A Study of the Binding of Mn$^{2+}$ to Bovine Pancreatic Deoxyribonuclease I and to Deoxyribonucleic Acid by Electron Paramagnetic Resonance

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SUMMARY

EPR studies of Mn$^{2+}$ binding to bovine pancreatic deoxyribonuclease I show that the enzyme can bind three Mn$^{2+}$ ions at pH 7.5 and 25°C. Two sites bind Mn$^{2+}$ strongly, with a $K_d$ of $10^{-4}$ M, and the third binds Mn$^{2+}$ weakly, with a $K_d$ of $10^{-3}$ M. Ca$^{2+}$ competes with the two strong sites, whereas Mg$^{2+}$ competes only with one of them, indicating that both sites are not equivalent.

Mn$^{2+}$ binding to DNA has been confirmed by EPR measurements. Two types of sites, with different affinities for Mn$^{2+}$ binding, were found on DNA molecules, one with a $K_d$ of $1.2 \times 10^{-4}$ M and the other with a $K_d$ of $10^{-3}$ M. Mg$^{2+}$ ions can displace Mn$^{2+}$ from the high affinity sites, but not from the low affinity sites. These results suggest that Mn$^{2+}$ binds not only to the phosphate groups, but also to the electron donor groups of the base rings.

Bovine pancreatic deoxyribonuclease, DNase I, requires the presence of divalent cations for activity (1, 2); however, the role of the metal ions is not understood. Recently, it has been demonstrated that Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ can interact directly with the protein molecule (3). On the other hand, evidence has been presented that divalent cations combine with DNA forming a metallosubstrate (4-7). The result of these interactions gives a particular type of cleavage of the DNA molecule, depending on the presence of a given metal. Bollum (8) showed that the nature of the activating metal ion qualitatively affects the specificity of the enzyme, and Melgar and Goldthwait (9, 10) found that the DNase I can cleave only one or both strands of DNA per encounter, depending on the divalent cation present in the solution.

These findings suggest a specific role for each metal in the enzymatic reaction of DNase I. In the work described here we have used electron paramagnetic resonance to examine the number of binding sites for Mn$^{2+}$ on DNase I and on DNA, the binding affinity and the specificity of these sites, and the competition by other metal ions.

EXPERIMENTAL PROCEDURE

Materials—Crystalline bovine pancreatic DNase I and calf thymus DNA were obtained from Sigma Chemical Co. Analytical reagent grade KCl, CaCl$_2$, MnCl$_2$, MnSO$_4$, MgSO$_4$, and TMA$^+$ were used. All solutions employed in these experiments were prepared using quartz-distilled water.

Tris-HCl buffer, 50 mM in Tris, pH 7.5, was used throughout. At the concentrations of manganese used the complex formed between the metal and the buffer was considered negligible (11). The solutions containing manganese were prepared immediately before using. The dilutions of the enzyme were made in cold buffer (4°C) just before use; under these conditions loss of activity was not observed in the course of the experiments.

DNase concentrations were calculated assuming a molecular weight of 31,000 for the protein (12, 13). Concentrations of DNA were determined by the absorption at 260 nm in a Beckman DU spectrophotometer assuming an $e_{260} = 6.6 \times 10^4$ (4).

EPR Measurements of Free and Bound Manganese—The free manganese ions were measured by recording the intensity of the X-band EPR spectra with a Varian 4502 spectrometer provided with a temperature control attachment V-4557. The temperature was maintained at 25°C ± 0.5°C by a flow of nitrogen and recorded by a Cu-Constantan thermocouple.

The aqueous samples were placed in 1-mm inner diameter capillary quartz tubes and introduced into the cavity. The same tube was used in each series of experiments, after a previous calibration with a manganese standard and the position of the tube in the cavity was carefully reproduced.

The manganese bound to DNA or to enzyme does not give a paramagnetic signal (14). This situation permits the measurement

1 The abbreviation used is: TMA, tetramethylammonium bromide.
of the bound manganese by difference (15). In order to obtain absolute concentrations of MnZ+, a correction was made for the spectral changes produced by the variation in the viscosity when DNA or DNase I were present in the solutions.

Analysis of Data—The parameters for manganese binding to DNase I have been determined by the graphic method of Hughes and Klotz (16). The association constant for manganese, \( K_a \), is defined in Equation 1:

\[
K_a = \frac{(Mn)_b}{(Mn)_f} \times \frac{n}{(DNase)_t - (DNase)_a} = \frac{1}{K_d}
\]

where \((Mn)_f\) and \((Mn)_b\) are respectively the molarities of the free and bound manganese, \(n\) the number of binding sites on the enzyme, \((DNase)_t\) the total concentration of the enzyme, and \(K_d\) the dissociation constant of manganese from the protein.

