Hydrolysis of *p*-nitrophenyl phenylphosphonate catalysed by bovine pancreatic deoxyribonuclease

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The ability of bovine pancreatic DNAase to hydrolyse the synthetic substrate *p*-nitrophenyl phenylphosphonate (NPPP) is intrinsic and is not due to the contamination of the DNAase preparation by non-specific phosphodiesterases because the activities of DNA and NPPP hydrolysis are co-eluted from a DEAE-cellulose column with use of the Ca^{2+} -affinity elution method and because the two activities are decreased simultaneously when the purified enzyme is treated with Cu^{2+} /iodoacetate, an active-site-labelling agent for DNAase. NPPP hydrolysis is facilitated by the metal ion–DNAase. At relatively high Na^+ concentrations, where the metal ion–DNA interaction is weak, DNA hydrolysis is also facilitated by the metal ion–DNAase. With NPPP as substrate the Michaelis constants are K_m 3.7 mM for Mn^{2+} and K_m 49 mM for Mg^{2+} in 0.2 M-Tris/HCl buffer, pH 7.2. Ca^{2+} competes with Mn^{2+} , with K_i 64 mM. Free Cu^{2+} ions non-competitively inhibit DNAase-catalysed DNA or NPPP hydrolysis in the presence of Mn^{2+} or Mg^{2+} and the inhibition is not relieved by Ca^{2+} . The affinity of Cu^{2+} for free DNAase is higher than that for Mn^{2+} –DNAase. Mn^{2+} is not bound to DNAase via a simple ionic interaction, as Mn^{2+} remains bound in the presence of relatively high Na^+ concentrations and induces a near-u.v. difference absorption spectrum. The kinetics of NPPP hydrolysis catalysed by Mn^{2+} –DNAase are sigmoidal. From the Hill equation, $h = 2.0$ is obtained, suggesting that more than two NPPP molecules are bound per molecule of DNAase with a certain amount of co-operativity. Because DNAase in solution is a monomer with a single catalytic site, the multiple NPPP molecules on a single protein molecule are probably in one location, resulting in a co-operative interaction that may resemble that in the stacked base-pairs of double-helical DNA.

INTRODUCTION

Several *p*-nitrophenyl esters of the thymidine phosphates have been reported as synthetic substrates for bovine pancreatic DNAase (Liao, 1975). This observation established that DNAase is a phosphodiesterase acting not only on large DNA molecules but also on small synthetic substrates. In our present study we have found that *p*-nitrophenyl phenylphosphonate (NPPP), which has no nucleotide structure and is smaller than any of the previously reported synthetic substrates, also can be hydrolysed by DNAase. DNA hydrolysis requires bivalent metal ions (McCarty, 1946), as does NPPP hydrolysis. However, in DNA hydrolysis free DNAase acts on the metal ion–DNA (Wiberg, 1958), whereas in NPPP hydrolysis metal ion–DNAase catalyses the hydrolysis of free NPPP because there is limited binding of metal ions to univalent NPPP. In the present paper we describe the use of NPPP as substrate kinetically to establish, on the DNAase molecule, the catalytic metal-ion site and a non-competitive Cu^{2+} site as well as the correlation of these sites with the DNAase-catalysed DNA hydrolysis.

MATERIALS AND METHODS

Materials

NPPP (free acid) was obtained from Sigma Chemical Co. For the preparation of 0.2 M-NPPP the acid was dissolved in 0.2 M-Tris and a few drops of 1 M-NaOH. The solution, titrated to pH 5 with acetic acid, was

extracted with benzene several times until the organic phase no longer released yellowish *p*-nitrophenol on back-extraction with 0.1 M-NaOH. The NPPP solution was standardized after hydrolysis in 1 M-NaOH by measuring the amount of *p*-nitrophenol ($\epsilon_{400} = 18\,320 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Kezdy & Bender, 1963). DNAase was prepared from the pancreatic DNAase supplied by Cooper Biomedical (code DP) by applying the chromatographic procedure of Liao (1974) on a DEAE-cellulose column (Fig. 1). Acetone-dried bovine pancreas powder and ethidium bromide were from Sigma Chemical Co.

