Active-site characterization of S1 nuclease II

Involvement of histidine in catalysis

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Modification of the histidine residues of purified S1 nuclease resulted in loss of its single-stranded (ss)DNAase, RNAase and phosphomonoesterase activities. Kinetics of inactivation indicated the involvement of a single histidine residue in the catalytic activity of the enzyme. Furthermore, histidine modification was accompanied by the concomitant loss of all the activities of the enzyme, indicating the presence of a common catalytic site responsible for the hydrolysis of ssDNA, RNA and 3'-AMP. Substrate protection was not observed against Methylene Blue- and diethyl pyrocarbonate (DEP)-mediated inactivation. The histidine (DEP)-modified enzyme could effectively bind 5'-AMP, a competitive inhibitor of S1 nuclease, whereas the lysine (2,4,6-trinitrobenzenesulphonic acid)-modified enzyme showed a significant decrease in its ability to bind 5'-AMP. The inability of the substrates to protect the enzyme against DEP-mediated inactivation, coupled with the ability of the modified enzyme to bind 5'-AMP effectively, suggests the involvement of histidine in catalysis.

INTRODUCTION

Single-strand specific nuclease from *Aspergillus oryzae* (S1 nuclease, EC 3.1.30.1) is an analytically important enzyme, used extensively for the characterization of nucleic-acid structure (Rushizky, 1981). However, very little information is available regarding the nature of its active-site. Recently, we have shown the involvement of lysine in the catalytic activity of S1 nuclease (Gite *et al.*, 1992). In the case of RNAases such as RNAase T1 (Irie, 1970; Takahashi, 1971), RNAase T2 (Kawata *et al.*, 1990) and RNAase A (Gundlach *et al.*, 1959; Crestfield *et al.*, 1963a,b), as well as in pancreatic DNAase (Price *et al.*, 1969), histidine has been implicated in the active site of the enzyme. Since S1 nuclease also acts on single-stranded DNA (ssDNA) and RNA, chemical modification of histidine was carried out to evaluate its role in the catalytic activity of the enzyme, the results of which are presented in this paper.

MATERIALS AND METHODS

Materials

Bio-Gel P-10 (Bio-Rad, Richmond, CA, U.S.A.); RNA (Sisco Research Laboratories, Bombay, India); Methylene Blue and hydroxylamine hydrochloride (BDH, Bombay, India); diethyl pyrocarbonate (DEP), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 3'-AMP, 5'-AMP, imidazole, *N*-acetylimidazole and BSA (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used. All other chemicals used were of analytical grade. High-molecular-mass DNA from buffalo liver was isolated according to the method of Mehra & Ranjekar (1979).

Enzyme assays

The ssDNAase and RNAase activities of S1 nuclease were determined as described earlier (Gite *et al.*, 1992). The amounts of acid-soluble nucleotides liberated following the hydrolysis of ssDNA or RNA, at pH 4.6 and 37 °C, were calculated by assuming a molar absorption coefficient of 10000 M⁻¹·cm⁻¹ and

10600 M⁻¹·cm⁻¹ for deoxyribonucleotide and ribonucleotide mixtures respectively (Curtis *et al.*, 1966). One unit of ssDNAase or RNAase activity is defined as the amount of enzyme required to liberate 1 μmol of acid-soluble nucleotides per minute under the assay conditions.

The phosphomonoesterase activity of S1 nuclease was assayed by measuring the amount of inorganic phosphate liberated following the hydrolysis of 3'-AMP, at pH 4.6 and 37 °C (Gite *et al.*, 1992). One unit of phosphomonoesterase activity is defined as the amount of enzyme required to liberate 1 μmol of inorganic phosphate per minute under the assay conditions.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951), using BSA as a standard.

Purification of S1 nuclease

S1 nuclease was purified to homogeneity as reported previously (Gite *et al.*, 1992).

Chemical modification studies

In chemical modification studies, the residual activity of the modified enzyme was determined using all three substrates: i.e. ssDNA, RNA and 3'-AMP. Unless otherwise mentioned, all the modification reactions were carried out at room temperature (26 ± 1 °C).

Photo-oxidation. This was carried out by exposing 200 μg of the purified enzyme, in 1 ml of 50 mM-sodium maleate buffer, pH 7.5, in a glass test-tube (10 mm × 100 mm) containing different concentrations of Methylene Blue, to a 200 W flood-light bulb held at a distance of 12 cm for 30 min, followed by estimation of the residual activities. Enzyme samples treated under identical conditions, but in the dark, served as the control.

Reaction with DEP. S1 nuclease (200 μg), in 1 ml of 50 mM-sodium maleate buffer, pH 6.8, was incubated for 20 min with various concentrations of DEP, freshly diluted with absolute ethanol. Aliquots were withdrawn at suitable intervals and the

Abbreviations used: ssDNA, single-stranded DNA; TNBS, 2,4,6-trinitrobenzenesulphonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DEP, diethyl pyrocarbonate.

