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INHIBITION OF LYSOZYME BY POLYVALENT METAL IONS

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Summary

The rate of hen egg-white lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17), catalysis was determined in the presence of various metal ions (Co^{2+}, Zn^{2+} and eight of the trivalent lanthanide ions). In the assay system employed, the lanthanides were found to inhibit more strongly than either Zn^{2+} or Co^{2+}. The inhibition data was fitted to several models of the interactions of the metal ion with the enzyme. These models ranged in complexity from a single inhibitory metal binding site on the enzyme (two-parameter fit) to the presence of two non-independent and non-equivalent inhibitory metal binding sites (five-parameter fit). The more complicated models did not fit the data more precisely than the simplest one-site model, suggesting that the adoption of the simpler model is warranted. The fact that the association constants obtained from the simplest analysis for Co^{2+} ($1.3 \pm 1.9 \cdot 10^2$ M^{-1}) and Gd^{3+} ($7.0 \pm 2.6 \cdot 10^3$ M^{-1}) are consistent with literature values determined from spectroscopic measurements further supports the validity of the simplest model.

Introduction

Hen egg white lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17) catalyzes the cleavage of the *N*-acetylmuramyl bonds of an alternating *N*-acetylglucosamine-*N*-acetylmuramic acid copolymer found in the cell walls of bacteria. The structures of lysozyme and lysozyme inhibitor complexes, determined by X-ray crystallography, suggest that the substrate-binding

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Abbreviation: MES, 2-(*N*-morpholino)-ethanesulfonic acid.

region consists of a cleft which can accomodate six hexose residues [1—3]. These six sites have been labelled A to F. Enzymatic cleavage of an *N*-acetyl-glucosamine hexasaccharide occurs at the glycosidic bond between the carbon 1 of the pyranoside occupying site D and the oxygen atom at carbon 4 of the hexose occupying site E. The carboxyl side chains of Asp-52 and Glu-35 lie in close proximity to this bond and have been shown to participate in the catalysis. Detailed mechanisms of action for this enzyme have been proposed [4—12].

Kurachi et al. [13] have shown that Gd^{3+}, Co^{2+} and Mn^{2+} bind to the two carboxyl groups at the active site in the crystal. There have been numerous solution studies which have shown that lysozyme can bind polyvalent metal ions [14—28]. However, little data has been presented on the effects of metal complexation on the rate of lysozyme catalysis. Dwek [29] has reported that Gd^{3+} inhibits lysozyme. Secemski and Lienhard [23] have presented evidence that the Gd^{3+}-lysozyme complex has a lower affinity for various substrates and inhibitors than the metal-free enzyme.

In this report, we have determined the rate of lysozyme catalysis in the presence of various metal ions. This inhibition data was fit to several models for the interaction of the metal ions with lysozyme. A simple model in which there is one metal ion binding site on the enzyme was found to be consistent with the experimental results.

Experimental section

Materials. Lysozyme (from hen egg white, EC 3.2.1.17) was purchased from Worhtington Biochemicals Corp. (Freehold, N.J.) and used without further purification. The rare earths were obtained from Alfa Corp. (Danvers, Mass.) in their acetate, chloride or nitrate forms. Morpholinoethane sulfonic acid was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Methods. Enzymatic activity was determined using a modification of a procedure reported by Gorin et al. [30]. This assay is based on the ability of lysozyme to clear a suspension of lyophilized *Micrococcus lysodeikticus.* The incubation mixture contained 25 mg/l lysozyme, 0.154 M NaCl, 150 mg/l *M. lysodeikticus* and 2% Triton X-100 and various concentrations of metal salts in 0.01 M MES (pH 6.1). The Triton X-100 was included because it was found that upon the addition of some of the metal salts studied, a precipitate formed which interfered with the turbidometric assay. The addition of this compound to the incubation mixture prevented the formation of any precipitate without affecting the enzymatic activity appreciably. The initial rates of clearing of the suspension were measured at 25°C and 560 nm in a Beckman model 25 spectrophotometer.

Results and Discussion

The percent inhibition of lysozyme activity due to the presence of metal ions is defined as

$$\%I_M = 100\, v_M/v_0 \qquad (1)$$

TABLE I

THE PARAMETERS RESULTING FROM ANALYSIS OF THE INHIBITION DATA FOR VARIOUS METAL IONS IN TERMS OF A 1 : 1 BINDING MODEL

These parameters are the results obtained from unbounded fits of the data. Although values of $\%I_{max}$ of larger than 100% were obtained in this way, these values may reflect the relative imprecision of the assay.