SDS/polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of SDS was essentially according to the method of Laemmli (1970). Slab gels, consisting of 10% polyacrylamide in the running gel and 3% in the stacking gel, were used.

Assays

The hydrolysis of NPPP by DNAase or by non-specific phosphodiesterases was monitored kinetically at 400 nm on a spectrophotometer (Hitachi model 100-10) equipped with a strip-chart recorder (linear model 535). The hydrolysis of DNA was measured by the hyperchromicity method of Kunitz (1950). Because of the relatively high molar absorbance of Cu^{2+} at 260 nm, for assays with Cu^{2+} on ethidium bromide/DNA solution ($[\text{DNA}] = 0.125 \text{ mg/ml}$; $[\text{ethidium bromide}] = 0.0125 \text{ mg/ml}$) the absorbance change at 465 nm was measured. Unless otherwise indicated the assay buffer is

Abbreviations used: NPPP, *p*-nitrophenyl phenylphosphonate; TDNPP, thymidine 3',5'-di-*p*-nitrophenyl phosphate.

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Fig. 1. Chromatography of DNAase on DEAE-cellulose

Approx. 100 mg of DNAase (code DP, obtained from Cooper Biomedical) was dissolved in 10 ml of 20 mM-Tris/HCl buffer, pH 8.0, containing 5 mg of phenylmethanesulphonyl fluoride. The solution was loaded on a DEAE-cellulose column (1.7 cm × 15 cm) previously equilibrated with 2 mM-CaCl₂ in 20 mM-Tris/HCl buffer, pH 8.0. The column was then washed with 1 column volume of the equilibrating buffer. DNAase was eluted with a linear gradient of CaCl₂ (----) in 20 mM-Tris/HCl buffer, pH 8.0 (2 mM- to 20 mM-CaCl₂, 200 ml of each). The fractions near 180 ml of effluent were assayed for the enzymic activity with DNA (▲) or NPPP (▼) as substrate. For the definition of specific activity see the text. ●, A₂₈₀.

1 mM-CaCl₂/0.2 M-Tris/HCl, pH 7.2, at 25 °C. One unit of DNA hydrolysis activity is defined as an increase of 1 absorbance unit/min per ml, and one unit of NPPP hydrolysis activity as an increase of 0.001 absorbance unit/min per 0.4 ml. Specific activity is designated as the number of units in 1 ml of DNAase solution per absorbance unit at 280 nm of that solution (units/A₂₈₀).

Computer analysis of data

The least-squares fit of data for the linear function in the double-reciprocal plots and the best fit of data for the Hill equation in the non-linear kinetic plots were analysed on an IBM PC/XT personal computer with the software of Symphony (Lotus Development Corp., Cambridge, MA, U.S.A.) and plotted on an Epson FX-80+ printer with the same software. The best-fit curve is obtained by non-linear regression. The regression analysis is formulated as a minimization of the regression function (Mannervik, 1981):

$$Y = \sum_{i=1}^n (v_i - v'_i)^2$$

where v_i and v'_i are the observed and calculated dependent variables respectively of the i th measurement.

RESULTS

Intrinsic phosphodiesterase activity of DNAase

The non-specific phosphodiesterase activities in many tissues and species can be measured with NPPP instead of bis-(*p*-nitrophenyl) phosphate as substrate (Kelly *et al.*, 1975; Kelly & Butler, 1975). To show that the DNAase-catalysed NPPP hydrolysis is not due to contamination by non-specific pancreatic phosphodiesterases (Terao & Ukyta, 1965) in the DNAase preparation, two experiments were carried out. First, DNAase in the major peak of the DEAE-cellulose chromatography (Fig. 1) was assayed with DNA or NPPP as substrate. Fig. 1 shows that the specific activity

is constant throughout the peak with either substrate. This type of chromatography is a method of purification utilizing Ca²⁺-affinity elution (Liao, 1974). DNAase in the major peak (Fig. 1), which was homogeneous on the basis of SDS/polyacrylamide-gel electrophoresis, was used throughout the study. Secondly, DNAase was inactivated by iodoacetate in the presence of Cu²⁺. In this process the essential His-134 residue is alkylated and the modified DNAase is inactive (Price *et al.*, 1969b; Liao *et al.*, 1973; Paudel & Liao, 1986). Fig. 2 shows that with either DNA or NPPP as substrate for monitoring