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reaction was arrested by the addition of 10 μ l of 10 mM-imidazole, pH 7.5. Subsequently, the residual activities were determined under standard assay conditions. Enzyme samples incubated in the absence of DEP served as controls. The DEP concentration in the diluted samples was determined by mixing an aliquot of the diluted sample with 3 ml of 10 mM-imidazole (pH 7.5), followed by monitoring of the increase in the absorbance at 230 nm. The amount of *N*-carbethoxyimidazole formed was calculated by using a molar absorption coefficient of $3000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Melchior & Fahrney, 1970). The concentration of the diluted stock DEP solution was 10 mM. The ethanol concentration in the reaction mixture did not exceed 2% (v/v) and had no effect on the activity and stability of the enzyme during the incubation period. S1 nuclease modification by DEP was also monitored spectrophotometrically by measuring the change in absorbance at 240 nm as described by Ovadi *et al.* (1967).

Reaction with hydroxylamine. Decarboxylation was carried out according to the method of Miles (1977). Samples of DEP-modified enzyme were incubated with 500 mM-hydroxylamine hydrochloride at pH 7.0 and 4 °C for 15 h and the enzyme activities were determined under standard assay conditions.

Reaction with *N*-acetylimidazole. S1 nuclease (100 μ g) in 1 ml of 50 mM-sodium borate buffer, pH 7.5, was incubated with 1 mM-*N*-acetylimidazole for 20 min followed by estimation of the residual activities under standard assay conditions. The enzyme incubated in the absence of *N*-acetylimidazole was taken as the control. The number of tyrosine residues modified was calculated by using a molar absorption coefficient of $1160 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 278 nm (Means & Feeney, 1971).

Reaction with DTNB. The enzyme (100 μ g), in 1 ml of 50 mM-Tris/HCl buffer, pH 7.9, was incubated with 2 mM-DTNB for 20 min and the residual activities were determined under standard assay conditions. Enzyme incubated in the absence of DTNB served as control. The number of cysteine residues modified were determined at 412 nm, using a molar absorption coefficient of $13600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Means & Feeney, 1971).

Substrate protection. The effect of substrate protection was studied by preincubating the enzyme with an excess of ssDNA, RNA and 3'-AMP, followed by treatment with the modifying reagents.

Inhibitor binding studies. The inhibitor binding studies on native and modified enzyme samples were carried out according to Hummel & Dreyer (1962). The DEP-modified enzyme (200 μ g) in 1 ml of 30 mM-sodium acetate buffer, pH 4.6, (containing 1 mM- ZnSO_4 , 50 mM-NaCl, 5% (v/v) glycerol and 20 μ M-5'-AMP), was passed through a Bio-Gel P-10 column (1 cm $\times$ 25 cm) equilibrated with the above buffer, at a flow rate of 0.4 ml/min. Fractions (2 ml) were collected and the absorbance at 260 nm was measured. Unmodified enzyme subjected to similar treatment was taken as control. For comparison, an identical experiment was carried out with the TNBS-modified enzyme. TNBS modification was carried out as described previously (Gite *et al.*, 1992).

C.d. measurement

C.d. measurements were carried out on a Jasco J-500 A spectropolarimeter, at 20 °C, in the range 200–240 nm.

RESULTS

Purified S1 nuclease, when subjected to photo-oxidation in presence of 0.2% Methylene Blue, showed a pH-dependent inactivation, and the maximum loss of activity was observed at

pH 7.5 (Fig. 1). When the enzyme was irradiated with 0.2% Methylene Blue at pH 7.5 for 30 min, it lost 70% of its initial activity towards ssDNA, RNA and 3'-AMP and the inactivation was dependent on the concentration of the reagent (Fig. 2). However, no loss of activity was observed in the controls.

Carbethoxylation of S1 nuclease at pH 6.8 for 20 min resulted in 65–75% loss of its initial activity and the inactivation was concentration-dependent. No loss of activity was observed in the control samples. The logarithm of the residual activity plotted as a function of time at various DEP concentrations was linear up to 27%, 24% and 34% of the initial activity towards ssDNA, RNA and 3'-AMP respectively (Fig. 3). The DEP-mediated inactivation followed pseudo-first-order kinetics at any fixed concentration of reagent. The pseudo-first-order rate constants

Fig. 1. Effect of pH on photo-oxidation of S1 nuclease

The enzyme (100 μ g/ml) was incubated at different pH (4.5–8.0) in the presence of 0.2% Methylene Blue at room temperature for 30 min as described in the Materials and methods section. An identical sample at each pH value was kept in the dark to serve as a control. Enzyme activity was measured using ssDNA as the substrate.

