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KINETIC STUDIES ON THE MECHANISM OF MG(II) ACTIVATION OF DEOXYRIBONUCLEASE I

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SUMMARY

When a magnesium salt of bovine spleen DNA (Mg:2P = 1.0) was used as the substrate for the deoxyribonuclease I (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5) reaction, the data obtained for Mg(II) activation was not as complicated as those obtained with NaDNA as substrate. The Mg(II) activation curve was biphasic, indicating that the activation occured at two sites on the protein. Kinetic analysis of the data shows that Mg(II) activation followed sigmoidal saturation kinetics for each site, with $n = 2$ for each site. Free Mg^{2+} was required for enzyme activity, confirming that a metalloenzyme as well as a metallosubstrate was necessary for deoxyribonuclease I activity.

INTRODUCTION

Pancreatic deoxyribonuclease I (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5) requires the presence of divalent cations for activity[1]. Evidence has been presented that divalent cations (*i.e.* Mg(II)) may activate by forming a metallosubstrate; Kunitz[2], Price *et al.*[3] and Wiberg[4] reported that, for maximum activity, Mg^{2+} concentration must be increased with increase in DNA concentration. However, Kunitz[2], Price *et al.*[3], and others[5,6] also found at low Mg(II) concentrations that the reaction velocity decreased with increased substrate concentrations. Erkama and Suutarinen[7], using MgDNA (Mg:2P = 1.28) prepared from NaDNA, found that added Mg(II) was still required for maximum activity of deoxyribonuclease I, and they concluded that perhaps a metallo-enzyme as well as a metallo-substrate was necessary for activity. Their investigation was limited to a single concentration of added Mg(II) and its effect on the pH profile of the enzyme.

The availability of a relatively pure preparation of a Mg(II) salt of DNA (Mg:2P = 1.04) made it possible for us to study the Mg(II) requirements for deoxyribonuclease I activity with NaDNA and MgDNA as substrates. We found it possible to do a kinetic study of the activation of deoxyribonuclease I by Mg(II) with MgDNA

as substrate; similar studies with NaDNA as substrate are too complicated to evaluate.

EXPERIMENTAL PROCEDURE

A magnesium salt of bovine spleen DNA was prepared by substituting $MgCl_2$ for the NaCl–sodium citrate medium in a standard DNA preparation procedure[8]. Sodium xylene sulfonate (Naxonate G) was added to remove protein, and the MgDNA was purified by reprecipitating three times with cold 2-propanol. The final precipitate was dried by washing, first with ethanol and then with acetone. When the purified MgDNA was dissolved in $5 \cdot 10^{-4}$ M $MgCl_2$, the usual DNA absorbance spectrum was obtained, with a maximum at 260 nm, the ratio of A_{260} to A_{235} was 2.28. MgDNA, dissolved in deionized water, produced a melting curve with a steep slope and a T_m at 86 °C. The final product had a hyperchromicity in deionized water of 43%, and an Mg(II):2DNA–P ratio of 1.04. Magnesium was assayed with a Perkin–Elmer model 303 atomic absorption spectrophotometer. For the phosphate assay, the DNA sample was first heated with 60% $HClO_4$, to destroy organic matter; phosphate was then determined by a standard method of Fiske and SubbaRow. The sodium salt of calf thymus DNA was obtained from Sigma Chemical Co., St. Louis, Mo. Stock solutions of both DNAs were prepared at 4 °C by dissolving 10 mg DNA in 50 ml deionized water; and the solutions were stored at 4 °C. Stock solutions of pancreatic deoxyribonuclease I (Worthington Biochemical Corporation, Freehold, N.J.) of 5 mg in 25 ml deionized water were stored at −20 °C, these solutions were defrosted and used as needed, and remained active for at least two months.

Assays were carried out with a Beckman DB split-beam spectrophotometer, equipped with a circulating constant temperature bath set at 25 ± 0.01 °C. Enzyme activity was measured by following the change in absorbance at 260 nm for 15 min (ref. 2) with an expanded scale recorder. An active sample thus produced a total increase in absorbance of 0.1 to 0.2 A units. Initial velocity was calculated from the tangent to the steepest segment of the curve. Both the sample and reference cuvettes contained 0.10 M sodium acetate buffer, pH 5.0, which included the indicated quantities of Mg(II), as $MgCl_2$, and DNA. The reaction was initiated by adding 10 μg of deoxyribonuclease I to the assay cuvette and an equivalent amount of water to the reference cuvette. All solutions were equilibrated at 25 ± 0.01 °C for at least 30 min before mixing.

RESULTS AND DISCUSSION

When NaDNA was used as the substrate for the deoxyribonuclease I reaction, added Mg(II) was necessary for any measurable reaction to occur. In the range 1 to 6 mM Mg(II), the initial velocity of reaction decreased as the DNA concentration increased (Fig. 1) confirming previous reports[1,5,6]. These effects of DNA and Mg(II) concentrations on the velocity of reaction, indicate a complicated interaction of Mg(II) with both the substrate and enzyme.