Fig. 2. Inactivation of DNAase by iodoacetate

To the DNAase solutions (1 mg/ml) in the 0.2 M-Tris/HCl buffer, pH 7.2, containing 4 mM-CuCl₂ were added various amounts of iodoacetate. At 10 min intervals samples were taken and diluted into 0.1 M-sodium acetate buffer to stop the reaction. These solutions were then assayed for enzymic activities with DNA (▼) or NPP (○) as substrate. Assay conditions: for DNA hydrolysis, see Liao (1974); for NPPP hydrolysis, 20 mM-NPPP, 50 mM-MnCl₂, 0.4 ml per assay. The inactivation rate is the loss of the fractional activity per unit time, expressed as %/min.

enzyme inactivation the fraction of DNAase inactivated per unit time is the same at each iodoacetate concentration. However, one could obtain the same result if a contaminating non-specific phosphodiesterase and DNAase are inactivated by iodoacetate at an equal rate. To rule out this possibility, the extract of the acetone-dried bovine pancreatic powder containing non-specific phosphodiesterase was mixed with a purified DNAase solution and this mixture was treated with iodoacetate as defined in the legend to Fig. 2. The results were that, after treatment, the non-specific phosphodiesterase activity, measured with NPPP as substrate without metal ions, remained unchanged, whereas the DNAase activity, measured by the hyperchromicity method of Kunitz (1950), was decreased.

Effect of Mn^{2+} or Mg^{2+} on the DNAase-catalysed NPPP hydrolysis

With a constant amount of NPPP, the Michaelis constant K_m $3.7(\pm 0.2)$ mM for Mn^{2+} is calculated from the intercept of the double-reciprocal plot on the $1/[Mn^{2+}]$ axis (Fig. 3b, line A). A similar plot was obtained for Mg^{2+} with an estimated K_m value of $49(\pm 5)$ mM. A close value of K_m $2.1(\pm 0.2)$ mM for Mn^{2+} with TDNPP as substrate is estimated from data in Fig. 3 of Liao (1975). On the basis of these kinetic data it is concluded that the activation by metal ions of the DNAase-catalysed NPPP or TDNPP hydrolysis is due to binding of Mn^{2+} or Mg^{2+} at or near the active centre of the enzyme. This metal-ion-binding site is thus termed the catalytic metal ion site.

Effect of Ca^{2+} on the DNAase-catalysed NPPP hydrolysis

The DNAase-catalysed NPPP hydrolysis was not detectable when the reaction was activated by Ca^{2+} alone. However, Ca^{2+} is a competitive inhibitor of Mn^{2+} , as shown in Fig. 3(a). The double-reciprocal plots of the reactions with (line B) and without (line A) 50 mM- $CaCl_2$ intersect at the same point on the $1/v$ axis. To compensate for the increase in ionic strength due to the 50 mM- $CaCl_2$, the reactions without $CaCl_2$ (line A) were measured in the presence of 0.15 M-NaCl. From Fig. 3(a) the Michaelis constants K_m $3.4(\pm 0.4)$ mM and $1.9(\pm 0.2)$ mM for Mn^{2+} with and without 50 mM- $CaCl_2$ respectively are calculated (the slightly lower value of K_m with 0.15 M-NaCl than K_m 3.7 mM without NaCl is discussed below). The competitive inhibition constant, K_i 64 mM for Ca^{2+} , is estimated by using the two K_m values on the basis of the formula derived by Segel (1976).

It should be noted that for all the data obtained, unless otherwise noted, 1 mM- $CaCl_2$ was included in the assay buffer. In the complete absence of Ca^{2+} rates of the DNAase-catalysed NPPP hydrolysis were decreased by about 10%. A similar 10% decrease was also noted with TDNPP as substrate (Liao, 1975).