Fig. 2. Effect of Methylene Blue concentration on the residual activity of purified S1 nuclease

Purified enzyme (200 μ g) was incubated at pH 7.5 at room temperature with various concentrations of Methylene Blue for 30 min as described in the Materials and methods section. Key to symbols: ●, ssDNAase; ▲, RNAase; ×, phosphomonoesterase.

Fig. 3. Pseudo-first-order plots for the inactivation of S1 nuclease by DEP (a), ssDNAase; (b), RNAase; (c), phosphomonoesterase. Concentrations of DEP were: 0 mM (●), 0.05 mM (▲), 0.1 mM (×), 0.15 mM (○) and 0.2 mM (■). Insets: second-order plots of the pseudo-first-order rate constants of inactivation (k_{app}) (min^{-1}) at different concentrations of DEP.

Fig. 4. Plot of percentage residual activity versus number of histidine residues modified. The number of histidine residues modified was estimated as described in the text. ssDNAase (●), RNAase (▲) and phosphomonoesterase (×).

were calculated from the slope of plots of log (percentage residual activity) versus reaction time, and the order was determined from the slope of the plots of $\log(k_{app})$ against $\log[\text{DEP}]$. These plots (insets, Fig. 3) indicated that the loss of enzyme activity towards all three of the substrates occurred as a result of modification of a single histidine residue per molecule of

Table 1. Effect of different modifying reagents on the activity of S1 nuclease

Modification reaction	Number of residues modified	Residual activity (%)		
		ssDNAase	RNAase	AMPase*
Control	0	100	100	100
Histidine (DEP)	1.0	38	35	38
Decarboxylation (hydroxylamine)	—	86	82	90
Tyrosine (<i>N</i> -acetylimidazole)	6.0	100	100	100
Cysteine (DTNB)	1.0	100	100	100

* Phosphomonoesterase activity.

the enzyme. Furthermore, carbethoxylation of the enzyme, as a result of DEP treatment, was accompanied by an increase in the absorbance of the modified protein at 240 nm. Based on a molar absorption coefficient of $3200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for carbethoxyhistidine at 240 nm (Ovadi *et al.*, 1967) and an M_r for S1 nuclease of 32000 (Iwamatsu *et al.*, 1991; Gite *et al.*, 1992), the total number of histidine residues modified was found to be 1.6. However, the plot of percentage residual activity versus the number of histidine residues modified revealed that the loss of activity towards ssDNA, RNA and 3'-AMP, resulted from the modification of a single histidine residue (Fig. 4). Incubation of the DEP-modified enzyme with 500 mM-hydroxylamine at pH 7.0 and 4 °C for 15 h restored 82–90% of its original activity (Table 1).

Methylene Blue- and DEP-mediated inactivation of S1 nuclease could not be prevented by incubating the enzyme with excess amounts of ssDNA, RNA and 3'-AMP before the modification reaction. In addition, the histidine-modified enzyme, which had very little catalytic activity, could bind 5'-

Table 2. Influence of histidine modification on the activity of S1 nuclease: substrate protection and inhibitor binding studies

For experimental details please refer to the Materials and methods section. Abbreviation: MB, Methylene Blue.

Incubation mixture	Residual activity (%)	5'-AMP binding (%)
Enzyme	100	100
Enzyme + TNBS (0.5 mM)	32	33
Enzyme + DEP (0.2 mM)	30	100
Enzyme + ssDNA (1 mg) + DEP	32	—
Enzyme + RNA (1 mg) + DEP	30	—
Enzyme + 3'-AMP (1 mM) + DEP	32	—
Enzyme + MB (0.2 %)	43	—
Enzyme + ssDNA (1 mg) + MB	46	—
Enzyme + RNA (1 mg) + MB	50	—
Enzyme + 3'-AMP (1 mM) + MB	50	—

Fig. 5. The c.d. spectra of S1 nuclease

The c.d. measurements were performed in a 1 mm cell at an enzyme concentration of 200 μ g/ml. Native enzyme (—) and DEP-treated enzyme (-----) spectra are shown.

AMP in a ratio of 1:1, similar to that of the unmodified enzyme. The lysine-modified enzyme, on the other hand, showed a decrease of approx. 70 % in its ability to bind 5'-AMP (Table 2). Moreover, the c.d. spectra of both unmodified and histidine-modified S1 nuclease were almost identical (Fig. 5), indicating that modification does not result in a gross change in the conformation of the enzyme.

Modification of tyrosine by treating the enzyme with *N*-acetylimidazole, though resulting in the modification of six residues out of a total of 16 (Iwamatsu *et al.*, 1991), did not have any effect on the activity of the enzyme, suggesting that tyrosine may not have a role in the catalytic activity of S1 nuclease (Table 1). Similarly, modification of the cysteine residues of the enzyme (by treating the enzyme with DTNB), though resulting in the modification of the only available cysteine residue (Iwamatsu *et al.*, 1991), did not affect the activity of the enzyme, showing that cysteine too may not have a role in the catalytic activity of S1 nuclease (Table 1).