The reciprocal form of Equation 1 is Equation 2:

\[
\frac{(DNase)_t}{(Mn)_f} = \frac{1}{n} \times \frac{1}{(DNase)_t} = \frac{1}{(Mn)_f} = \frac{1}{K_d}
\]

from this equation \(n\) and \(K_a\) can be obtained as the intercepts, plotting the variation of \((DNase)_t/(Mn)_f\) as a function of \(1/(Mn)_f\).

An alternate method for finding \(n\) and \(K_a\) is the Scatchard (17) using Equation 3:

\[
\frac{(Mn)_b}{(DNase)_t} = \frac{n}{(Mn)_f} = \frac{1}{(DNase)_t} = \frac{K_d}{(Mn)_f}
\]

Plotting

\[
\frac{(Mn)_b}{(DNase)_t} = \frac{1}{(Mn)_f} = \frac{K_d}{(Mn)_f}
\]

as a function of

\[
\frac{(Mn)_b}{(DNase)_t} = \frac{1}{(Mn)_f} = \frac{K_d}{(Mn)_f}
\]

the intercepts are \(n\) and \(n/K_d\) if the line is straight and only one type of site exists. If there exist two or more independent classes of sites, with different dissociation constants, the plot may be curved or may consist of a broken line, and the \(x\)-intercept will be the total number of sites (18). This method has been used also for the treatment of the results obtained with DNA; \((DNase)_t\) were the molar concentration of the phosphate contained in the DNA.

The dissociation constant for magnesium was measured by competition with manganese (19). Magnesium was added in increasing amounts to solutions containing manganese and DNase I, and the changes in the free MnZ+ were measured by EPR; the dissociation constant, \(K_{DM}\), was calculated by the method of Hastings et al. (20), assuming that the competition occurs only for one type of site, from the following equations:

\[
K_{DM} = \frac{[Mg]_t - [MgDNase]_t}{[MgDNase]_t}
\]

where \([MgDNase]_t\) is the concentration of the Mg-DNase complex and \([DNase]_t\) the unbound DNase. If MgZ+ competes with MnZ+, then:

\[
[MgDNase]_t = [DNase]_t - [DNase]_a - [MnDNase]_t
\]

where \([MnDNase]_t\) is the concentration of the Mn-DNase complex and may be evaluated from the relationship:

\[
[MnDNase]_t = (Mn)_t - (Mn)_a = (Mn)_b
\]

\([DNase]_t\) may then be determined in terms of the known \(K_{DM}\):

\[
[DNase]_t = \frac{K_{DM} \times (Mn)_b}{(Mn)_f}
\]

A similar treatment was applied to the binding of magnesium to DNA.

RESULTS

Binding of MnZ+ to DNase I—A solution containing \(1.5 \times 10^{-4} \text{M DNase}\) I was titrated with variable amounts of manganese chloride (5 \(\times\) \(10^{-5}\) to 2.8 \(\times\) \(10^{-5}\) M) at 2°C. The results are shown in Fig. 1. The Scatchard (17) plot shows an inflexion of the curve indicating the presence of two types of binding sites with different binding constants. Extrapolation of the linear segments indicates that DNase I binds tightly two (1.7) manganese ions with a dissociation constant of \(10^{-4} \text{M}\), and weakly only one (0.8) manganese ion with a dissociation constant of \(10^{-5} \text{M}\).

Effect of Other Metal Ions on MnZ+ Binding to DNase I—The effect of CaZ+ and MgZ+ ions on the manganese binding to DNase I was observed by adding increasing amounts of CaZ+ or MgZ+ to a solution containing \(1.1 \times 10^{-4} \text{M DNase} I\) and 2 \(\times\) \(10^{-4}\) M MnSO4. When the concentration of calcium or magnesium was raised, the manganese was released from the protein (Fig. 2); the displacement started when the molarity of the competing ion was similar to that of manganese and was complete at 1.5 \(\times\) \(10^{-4}\) M for MgZ+ and at 2.5 \(\times\) \(10^{-4}\) M for CaZ+. These results can not be attributed to a dielectric effect produced by the increase in the ionic strength. Replacement of MgZ+ or CaZ+ ions by 0.1 M TMA+ ions produced a decrease of only 5% of the enzyme-bound manganese (Fig. 2).