Effect of Cu^{2+} on the DNAase-catalysed NPPP hydrolysis

As shown by Price *et al.* (1969b), the binding of either Mn^{2+} or Cu^{2+} to the DNAase molecule at or near the active centre is required for inactivation of DNAase by iodoacetate. If this metal-ion-binding site is the above-mentioned catalytic metal-ion site, one should observe Cu^{2+} competing with Mn^{2+} for NPPP hydrolysis.

Fig. 3. Inhibition by Cu^{2+} and Ca^{2+} against Mn^{2+} on the DNAase-catalysed NPPP hydrolysis

The reciprocal of the NPPP hydrolysis rate ($1/v$) is plotted as a function of the reciprocal of the Mn^{2+} concentration. Assay conditions: for panel (a), line A, 10 mM-NPPP, 0.15 M-NaCl, 0.02 mg of DNAase, 0.4 ml per assay; for panel (a), line B, 10 mM-NPPP, 50 mM- $CaCl_2$, 0.02 mg of DNAase, 0.4 ml per assay; for panel (b), line A, 10 mM-NPPP, 0.02 mg of DNAase, 0.4 ml per assay; for panel (b), line B, 10 mM-NPPP, 0.4 mM- Cu^{2+} , 0.02 mg of DNAase, 0.4 ml per assay.

However, Fig. 3(b) shows that the double-reciprocal plots of the Cu^{2+} -inhibited (line B) and -uninhibited (line A) reactions intersect to the left of the $1/v$ axis. The kinetics of this type are usually considered as the mixed-type inhibition or a form of non-competitive inhibition, and the inhibitor, Cu^{2+} , must bind to a site other than the active site (Segel, 1976). On the basis of the formulae derived by Segel (1976), the two dissociation constants, K_i 0.3 mM for the complex of Cu^{2+} and the free DNAase and αK_i 1.3 mM for the complex of Cu^{2+} and the Mn^{2+} -DNAase, are calculated by using the slopes of the plots and the intercepts on the two axes (Fig. 3b). The factor α is approximately 4, indicating that the affinity of the free DNAase for Cu^{2+} is much higher than that of the Mn^{2+} -DNAase. Plots similar to those shown in Fig. 3(b) were obtained when Mg^{2+} was used instead of Mn^{2+} .

When the $CaCl_2$ concentration in the assay buffer was increased from 0 to 10 mM, the inhibition kinetics of NPPP hydrolysis of Cu^{2+} , in the range of 0.1–5 mM, were unchanged. As shown by Price (1975), the two high-affinity Ca^{2+} -binding sites, with an average dissociation constant of about 0.01 mM (Price, 1972), are essential to the enzymic activity. At 10 mM- Ca^{2+} the high-affinity Ca^{2+} -binding sites (Price, 1975) must be saturated with Ca^{2+} , yet Ca^{2+} does not affect the Cu^{2+} inhibition

kinetics, indicating that the non-competitive Cu^{2+} site is not one of the high-affinity Ca^{2+} -binding sites.

With 0.1 M-iodoacetate, pH 7.2, the rate of inactivation of DNAase is increased with an increase of the Cu^{2+} concentration and the maximum is reached at about 5 mM- Cu^{2+} ; it is decreased to about 15% of the maximal rate at 10 mM- Cu^{2+} and not detectable above 20 mM- Cu^{2+} . By using the first part of the inactivation kinetic data (0–5 mM- Cu^{2+}) and the double-reciprocal plot method (Segel, 1976), the dissociation constant of Cu^{2+} -DNAase responsible for the iodoacetate inactivation is estimated to be about 35 mM, over 100 times higher than the dissociation constant of Cu^{2+} responsible for the non-competitive inhibition (see the above K_i for Cu^{2+}). Therefore the rapid decrease in the iodoacetate inactivation rate at above 5 mM- Cu^{2+} suggests that the first Cu^{2+} ion, bound to the high-affinity non-competitive Cu^{2+} site, prevents DNAase binding of a second Cu^{2+} ion at the low-affinity iodoacetate-inactivated Cu^{2+} site.

