

Chemical Modifications and Kinetic Study of Ribonuclease Sa Active Site

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Abstract. Chemical modification experiments and kinetic measurements have been carried out to identify the active site components of guanylspecific ribonuclease Sa (E. C. 3.1.4.8.) from *Streptomyces aureofaciens*. Modification experiments with phenylglyoxal and diketene showed that neither arginine nor lysine residues, which could bind the negatively charged phosphate group of the substrate, belonged to the active site of the enzyme. The pH dependence of the kinetic constants k_{cat} and K_m in the hydrolysis of Guo-P-Cyd and Guo-P-Cyd-P with ribonuclease Sa indicated that a histidine residue could have the above mentioned function. Remarkable reduction (87 per cent) of the enzyme activity after modification with tetranitromethane was found. With these properties ribonuclease Sa considerably differs from ribonuclease T₁.

Key words: Guanyloribonuclease Sa — Active site — Chemical modifications — Kinetic measurements

Introduction

Ribonuclease Sa (E.C. 3.1.4.8) is an extracellular enzyme of *Streptomyces aureofaciens* isolated from the cultivation medium (Bačová et al. 1971). This enzyme catalyzes the cleavage of internucleotide bonds in RNA and in oligonucleotides only between guanosine-3'-phosphate and 5'-OH group of the ribose of the adjacent nucleotide. The first step is transphosphorylation and the product of this step is an oligonucleotide terminated by guanosine-2',3'-cyclic phosphate. In the second step the guanosine-2',3'-cyclic phosphate is hydrolysed to guanosine-3'-phosphate as a final product (Zelinková et al. 1971). With these properties

Abbreviations: Guo-3' P, Guanosine 3'-phosphate; Guo-2' P, Guanosine 2'-phosphate; Guo, Guanosine; Guo-P-Cyd, Guanylyl-3', 5'-Cytidine; Guo-P-Cyd-P, Guanylyl-3', 5'-Cytidine-3'-phosphate; Ino-3'-P, Inosine-3'-phosphate; Ado-3'-P, Adenosine-3'-phosphate.

the enzyme belongs to a group of guanyloribonucleases among which ribonuclease T₁ from *Aspergillus oryzae* (Egami et al. 1964) is the best known. The molecular weight of ribonuclease Sa is 10 000 (Gašperík 1980).

Recently, guanyloribonucleases have been intensively studied as suitable objects for the study of molecular basis of specific enzyme action because of their narrow specificity, good stability and low molecular weight (Bezborodova and Bezborodov 1979). This paper deals with the identification of ribonuclease Sa active site components and with the characterisation of interactions between the active site and ligands.

Material and Methods

Ribonuclease Sa was isolated and purified to a chromatographically pure state according to Bačová et al. (1971) slightly modified by applying rechromatography on phosphocellulose. For experiments lyophilized samples of the enzyme with specific activity around 200 000 units/mg were used. Substrates Guo-P-Cyd and Guo-P-Cyd-P were from Zellstoff (Waldhof, F.R.G.) and inhibitors Guo, Guo-2'-P, Guo-3'-P, Ino-3'-P and Ado-3'-P were obtained from Sigma (Missouri, U.S.A.). Other reagents were of p. a. grade.

Inactivation of ribonuclease Sa by inactivators was determined by detection of the enzyme activity change after 24 hours of incubation of the enzyme-inactivator mixture at 37°C. In the case of tetranitromethane the samples for activity determinations were taken also after 5, 10, 30 and 60 minutes and 30 hours of incubation. Free RNase incubated at the same conditions served as the standard. The degree of inactivation was determined from the ratio of the activity of the inactivated enzyme and the standard. For determination of the enzyme activity the method of Egami et al. (1964) was used. The composition of buffers and their pH values were chosen according to Means and Feeney (1971).

For kinetic measurements the stock enzyme solution was prepared by dissolving the lyophilized enzyme sample in 0.2 mol.l⁻¹ NaCl in redistilled H₂O. The concentrations of the stock substrate solutions were determined spectrophotometrically using the known molar extinction coefficients of the substrates. As a buffer a solution of 0.2 mol.l⁻¹ DMG — Tris (0.1 mol.l⁻¹ dimethylglutaric acid — 0.1 mol.l⁻¹ Tris and concentrated HCl to adjust the pH) was used. The temperature of the reaction