Metal ion	Association constant, K (M^{-1})	$\%I_{max}$	S.D.
Co2+	$1.3 \pm 1.9 \cdot 10^2$	80 ± 50	14.3
Zn^{2+}	$1.3 \pm 0.5 \cdot 10^2$	131 ± 24	7.1
La^{3+}	$5.0 \pm 1.4 \cdot 10^3$	98 ± 9	5.3
Pr^{3+}	$7.5 \pm 3.7 \cdot 10^3$	104 ± 15	12.5
Nd^{3+}	$3.7 \pm 2.1 \cdot 10^3$	138 ± 39	5.5
Gd^{3+}	$7.0 \pm 2.6 \cdot 10^3$	120 ± 15	7.9
Tb^{3+}	$4.0 \pm 1.7 \cdot 10^3$	113 ± 27	6.9
Dy^{3+}	$8.7 \pm 4.0 \cdot 10^3$	106 ± 17	10.6
Er^{3+}	$6.6 \pm 1.5 \cdot 10^3$	107 ± 6	5.5
Tm^{3+}	$4.1 \pm 1.9 \cdot 10^3$	113 ± 27	11.0

where $\%I_M$ is the percent inhibition at the metal ion concentration, M, v_M is the initial rate of clearing of the assay mixture containing a given metal ion concentration, M, and v_0 is the rate of clearing of the assay mixture with no metal ions present. Inhibition experiments were performed for the various metal ions listed in Table I. In general, the trivalent lanthanide ions have similar binding and inhibition parameters. The results for a typical example, Gd^{3+}, are shown in Fig. 1. For Co^{2+} and Zn^{2+}, a higher concentration was necessary to produce a level of inhibition equivalent to the lanthanides. This is clear from the inhibition observed in the presence of various concentrations of Zn^{2+}, shown in Fig. 2.

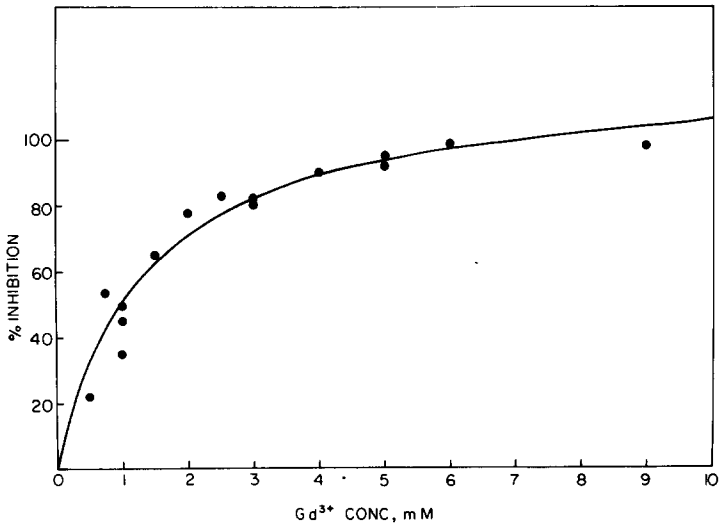

Fig. 1. Variation in the percent inhibition of lysozyme activity as a function of Zn^{2+} present.

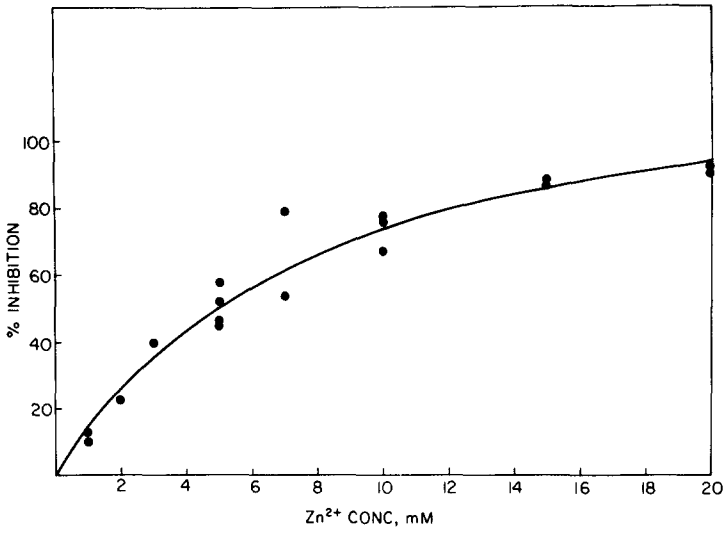

Fig. 2. Variation in the percent inhibition of lysozyme activity as a function of Gd^{3+} present.

Since there is evidence that metal complexation occurs at the active site carboxyl groups [13], we analyzed the inhibition data in terms of a model in which there is one inhibitory metal binding site in lysozyme. For this case, the percent inhibition at each metal concentration, M_t, is given by

$$\%I_M = \%I_{max}[E \cdot M]/E_t \quad (2)$$

where E · M refers to the metal-lysozyme complex [] dnotes equilibrium concentration, $\%I_{max}$ is the percent inhibition observed when all of the enzyme is complexed and E_t is the total concentration of lysozyme present. The concentration of the metal-lysozyme complex is given by

$$[E \cdot M] = K(E_t - [E \cdot M])(M_t - [E \cdot M]) \quad (3)$$

where K is the association constant for the metal-lysozyme complex and E_t and M_t refer to the total molar concentrations of lysozyme and metal ions present, respectively. Note that if K is known, the concentration of the metal-lysozyme complex can be calculated by solving the quadratic equation in [E · M] resulting from Eqn. 3. It is clear from Eqns. 2 and 3 that the inhibition data for a given metal can be related to its concentration through the two parameters, $\%I_{max}$ and K.