Cu^{2+} interacts with Tris in dilute solutions, the first dissociation constant of the Cu^{2+} -Tris complex being in the 0.1 mM region (Hanlon *et al.*, 1966). In all the above assays 0.2 M-Tris was used. Thus the number of free Cu^{2+} ions in the assay buffer is less than one-thousandth the number of total Cu^{2+} ions. To examine whether the free or Tris-complexed form of Cu^{2+} was responsible for the non-competitive inhibition, the assay buffer was changed to 0.2 M-Hepes. The result was that, for the same amount of inhibition, the Cu^{2+} concentration in Hepes was also less than one-thousandth that in Tris. Therefore it is the free Ca^{2+} ion and not the Cu^{2+} -Tris complex that inhibits DNAase, and all the above dissociation constants of Cu^{2+} should be considered as the apparent constants.

Effect of NaCl and bivalent ions on DNA hydrolysis

With DNA as substrate the double-reciprocal ($1/v$ versus $1/[\text{Mn}^{2+}]$) plot was not linear (Fig. 4a). The non-linearity of the double-reciprocal plot is probably due to the small dissociation constant of the Mn^{2+} -DNA complex (Wiberg & Neuman, 1957). In other words, under the DNA-assay conditions the kinetics probably reflect the mixture of the two reactions, the DNAase-catalysed Mn^{2+} -DNA hydrolysis and the Mn^{2+} -DNAase catalysed DNA hydrolysis. If the number of Mn^{2+} ions bound to DNA could be decreased to a minimum, for example by including a relatively high concentration of NaCl (Shack & Bynum, 1959), DNA hydrolysis by DNAase would be under a situation analogous to NPPP hydrolysis, assuming that NaCl has very little effect on Mn^{2+} binding to DNAase (this in fact is true, as shown below). Indeed, when 0.5 M-NaCl was included in the assay buffer, the double-reciprocal plot became linear and the apparent K_m value was increased to $2.1(\pm 0.3)$ mM (Fig. 4b), much closer to that with NPPP as substrate (see above). Thus it is likely that the very same Mn^{2+} ion bound to DNAase is required for either the NPPP or the DNA hydrolysis.

Plots similar to those shown in Fig. 3(b) were obtained with DNA instead of NPPP as substrate. The inhibition kinetics of DNA hydrolysis again showed that Cu^{2+} is a non-competitive inhibitor.

Kinetics of NPPP hydrolysis catalysed by DNAase

Under saturation conditions for Mn^{2+} the plot of the hydrolysis rate as a function of NPPP concentration is

Fig. 4. Effect of ionic strength on the kinetic parameters of DNAase for Mn^{2+} with DNA as substrate

The DNA hydrolysis was measured in 0.1 M-Tris/HCl buffer, pH 7.2, containing 1 mM- CaCl_2 and 0.05 mg of DNA/ml. Panel (a), no NaCl; panel (b), 0.5 M-NaCl.

sigmoidal (Fig. 5). The Hill coefficient, $h = 2.0 (\pm 0.1)$, and $V_{\max}$ $0.12 (\pm 0.02)$ absorbance unit/min are obtained by computer regression analysis of the data and the substrate concentration for the half-maximal activity is 143 mM-NPPP. A similar sigmoidal curve was also obtained in the DNAase-catalysed TDNPP hydrolysis (Liao, 1975). The h value in TDNPP hydrolysis, within the standard-error estimates, is the same as that in NPPP hydrolysis, but the substrate concentration for the half-maximal activity is decreased to 12.1 mM-TDNPP [obtained from the data of Fig. 1 in Liao (1975) by the computer regression analysis].