Added Mg(II) was also necessary for the deoxyribonuclease I reaction with MgDNA as substrate (Fig. 2), however in this case, initial velocities increased as the DNA concentration increased. At low Mg(II) concentrations, (4.5 mM), there was no

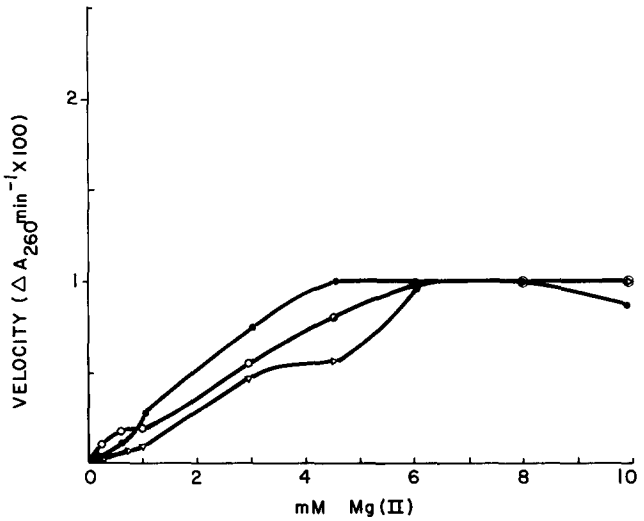

Fig. 1. Mg(II) activation of deoxyribonuclease I with NaDNA as substrate. ●—●, 6.6 μg/ml NaDNA; ○—○, 10 μg/ml NaDNA; △—△, 20 μg/ml NaDNA.

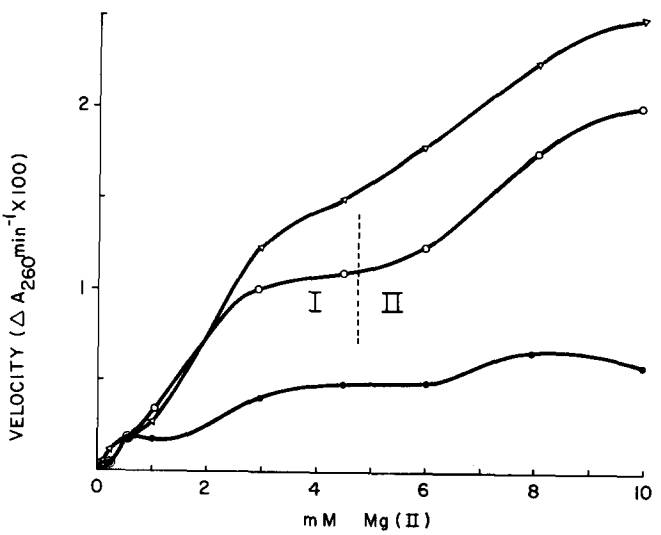

Fig. 2. Mg(II) activation of deoxyribonuclease I with MgDNA as substrate. ●—●, 6.4 μg/ml MgDNA; ○—○, 13 μg/ml MgDNA; △—△, 20 μg/ml MgDNA.

competition between the MgDNA and the enzyme for the available Mg(II), in contrast to the results obtained with NaDNA. The fact that free Mg(II) must be present for any reaction to occur with MgDNA, indicates that a metallo-substrate and a metallo-enzyme must both be present as suggested by Erkama and Suutarinen[7]. The Mg(II) activation curves for deoxyribonuclease I are biphasic for all concentrations of MgDNA tested; this is particularly apparent for the middle curve in Fig. 2 (13 μg/ml, MgDNA). The data responsible for this curve were therefore selected for further kinetic analysis. The curve was divided into two parts, as indicated in Fig. 2, and

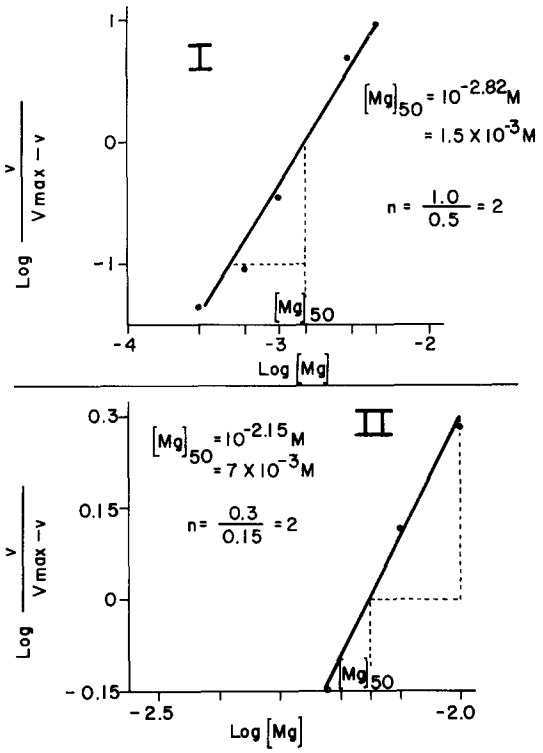

Fig. 3. Hill plots of the two sections of the 13 μg/ml MgDNA curve of Fig. 2.

each part treated separately. We were thereby assuming that there were two sites of reaction of Mg(II) with the enzyme protein.

Double-reciprical plots of Curves I and II, both resulted in concave-upward curves, indicating a multiple combination of the activator with the enzyme, at each site. Since the Mg(II) activation curves did not follow hyperbolic saturation kinetics, the possibility that these curves could be analyzed using sigmoidal saturation kinetics was then investigated. Fig. 3 shows the separate plots for Section I and II of the middle curve in Fig. 2, using the straight line form of the Hill equation:

$$\log \frac{v}{V - v} = n \log [\mathrm{Mg(II)}] - \log K'$$

In each case, V was estimated by considering the initial velocity at the point of inflection of the experimental curve as $V/2$. The slope of the resultant straight line is n in the Hill equation:

$$v = \frac{V [\mathrm{Mg(II)}]^n}{K' + [\mathrm{Mg(II)}]}$$

From Fig. 3, n is 2.0 for both sites I and II, indicating multiple sequential combinations of Mg(II) at each site.

To our knowledge, this is the first reported instance of enzyme activation which produces a double Hill plot.

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