Table 1. Composition of reaction mixtures for inactivation of ribonuclease Sa

Inactivator	Inactivator conc. (mol/l)	Enzyme conc. (mol/l)	Buffer (mol/l)	pH
Iodoacetate	3.3×10^{-4}	3.3×10^{-7}	DMG-Tris 0.05	1.5—8.5
Tetranitromethane	2×10^{-2}	2×10^{-5}	Tris-HCl 0.1	8.5
Phenylglyoxal	3.3×10^{-4}	3.3×10^{-7}	Tris-HCl 0.1	7.8
Diketene	2.6×10^{-2}	2.6×10^{-5}	Tris-HCl 0.1	8.5

mixture was kept at $25 \pm 0.1^\circ\text{C}$. The measurements were carried out by spectrophotometric detection of the time course of guanylic substrate splitting at 280 nm using the Varian Cary spectrophotometer model 15 (Cary Instruments, U.S.A.). To determine the kinetic constants at given pH value, 6–8 progress curves were registered at different initial concentrations of the substrate. Kinetic constants k_{cat} and K_m were obtained from plots of Lineweaver and Burk (1934). As a substrate for inhibition constant measurements Guo-P-Cyd was used.

Results

Chemical modifications. The aim of chemical modifications was the selective blocking of functional groups of amino acid residues of ribonuclease Sa. The participation of these residues in the active site was estimated from the activity change of the modified enzyme.

Composition of the reaction mixtures for inactivation is given in Table 1. Since the 1000-fold molar excess of diketene and phenylglyoxal did not cause any inactivation of ribonuclease Sa, this molar excess was used for all inactivators. In Fig. 1 the pH dependence of inactivation of ribonuclease Sa by iodoacetate is

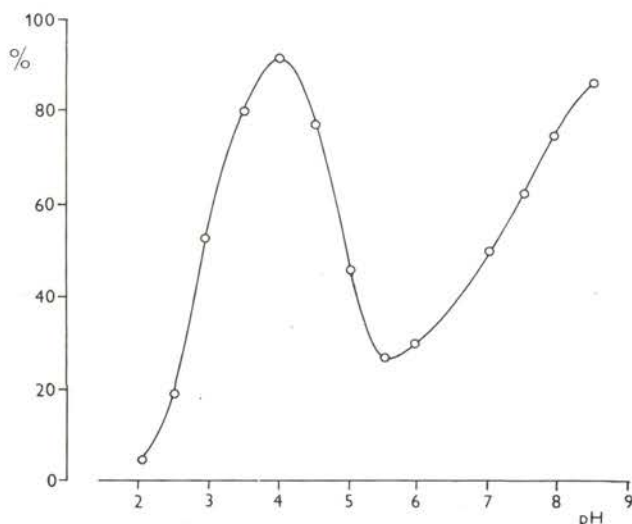


Fig. 1. pH dependence of inactivation by iodoacetate

shown. This dependence has a maximum at pH 4 and a minimum around pH 5.5. When the enzyme was incubated with tetranitromethane, 13% of residual enzyme activity was still observed after 30 hours of incubation (Fig. 2). The results of further modification experiments are presented in Table 2 and it can be seen that diketene and phenylglyoxal did not cause any inactivation of ribonuclease Sa.

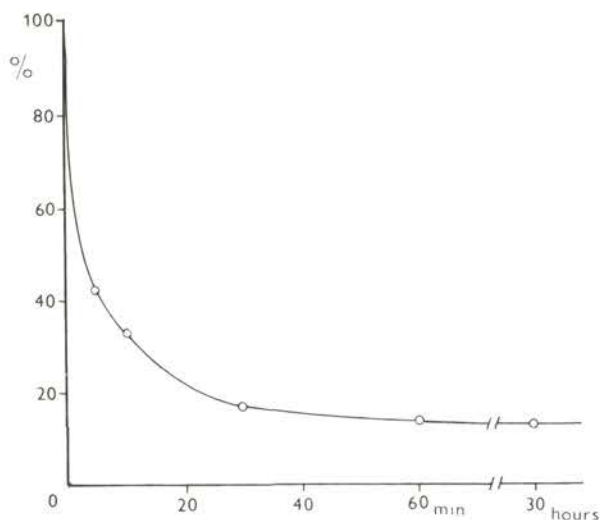


Fig. 2. Time dependence of inactivation by tetranitromethane

Table 2. Modification reactions of ribonuclease Sa

Inactivator	Inactivation	Residue to be modified
Iodoacetate	+	?
Tetranitromethane	+	Tyr
Phenylglyoxal	—	Arg
Diketene	—	Lys

Kinetic measurements. pH dependence, of kinetic constants k_{cat} and K_m in the reaction of Guo-P-Cyd and Guo-P-Cyd-P hydrolysis was used to estimate the ionisation constants of those groups of the enzyme which are responsible for its activity. In the case of Guo-P-Cyd (Fig. 3) the course of k_{cat} is typically bellshaped in the range of pH from 4.5 to 6 and a small perturbation occurs in the pH range from 6 to 7.5. The value of K_m is approximately constant in the range from pH 4 to pH 7, however, it considerably increases above pH 7. The pH optimum of Guo-P-Cyd hydrolysis is pH 5.0. Similar courses of k_{cat} and K_m are seen in Fig. 4 for Guo-P-Cyd-P, but the next phosphate group at Guo-P-Cyd-P shifts the pH optimum of k_{cat} to pH 6.