Although the kinetics are sigmoidal, DNAase cannot be considered an allosteric enzyme of multiple subunits with multiple active sites because the enzyme in solution exists as a monomeric protein (Lindberg, 1967) containing a single essential histidine residue (Price *et al.*, 1969b) and a single catalytic Mn^{2+} site, as shown above. However, the value of $h = 2.0$ indicates that more than one NPPP molecule must be bound to a DNAase molecule before NPPP hydrolysis and that a co-operativity exists during the binding of these NPPP molecules (Segel, 1976). The amount of co-operativity will be relatively high if there are only two NPPP molecules bound per molecule of DNAase (Segel, 1976). If the co-operative interaction among NPPP molecules on the same protein does indeed exist, it may be via π -electrons of the aromatic rings of NPPP, resembling that in the stacked base-pairs of double-helical DNA (Kornberg, 1980), the biological substrate of DNAase.

The large difference between the amounts of NPPP and TDNPP required for the half-maximal activity (see

Fig. 5. Kinetics of the DNAase-catalysed hydrolysis of NPPP

The NPPP hydrolysis rates were measured as the absorbance change at 459 nm for NPPP concentrations above 50 mM and at 400 nm when below 50 mM because the NPPP solutions above 50 mM absorbed a significant amount of light at 400 nm. Assay conditions: 50 mM- MnCl_2 , 0.01 mg of DNAase, 0.4 ml per assay. The experimental points obtained from absorbance changes at 400 nm were corrected for the absorbance change at 459 nm. The curve is generated by the computer on the basis of the Hill equation, $v = (V_{\max} \times [S]^h) / (K' + [S]^h)$, with $K' = 16000$ mM, $h = 1.95$ and $V_{\max} = 0.121$ absorbance unit/min. The Hill plot (inset) is obtained by using these parameter values.

Fig. 6. Mn^{2+} -induced difference absorption spectrum of DNAase

The spectrum was obtained on a Hitachi double-beam spectrophotometer (model U-3200). Reference cell, no MnCl_2 ; sample cell, 20 mM- MnCl_2 ; scanning speed, 15 nm/min; band width, 2.0 nm; response, medium. DNAase (1 mg/ml) was in 0.2 M-Tris/acetate buffer, pH 6.0, containing 1 mM- CaCl_2 . A difficulty was encountered for measuring spectra of solutions at pH 7.2 because Mn^{2+} produced an increase in absorbance with time.

protein. However, the orientation of the stacked TDNPP molecules may differ from that of the stacked base-pairs in DNA because the phosphodiester bond of TDNPP that is cleaved is in the 3'-position (Liao, 1975) whereas the one of DNA that is cleaved is in the 5'-position (Laskowski, 1971).

Nature of the catalytic Mn^{2+} site

When NaCl was included in the assay buffer 1 M-NaCl caused a 30 % increase in the NPPP hydrolysis rate with 10 mM-NPPP and 2 mM- MnCl_2 . This NaCl effect is due to a decrease in the K_m for Mn^{2+} rather than an increase in $V_{\max}$. As shown in Fig. 3 [compare panel (a), line A, with panel (b), line A], 0.15 M-NaCl can cause the K_m value to change from 3.7 mM to 1.9 mM. Thus the charge interaction probably does not play an important role in the binding of Mn^{2+} to DNAase. In fact, the Mn^{2+} -induced difference spectrum of DNAase (Fig. 6) shows two major positive peaks, one at 275 nm and the other 282 nm. The spectrum is similar to that of the Ca^{2+} -induced difference spectrum (Poulos & Price, 1972). On the basis of the observations reported by Yanari & Bovey (1960), this shift in the spectrum is characteristic of aromatic amino acid residues that are exposed to a more hydrophobic environment. Thus the catalytic metal ion site is probably located at or near Trp-155 and Tyr-172, the two crucial aromatic amino acid residues of DNAase (Sartin *et al.*, 1980; Liao *et al.*, 1982). The binding of Mn^{2+} shields these side chains from the aqueous environment.

