Protection of Nuclease P₁ against Thermal Inactivation by Zinc Ion[†]

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Nuclease P₁ is a zinc metalloenzyme whose catalytic function and secondary structure owe to zinc atoms.¹⁻⁶⁾ In the present paper, Zn²⁺ is demonstrated to protect purified nuclease P₁¹⁾ against thermal inactivation. The enzyme activity toward 3'-AMP was assayed as described previously.¹⁾

Thermostability of nuclease P₁ varied considerably, depending upon pH and the kind of compounds present in the enzyme solution. Addition of Zn²⁺ to the enzyme solution generally shifted the stable pH range of the enzyme to acid side and increased thermostability of the enzyme markedly. Examples are given in Fig. 1. It should be noted that without Zn²⁺ the enzyme was quite thermolabile in the presence of 5'-nucleotides at around pH 5, near its optimal pH for RNA and heat-denatured DNA.³⁾

As shown in Fig. 2, in the absence of Zn²⁺ the enzyme was rapidly inactivated during the reaction at 70°C with RNA and heat-denatured DNA and the degradation was incomplete, while in the presence of Zn²⁺ the thermal inactivation was markedly depressed and the complete degradation was observed.

The most effective concentration of Zn²⁺ for the protection of the enzyme against thermal inactivation during hydrolysis of RNA was 2×10^{-4} M: after reaction mixture (5 ml) containing 40 mg of RNA, 2.5 μ g of nuclease P₁, and Zn²⁺ at various concentrations was incubated at pH 4.8 at 65°C for 30 min, the reaction mixture (1 ml) was dialyzed against running water for 10 hr at 4°C, and the remaining activity toward 3'-AMP was assayed. In the presence of 0, 2×10^{-6} , 6×10^{-6} , 2×10^{-5} , 6×10^{-5} , 2×10^{-4} , 6×10^{-4} , and 2×10^{-3} M of Zn²⁺, the remaining activities were 8.6, 25.7, 50.6, 73.5, 71.7, 88.0, 84.9 and 83.3%, respectively.

Among other metal ions tested only Co²⁺ and Mn²⁺ exerted a slight protection of the enzyme during hydrolysis of RNA at 70°C for 30 min: in the presence of 2×10^{-4} M of SrCl₂, CoCl₂, CdCl₂, CaCl₂, MnCl₂,

FIG. 1. Effect of Zn²⁺ on Thermostability of Nuclease P₁ in the Presence of Various Compounds.

Nuclease P₁ (4 μ g) dissolved in 1 ml of water (a), 0.01 M NaCl (b), 0.01 M veronal-acetate buffer (c), amino acid mixture (Ajinomoto's calibration mixture) consisting of 5×10^{-5} M each of amino acids except proline (1×10^{-4} M) (d), 0.01 M AMP (e), 0.01 M GMP (f), 0.01 M CMP (g) or 0.01 M UMP (h) at the indicated pH in the presence or absence of 2×10^{-4} M ZnCl₂ was incubated at 65°C for 30 min. Except veronal-acetate buffer, pH of each solution was adjusted with HCl or NaOH solution. After incubation, the remaining activity toward 3'-AMP was assayed.

●—●, absence of Zn²⁺; ○---○, presence of Zn²⁺.

ZnCl₂, NiCl₂, MgCl₂, PbCl₂ and BaCl₂, the remaining activities were 9.7, 24.2, 8.3, 11.1, 16.8, 84.3, 8.1, 10.6, 8.0 and 10.8%, respectively. Without the addition, the remaining activity was 10.2%.

Alkaline phosphatase,⁷⁾ carbonic anhydrase,⁸⁾ carboxypeptidase,⁹⁾ leucine aminopeptidase,¹⁰⁾ phosphomannose isomerase¹¹⁾ and neutral proteases¹²⁾ have been known to be zinc metalloenzymes. However, so far as we know, there has been no report that Zn²⁺ increased their thermostability. Ca²⁺ is a potent stabilizer of neutral proteases¹²⁾ and α -amylase.¹³⁾ Take-amylase A molecule retains 10 atoms of calcium when the enzyme is exposed to Ca²⁺; ^{14,15)} one is firmly bound and plays a decisive role in preserving the integrity of the active conformation, and the others are loosely bound and make the enzyme molecule resistant to denaturation, suggesting that the formation of intramolecular calcium salt linkage stabilizes the tertiary and secondary structures. The protection of nuclease P₁ by Zn²⁺ may be also due to formation of the intramolecular zinc salt linkage. This view is supported by observations of Rokugawa *et al.*¹⁶⁾ that immobilized nuclease P₁ was more thermostable than

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FIG. 2. Formation of Uranyl Reagent-soluble Compounds and Stability of Nuclease P_1 during Course of Digestion of RNA (A) and Heat-denatured DNA (B) in the Presence or Absence of Zn^{2+} .

The reaction mixture (A) containing 4 ml of 3% RNA in M/35 veronal-acetate buffer, pH 4.8, and 2 ml of nuclease P_1 (2.5 μ g/ml), and the reaction mixture (B) containing 4 ml of 0.5% heat-denatured DNA in M/35 veronal-acetate buffer, pH 4.8, and 2 ml of nuclease P_1 (0.5 μ g/ml) were incubated at 70°C in the presence or absence of Zn^{2+} at the final concentration of 2×10^{-4} M. An aliquot was withdrawn at the indicated time and uranyl reagent-soluble products and the remaining enzyme activity were determined as follows: (1) Uranyl reagent-soluble products: the aliquot (0.5 ml) was added to 2 ml of cold uranyl reagent (0.25% uranyl acetate in 2.5% perchloric acid) and the mixture was allowed to stand in ice water for 30 min. The formed precipitate was centrifuged off and absorbance at 260 nm of the supernatant was determined. The value in the complete digestion, where nucleic acid was wholly converted into uranyl reagent-soluble products, was expressed as 100%. (2) Remaining enzyme activity: the aliquot (1 ml) was dialyzed against running water at 4°C for 10 hr. The dialyzed inner solution was assayed for the activity toward 3'-AMP.

●—●, remaining activity in the absence of Zn^{2+} ; ○—○, remaining activity in the presence of Zn^{2+} ; ▲—▲, uranyl reagent-soluble products in the absence of Zn^{2+} ; △—△, uranyl reagent-soluble products in the presence of Zn^{2+} .

the native enzyme and did not require Zn^{2+} for stabilization. In addition, there is not yet any explanation about the interesting phenomenon that the addition of Zn^{2+} shifted the stable pH range to acid side.

Sufficient amount of nuclease P_1 can degrade RNA and heat-denatured DNA at a low temperature.¹⁾ Although initial rate of hydrolysis at around 70°C is about 17-fold greater than that at 37°C,²⁾ the complete

digestion could not be attained with a small amount of nuclease P_1 at such a high temperature owing to rapid thermal inactivation of the enzyme during the hydrolysis. Now, the addition of Zn^{2+} as a stabilizer made it possible to accomplish practically the complete digestion of RNA and heat-denatured DNA even with a small amount of the enzyme at a high temperature.

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