The pH dependence of the inhibition constant K_i for Guo-2'-P and Guo-3'-P using Guo-P-Cyd as substrate is demonstrated in Fig. 5. Within the investigated pH interval Guo-3'-P is a stronger inhibitor than the 2' isomer of this nucleotide. Optimum inhibition is again at pH 5, as for hydrolysis of Guo-P-Cyd.

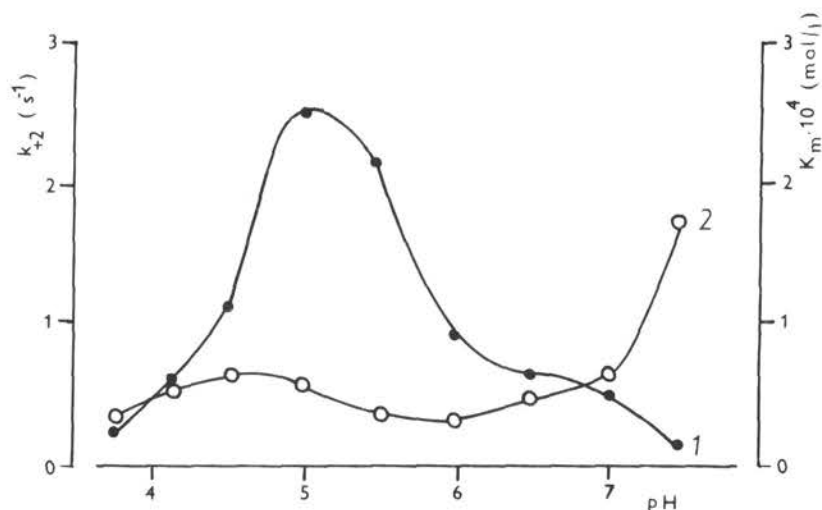


Fig. 3. pH dependence of k_{cat} (1) and K_m (2) for the hydrolysis of Guo-P-Cyd

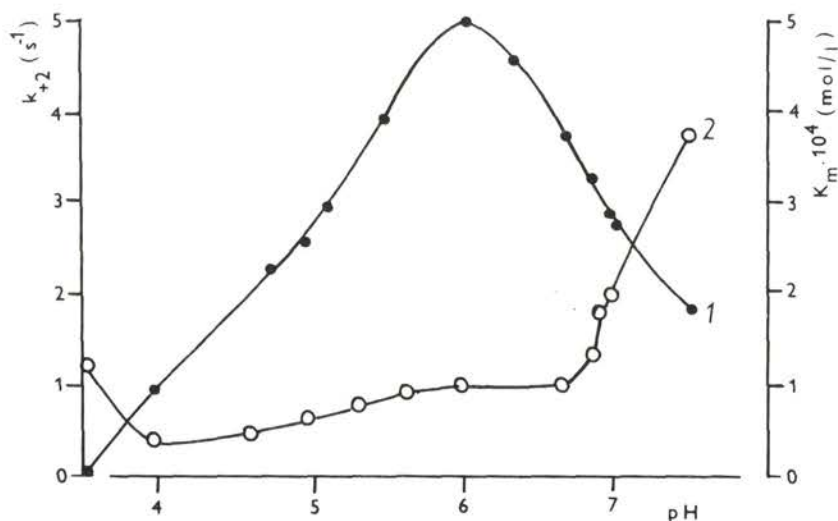


Fig. 4. pH dependence of k_{cat} (1) and K_m (2) for the hydrolysis of Guo-P-Cyd-P

In order to explain the character of interactions between the nucleotide and enzyme, the inhibition constants of some purine nucleotides and Guo were studied for the hydrolysis of Guo-P-Cyd (Table 3). All ligands are competitive inhibitors of this reaction. The enzyme shows the highest affinity to the guanylic base, the lowest to the adenylic one.