CaZ+ ions displaced all of the MnZ+ bound to the enzyme, whereas MgZ+ ions displaced only a portion of the MnZ+; 0.6 manganese ions still remained bound to the protein when the maximum effect of MgZ+ was achieved. This result was interpreted as evidence that MgZ+ competes for only one of the strong Mn binding sites of the enzyme.

Assuming that the magnesium competition occurs only for one of the two strong Mn binding sites, an apparent \(K_{DM}\) was calculated. A progressive increase of the constant was observed as the magnesium concentration increased, which indicates the existence of other Mg binding sites in addition to the site common with manganese. However, when the apparent dissociation constants were plotted as a function of the concentration of the Mg-DNase complex, a real \(K_{DM} = 6 \times 10^{-4} \text{M}\) was obtained by extrapolation to the ordinate axis (Fig. 3). A similar treat-
Dependence of the number of Mn$^{2+}$ ions bound per DNase molecule in the presence of other ions. The samples contained $1.1 \times 10^{-4}$ M DNase I, $2 \times 10^{-4}$ M MnSO$_4$, 50 mM Tris-HCl buffer, pH 7.5, and variable amounts of the competing ions. When the tetramethylammonium bromide was used, the MnSO$_4$ was replaced by $2 \times 10^{-4}$ M MnCl$_2$. Temperature 2$^\circ$.

**FIG. 2.** Dependence of the number of Mn$^{2+}$ ions bound per DNase molecule in the presence of other ions.

Variation of the apparent $K_{DM}$ in the function of the Mg-DNase complex. The values for $K_{DM}$ were calculated from the data on Fig. 2.

**FIG. 3.** Variation of the apparent $K_{DM}$ in the function of the Mg-DNase complex. The values for $K_{DM}$ were calculated from the data on Fig. 2.

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**Fig. 4.** Scatchard plot of the binding of Mn$^{2+}$ on DNA. The samples contained $1.4 \times 10^{-3}$ M DNA, 50 mM Tris-HCl buffer, pH 7.5, and variable amounts of MnSO$_4$. Temperature 2$^\circ$.

**Fig. 5.** Effect of other cations on the Mn$^{2+}$ bound to DNA. The samples contained $1.4 \times 10^{-3}$ M DNA, 50 mM Tris-HCl buffer, pH 7.5, and variable amounts of the competing ions. Temperature 2$^\circ$.

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Binding of Mn$^{2+}$ to DNA—When a solution containing $1.4 \times 10^{-3}$ M DNA was titrated with manganese sulfate ($10^{-4}$ to $3.3 \times 10^{-4}$ M) at 2$^\circ$, two types of binding sites for the metal ion were evident. The Scatchard plot, in Fig. 4, shows 0.19 strong binding sites per DNA-phosphate, with a $K_d = 10^{-4}$ M, and 0.13 weak binding sites, with a $K_d = 1.2 \times 10^{-3}$ M. These results were reproduced in a series of seven experiments which gave $0.16 \pm 0.04$ strong sites with a $K_d = (1.2 \pm 0.2) \times 10^{-4}$ M, and $0.11 \pm 0.04$ weak sites with a $K_d = (1.0 \pm 0.8) \times 10^{-3}$ M.

The number of sites and the dissociation constants are temperature dependent; when the experiments were run at 25$^\circ$, we found 0.26 strong sites with a $K_d = 6 \times 10^{-3}$ M, and 0.09 weak sites with a $K_d = 5 \times 10^{-4}$ M. Similar results were obtained by Eisinger et al. (5) using the enhancement of the relaxation time of the protons in the presence of manganous ions.

**Mg$^{2+}$ Competition for Mn Binding Sites on DNA**—The competition between Mn$^{2+}$ and the diamagnetic magnesium ions was studied adding increasing amounts of magnesium sulfate to a solution containing $1.4 \times 10^{-3}$ M DNA and $2 \times 10^{-4}$ M manganous ions. The results are shown in Fig. 5. Competition starts when the molarity of Mg$^{2+}$ equals the Mn$^{2+}$ concentration, and reaches a plateau at $2 \times 10^{-4}$ M magnesium. There was no further release of Mn$^{2+}$ from DNA at higher concentrations of competing ion. A similar effect was obtained replacing Mg$^{2+}$ by K$^+$ or TMA$^+$ ions, but the concentration required to start the release of Mn$^{2+}$ from DNA was 100-fold higher (Fig. 5). Since the effect of K$^+$ parallels that of TMA$^+$, this suggests that the potassium effect is due to the increase in the ionic strength. In the case of Mg$^{2+}$ the ionic strength effect was negligible.

