or Ca$^{2+}$. It has been shown that E. coli beta-galactosidase has one tightly bound Mg$^{2+}$ (or Mn$^{2+}$) per subunit, which is essential for enzyme activity. The bound Mg$^{2+}$ to the E. coli enzyme has been suggested to act as an electrophilic catalyst (4, 19, 20), whereas there is alternative evidence suggesting that it is important in maintaining proper structure (presumably for alignment of the catalytic carboxyl group (21)) at the active site and does not have a direct role during catalysis (5, 22). In the S. rectiivirgula enzyme, the catalytically important Mn$^{2+}$ should not be essential for primary catalysis such as electrophilic catalysis, because a heat-activated Ca$_{4}$ species that lacked the Mn$^{2+}$ ion had 23% of the activity of the Ca$_{4}$Mn$_{4}$ or Ca$_{4}$Mn$_{1}$ species. Although we could find no significant difference between Ca$_{4}$Mn$_{4}$ and Ca$_{4}$ species in UV, fluorescence, and CD spectra (data not shown), the Mn$^{2+}$ ion might be most likely to play a role in maintaining proper structure for activity.

Binding of Mn$^{2+}$ and Ca$^{2+}$ to class II sites had no effect on enzyme stability. We could find no specific biological significance of Ca$^{2+}$ ions bound to the class II sites.

Class III Sites—Equilibrium binding analyses as well as kinetic and thermostability studies showed that these four class III sites were specific for Mn$^{2+}$ and Mg$^{2+}$ and were apparently equivalent and independent of each other. We could find no significant difference between the Ca$_{4}$Mn$_{4}$ and Ca$_{4}$Mn$_{1}$ species in UV, fluorescence, and CD spectra (data not shown). Although binding of Mn$^{2+}$ or Mg$^{2+}$ to the class III sites was required for the maximum enzyme activity, it had only small ancillary effects on kinetic parameters (i.e. 1.8-fold increase in $V_{max}$ and 3-fold decrease in $K_{M}$), suggesting that metal ions bound to the class III sites do not play essential roles in the catalysis. Stability studies showed that binding of Mn$^{2+}$ and, to a lesser extent, Mg$^{2+}$ to class III sites confers thermostability as found at 60 °C and pH 7.2. The more Mn$^{2+}$ ions were bound to the class III sites, the more thermostable was the enzyme. One possible explanation for this is that a conformational change that occurs after the Mn$^{2+}$ binding stabilizes the enzyme. However, this can also be explained simply in terms of thermodynamics (Le Chatelier principle), which does not involve any structural changes in the protein (23-25). Loss of bound metals from class III sites below pH 7 may be explained in terms of protonation of amino acid ligands, and this causes loss of binding capacity for the metal. Alternatively the loss also could be ascribed to a reversible pH-dependent perturbation in the conformation of the metal-binding domain during titrations at pH 5.5-7.0.

It should be noted that S. rectiivirgula beta-galactosidase is an extracellular enzyme; it is secreted into the medium and should decompose lactose during the growth of the actinomyocyte at elevated temperatures (55 °C) for a prolonged time (3 days) (11). Because Mn$^{2+}$ and Mg$^{2+}$ ions are originally present in the medium (without supplemental Mn$^{2+}$) at concentrations of 2-4 and 200-320 μM, respectively, as found by ICPES, these metal ions should occupy class III sites to confer thermostability that permits the enzyme to retain sufficient activities during the actinomyceous growth. Indeed, after incubation under such growth conditions, the enzyme retained a higher residual activity (68%) in the medium than that in assay (5%) observed in a metal-depleted medium, which had been previously treated with Chelate Cellulofine. Therefore, metal binding to class III sites should be of biological significance in terms of maintaining thermostability rather than the maximum activity.

In summary, S. rectiivirgula beta-galactosidase is a heteronuclear multimetal enzyme (26) that requires multiple divalent metal ions for catalytic activity and thermostability. None of these metals appears to participate directly in primary catalysis. The bound metals are most likely to play a role in maintaining the native functional structure of the enzyme. Such metal ion requirements are clearly different from those of E. coli and R. melilotii beta-galactosidases and other glycosidases.

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REFERENCES

SCHEME 1. Divalent metal ion requirements of S. rectiivirgula beta-galactosidase. Double-headed arrows, right-headed arrows, and left-headed arrows indicate dialysis, treatment with Chelate Cellulofine, and addition of metal ion, respectively. Species names are shown in parentheses. For explanation of the site occupation, see legend of Table I.
Actinomycetous β-Galactosidase