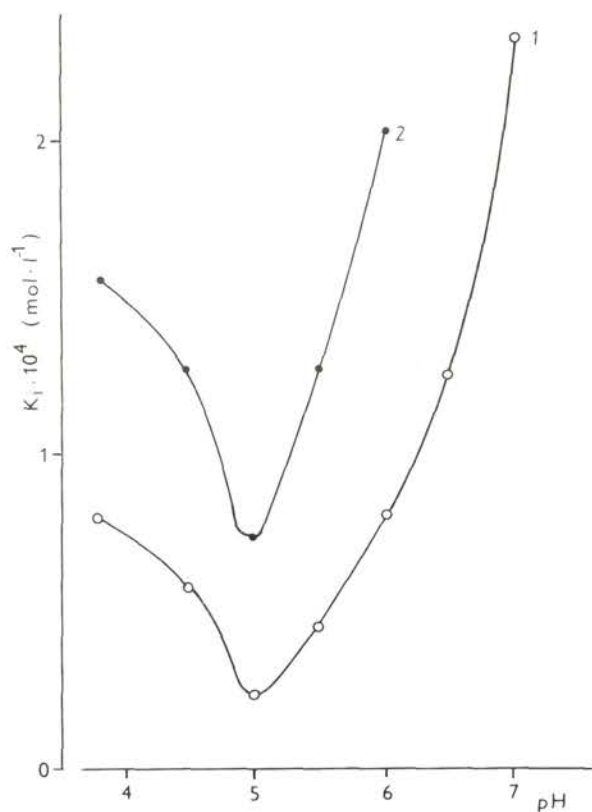


Fig. 5. pH dependence of K_i for Guo-3' P (1) and Guo-2' P (2)

Table 3. Inhibition constants of purine nucleotides for hydrolysis of Guo-P-Cyd by ribonuclease Sa at pH 5.

Inhibitor	K_i (10^{-5} mol/l)	Inhibitor	K_i (10^{-5} mol/l)
Guo-3'-P	2.2	Ino-3'-P	190
Guo-2'-P	7.1	Ado-3'-P	570
Guo	35		

Discussion

For the interpretation of the results of our modification and kinetic experiments we used the results of analogous studies on ribonuclease T₁.

The difference between pH dependence of ribonuclease T₁ inactivation by iodoacetate (Pongs 1968) and that of ribonuclease Sa by the same reagent indicates

that the composition of the active site components in these enzymes is different. Moreover, the carboxymethylated ribonuclease T_1 can be reactivated by a short treatment with $0.1 \text{ mol.l}^{-1} \text{ NaOH}$ (Takahashi 1967) while under these conditions the reactivation of ribonuclease Sa was not achieved, although native ribonuclease Sa retains activity under the same conditions. Then, it is questionable, whether in the case of ribonuclease Sa the carboxymethylation at pH 4 occurs at a carboxylic group of the enzyme. However, it cannot be excluded that in the carboxymethylated species a subsequent conformational change occurs preventing reactivation. Takahashi (1967) showed that the carboxymethylated residue of ribonuclease T_1 is Glu 58.

According to Takahashi (1968) phenylglyoxal, a specific reagent for arginine, inactivates ribonuclease T_1 . He found that phenylglyoxal blocks Arg 77 which binds the anionic phosphate group of the substrate (Takahashi 1970). In contrary to RNase T_1 , the activity of ribonuclease Sa is not affected by this reagent. Similarly diketene, a specific reagent for the second basic amino acid, lysine, does not inactivate ribonuclease Sa, either. Since we have eliminated arginine and lysine as the positively charged groups binding the anionic phosphate group, the increase of K_m at pH above 7 could indicate that histidine has this function.

Remarkable reduction of ribonuclease Sa activity after the treatment with tetranitromethane, an agent nitrating tyrosine, indicates that tyrosine could be a part of the active site. The residual 13% activity suggests that tyrosine plays only an auxiliary role in the active site. Tyrosine could be the hydrophobic site for the interaction of the base with the enzyme.

Differences in affinity of different purine nucleotides to ribonuclease Sa show that there are specific interactions between the nucleotide base and functional groups of the active site. These specific interactions are performed most probably through the formation of H-bridges between the donor — acceptor groups of the enzyme and the heterocyclic base. Comparison of inhibition constants for Guo-3'-P and Ino-3'-P shows that the exchange of NH_2 group in position 2 of the base for hydrogen atom causes a very remarkable decrease of nucleotide affinity to the enzyme. It does not mean that this group is directly involved in binding since also the basicity at N — 1 is lowered. An inevitable condition for the specific interaction seems to be the existence of the amide fragment which is formed by N(1) — H and C(6) = O of the heterocyclic base. In accordance with conclusions made by Uchida and Egami (1971) and Ivanova et al. (1974) it can be assumed that the presence of the mentioned amide fragment in the nucleotide base is the characteristic feature of the substrates of guanyloribonucleases.

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Received March 12, 1982 / Accepted March 31, 1982