At the low concentration of manganese used, it was assumed that the competition occurs only on the strong binding sites, and a $K_{DM} = (1.9 \pm 0.5) \times 10^{-4}$ M was calculated, as the mean value of 11 experiments. This value is very close to that obtained by Sander and Ts'o (4) at room temperature, who found a $K_{DM} = 1.7 \times 10^{-4}$ M.

Magnesium does not compete for the weak Mn binding sites on the DNA molecule. When a solution containing $1.4 \times 10^{-4}$ M DNA and $5 \times 10^{-4}$ M MgSO$_4$ was titrated with MnSO$_4$, the Hughes and Klotz (16) plot shows a straight line, indicating the presence of only one type of Mn binding site (Fig. 6). Under these conditions the dissociation constant, $K_d = 10^{-8}$ M, and the number of sites, n = 0.11 per phosphate, agree with the values expected for the weak Mn binding sites. Thus, the high...
The interaction of metal ions with nucleic acids and nucleotides has been extensively studied (26, 27). Two sites for metal binding have been found on DNA molecules: the phosphate moieties of the ribose-phosphate backbone and the electron-donor groups on the bases (6). Metals have different affinities for these sites; Mg²⁺ binds more specifically to the acidic groups of phosphate stabilizing the double helix, whereas Cu²⁺, Zn²⁺, and Mn²⁺ bind simultaneously to phosphates and base rings, producing a variable amount of destabilization in the ordered structure of DNA (6, 27–29). In the present work we found two types of Mn binding sites on DNA, one of which is freely available to Mg²⁺. Titration of DNA by Mn⁴⁺ in the presence of 5 × 10⁻⁴ M Mg²⁺ made apparent that magnesium prefers the strong Mn binding sites. From these results it can be inferred that the strong binding sites are the phosphates. On the other hand, the weak sites, available only for manganese are the electron-donor groups of the base rings, mainly the GC pairs (29).

Cohn (15) classified the enzymes activated by divalent metals in two categories: (a) those which bind the metal ion on the enzyme, activating directly or indirectly on the active site (enolase and pyruvate kinase), and (b) those which function as free enzymes binding specifically to metal-substrates (creatine kinase and yeast hexokinase). It is noteworthy that the enzymes of the first group are inhibited by Ca²⁺, and the enzymes of the second group are all activated by the same ion (15). DNase I could be considered in the second group. Shack and Dzymk (7) found that the metal ion requirement increases with the DNA concentration, which is evidence for the formation of a metal-DNA complex. In this line, we can assume as a first hypothesis that the metals may induce conformational changes in the DNA molecule, making the substrate recognizable by the enzyme. The differences in the binding of Mn⁴⁺ and Mg²⁺ on the DNA involve a particular form in the cleavage of the substrate. In both cases the enzyme would hydrolyze a single strand of the double helix, but in the case of Mn⁴⁺ the binding of the cation on the bases could hold the ternary complex together and facilitate the rupture of the second strand.

Another possibility is that Mn⁴⁺ would make the reaction less specific for a particular site on DNA. An argument in favor of this is that the replacement of Mg²⁺ by Mn⁴⁺ produces errors in the mechanism of reaction of the DNA and the RNA polymerases, such as the incorporation of ribonucleotides on DNA and deoxyribonucleotides on RNA, respectively (26, 30). Similarly the dC strand of the polymer dI:dC is hydrolyzed by DNase I in the presence of Mn⁴⁺, whereas in the presence of Mg²⁺ the dC strand appears resistant to the attack of the enzyme (8).

Nevertheless, these hypotheses can not explain why Ca²⁺ activates DNase I giving “single hit” kinetics (10, 31). Studies
of the binding of Ca$^{2+}$ to mononucleotides showed that this ion behaves as Mg$^{2+}$, binding only to phosphate groups (32). Assuming a similar situation in Ca$^{2+}$ binding to DNA, we have to admit an effect of the metal ion independent of the binding on DNA, which should be involved in changing the kinetics of the enzymatic reaction of DNase I. This effect could be related to the binding of Ca$^{2+}$ and Mn$^{2+}$ to the enzyme, but without any further experimental evidence we are not able to propose a model to explain this phenomenon.

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