The best fit values for K and $\%I_{max}$ for the metal ions examined are given in Table I. These parameters were determined using a non-linear least-squares minimization algorithm (Ostroy, F. and Garland, F., unpublished data). The error estimates given in Table I for these parameters are the 95% confidence intervals obtained from the correlation matrix using the assumption of linearity in the region of the minimum. The standard deviation at the minimum was defined as,

$$\text{S.D.} = [\sum_{i=1}^{N} (\text{obs} - \text{calc})^2/(N - P)]^{1/2} \quad (4)$$

where obs and calc refer to the observed and calculated percent inhibitions, respectively, N is the number of data points and P is the number of parameters in the fit. For the single site model, the fits were repeated using a linear grid search technique. For all of the metal ions given in Table I, the two different fitting procedures yielded identical sets of parameters.

McDonald and Phillips [14] have analyzed the shifts in the proton NMR spectrum of lysozyme induced by Co^{2+} in terms of a 1 : 1 association of the metal and enzyme. These authors have reported an association constant for Co^{2+} of $5.7 \cdot 10^1\ M^{-1}$ at 55°C and a p^2H of 5.5 [14]. A value of $3.7 \pm 1.5 \cdot 10^1\ M^{-1}$ at 55°C and a p^2H of 5.5 was recently obtained by Lenkinski et al. [28] from NMR studies. The association constant found from our analysis of inhibition data is $1.3 \pm 1.9 \cdot 10^2\ M^{-1}$ at 25°C and pH 6.1. Gallo et al. [16] have shown that, for Mn^{2+}, the association constant increases significantly with increasing pH over the pH range of 5—6.5. Since this variation has been interpreted in terms of a competing protonation of the carboxyl ligands of lysozyme, a similar trend should be expected for Co^{2+}. In addition, the association constant should show some temperature dependence. On the basis of these considerations, we conclude that the value for the association constant of Co^{2+} obtained from the present inhibition study is in agreement with those obtained from NMR experiments. In addition the value of the association constant for Co^{2+} is in agreement with estimates made from competition experiments with Mn^{2+} using electron paramagnetic resonance [16] ($1 \cdot 10^2\ M^{-1}$ at 25°C and pH 5.5) and from other inhibition experiments [16] ($4.0 \cdot 10^1$—$6.0 \cdot 10^1$ at 25°C and pH 5.5).

The binding of Gd^{3+} to lysozyme has been monitored by a magnetic resonance method [22] as well as by ultraviolet difference spectroscopy combined with proton release experiments [23]. There is a large difference between the association constants determined from these experiments. The NMR experiments yielded a value of $9.0 \cdot 10^1\ M^{-1}$ at 23°C and pH 5.05 while the ultraviolet results gave a value of $2.1 \cdot 10^3\ M^{-1}$ at 25°C and pH 6.15. This large difference may reflect the fact that the particular NMR method employed (water relaxation rate enhancement) may be more sensitive to the presence of a number of weak non-specific metal binding sites. The association constant found from our analysis of the Gd^{3+} inhibition data (cf. Fig. 1) was found to be $7.0 \pm 2.6 \cdot 10^3\ M^{-1}$ at 25°C and pH 6.1 which is in qualitative agreement with the result obtained from ultraviolet measurements.

The X-ray data of Kurachi et al. [13] have shown two distinct sites for Gd^{3+} which are 3.56 Å apart. Yonath et al. [31] have found two sites for Cu^{2+} binding to lysozyme in the crystal. For these reasons we fit the inhibition data for all of the metal ions tested to several more complicated models for the inhibitory process. We found that the use of these more complicated modes did not improve the precision of the fits significantly (i.e. the standard deviation did not vary by more than 10%) in all of the metals except Co^{2+}. We have therefore reported the binding constants obtained using the single site model in Table I.

Since the values of the association constants obtained from our analysis of inhibition data of Co^{2+} and Gd^{3+} are similar to literature values obtained from spectroscopic measurements, we suggest that the values contained in Table I for

the other metals are good estimates of their corresponding association constants. These data are the first direct evidence that other trivalent lanthanide ions inhibit lysozyme in a manner similar to gadolinium. On the basis of the findings of Epstein et al. [32] in the porcine trypsin system and from studies of lanthanide binding to nitrolotetraacetic acid [33], the association constants for the lanthanides are expected to vary systematically with the ionic radius of the metal ion. The reasons for these trends have been discussed in detail by Reuben [34,29]. Although we could not observe this sort of trend in the data contained in Table I, it is possible that this trend is masked by comparatively larger error limits obtained from the association constants.

We wish to point out that inhibition studies such as the present one may provide a means for monitoring lanthanide binding to other enzymes. These kinds of experiments can be used to estimate the association constants for some of the lanthanides whose binding is difficult to monitor using spectroscopic methods.

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