DISCUSSION

Since histidine has been implicated in the catalytic activity of several nucleases and DNA polymerase I (Pandey *et al.*, 1987), modification of histidine was carried out to evaluate its role in the catalytic activity of S1 nuclease. When purified S1 nuclease was incubated with Methylene Blue, at pH 7.5 and 26 °C for 30 min, it resulted in a 60–70 % loss of its initial activity towards ssDNA, RNA and 3'-AMP. The inhibition of the enzyme activity could be prevented by shielding the enzyme–Methylene Blue mixture from irradiation, indicating the presence of histidine at or near the active site. The pH-dependent inactivation of the enzyme was similar to that observed in a case of photo-oxidation of free histidine (Weil, 1965) and also in the photo-inactivation of several enzymes with histidine at their active site (Westhead, 1965; Martinez-Carrion *et al.*, 1967; Chattarjee & Noltmann, 1967), suggesting the presence of histidine at or near the active site of S1 nuclease (Fig. 1).

The involvement of histidine in the catalytic activity of S1 nuclease was further ascertained by modifying the enzyme with a histidine-specific reagent, i.e. DEP. Modification of the enzyme with DEP also resulted in a significant decrease in its activity towards ssDNA, RNA and 3'-AMP, indicating that histidine may have a role in the catalytic activity of S1 nuclease. Kinetic analysis of DEP inactivation revealed that the loss of activity towards all of the substrates was caused by the modification of a single histidine residue (Fig. 3). The DEP-mediated inactivation of the enzyme was accompanied by a significant increase in the absorbance of the modified enzyme at 240 nm, which is characteristic of ethoxycarboxylation of histidine residues. Determination of the number of essential histidine residues following DEP modification indicated that the modification of a single histidine is responsible for the loss of enzyme activity towards all the substrates (Fig. 4). Hydroxylamine treatment of the DEP-modified enzyme restored a significant amount of its activity towards all the substrates, substantiating the role of histidine in the catalytic activity of S1 nuclease (Table 1).

Though DEP is specific for histidine at or around neutral pH, it also reacts, to a lesser extent, with tyrosine, cysteine and lysine residues (Miles, 1977). However, *N*-acetylimidazole treatment of purified S1 nuclease did not bring about any decrease in the enzyme activity, suggesting that tyrosine may not have a role in activity of S1 nuclease (Table 1). The modification of tyrosine, as a result of DEP treatment, was further ruled out by the observation that there was no significant decrease in the absorbance of the modified protein at 278 nm. Though the above observations support the presence of histidine at or near the active site, they still do not rule out the possible involvement of cysteine. Hence, modification of cysteine was carried out with a cysteine-specific reagent, namely DTNB. DTNB-treated enzyme retained its full activity, ruling out the involvement of cysteine in the catalytic activity of S1 nuclease. Recently, we have shown the involvement of lysine in the catalytic activity of S1 nuclease (Gite *et al.*, 1992). However, in the present case, the loss of activity by S1 nuclease as a result of DEP treatment cannot be correlated to lysine modification, since the DEP-modified enzyme could recover a significant amount of its activity in the presence of hydroxylamine. Had the inactivation of S1 nuclease been caused by lysine modification, then hydroxylamine treatment would not have restored its activity.

Studies on substrate protection revealed that Methylene Blue- and DEP-mediated inactivation could not be prevented by incubation of the enzyme in the presence of excess amounts of ssDNA, RNA and 3'-AMP, prior to the modification reaction (Table 2). Additionally, DEP modification did not bring about any gross change in the enzyme structure, indicating that the loss

of enzyme activity is due to histidine modification rather than structural changes (Fig. 5). Through inhibitor binding studies on TNBS-modified S1 nuclease, we have shown that lysine is involved in the substrate binding (Gite *et al.*, 1992). In the present studies, the DEP-modified S1 nuclease fully retained its ability to bind 5'-AMP, a competitive inhibitor of the enzyme, whereas the TNBS-modified enzyme showed a significant decrease in its ability to bind 5'-AMP, under identical conditions (Table 2). These results point towards the involvement of histidine in catalysis rather than in substrate binding.

Recently, through lysine modification of S1 nuclease, we demonstrated the existence of a common catalytic site for the hydrolysis of both mononucleotides and polynucleotides (Gite *et al.*, 1992). In the present studies, the parallel loss of ssDNAase, RNAase and phosphomonoesterase activities after histidine modification, and their restoration on hydroxylamine treatment, confirm the presence of a common catalytic site for the hydrolysis of both monomeric and polymeric substrates.

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