DISCUSSION

From the foregoing descriptions of the metal ion activation of DNAase-catalysed hydrolyses of NPPP and DNA, one may outline the reaction scheme for the enzyme shown in Scheme 1. In this scheme E is DNAase and Ca and Mn are the bound forms of Ca^{2+} and Mn^{2+} respectively. Reaction 2, representing the Ca^{2+} binding to DNAase, produces a protein conformation change (Poulos & Price, 1972; Tullis & Price, 1974), protects DNAase from inactivation by proteinases (Price *et al.*, 1969a) and chemicals (Price *et al.*, 1969c; Hugli & Stein, 1971; Plapp *et al.*, 1974; Poulos & Price, 1974) and facilitates inactivation of DNAase by 2-nitro-5-thiocyanobenzoic acid (Liao & McKenzie, 1979) and *p*-nitrobenzenesulphonyl fluoride (Liao *et al.*, 1982). Whether Ca^{2+} is absolutely required for DNAase activity is under debate. In the complete absence of Ca^{2+} the NPPP and DNA hydrolysis activities of DNAase with Mn^{2+} are measurable. However, as compared with those with Ca^{2+} plus Mn^{2+} , DNA hydrolysis is decreased by one-half to two-thirds (Junowicz & Spencer, 1973; Price, 1975) and NPPP hydrolysis by 10 %.

Under the DNA-assay conditions of Kunitz (1950) and many others (Shack & Bynum, 1964; Melgar & Goldthwait, 1968; Eichhorn *et al.*, 1969; Junowicz & Spencer, 1973), the normal pathway of reactions 3 and 6 leading to the formation of quaternary complex (Ca-E-Mn-DNA) is predominant. The alternative pathway becomes important when reaction 6 is blocked with a relatively high concentration of NaCl. In this alternative pathway the Ca^{2+} -bound DNAase (Ca-E) binds Mn^{2+} (reaction 2) before the binding of the metal-ion-free DNA (reaction 4). The common quaternary complex (Ca-E-Mn-DNA) formed by either pathway is irre-

above) is expected, as TDNPP contains thymidine in its structure whereas NPPP has little resemblance to a nucleotide except for the phosphodiester bond. The much lower amount of TDNPP required for the half-maximal activity suggests that stacked TDNPP molecules are more stable than stacked NPPP molecules on the

Scheme 1. Proposed reaction pathways of the hydrolysis of DNA and NPPP catalysed by DNAase

versibly hydrolysed to products (reaction 5). It is thus evident that the metal ion may function to bridge the enzyme and substrate. Owing to the difficulty for the univalent NPPP to form a complex with Mn^{2+} , only the alternative pathway (reactions 2 and 4) can operate with NPPP. The reaction in which the free DNAase acts on the metal ion–DNA, as previously suggested for the DNAase mechanism (Wiberg, 1958), is only part of the reaction scheme.

According to Scheme 1, Mn^{2+} should bind to either the substrate or enzyme. Therefore one should see inhibition of DNA hydrolysis at relatively high Mn^{2+} concentrations, where significant amounts of Ca–E–Mn and Mn–DNA are formed. In fact, auto-inhibition at above 50 mM- Mn^{2+} has been observed by us, and by others with other metal ions (Junowicz & Spencer, 1973; Price, 1975).

Not shown in Scheme 1 is the binding of Cu^+ at the non-competitive Cu^{2+} site, leading to inactive forms of DNAase. The non-competitive Cu^{2+} site is not one of the initial activating Ca^{2+} -binding sites as Ca^{2+} cannot relieve the inhibition caused by Cu^{2+} . The binding of Cu^{2+} affects the binding of Mn^{2+} or Mg^{2+} at the catalytic metal-ion site and renders the enzyme inactive. The possibility of DNAase having this Cu^{2+} site was previously suggested but not demonstrated (Eichhorn *et al.*, 1969).

Thus it is concluded that there is a catalytic metal-ion site at or near the active centre of the DNAase molecule. To date Mn^{2+} ion is known to be the metal ion with the highest affinity. Since the site has not yet been located by X-ray crystallography (Suck *et al.*, 1984; Suck & Oefner, 1986), it is difficult to visualize how the metal ion functions during the enzymic catalysis. The metal ion may interact with the enzyme via co-ordination and may actually participate in the catalytic reaction.

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