The Number and Role of Histidine Residues in the Active Site of Guanyloribonuclease Sa

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Abstract. The number and role of histidine residues in the active site of extracellular guanyloribonuclease Sa produced by *Streptomyces aureofaciens* (RNAase Sa) were studied via chemical modification by ethoxyformic anhydride by means of circular dichroism measurements. It was shown that only one of two histidines of RNAase Sa is situated in the active site of the enzyme. Ethoxyformylation of RNAase Sa in the presence of Guo-3'-P, Guo-5'-P and dGuo-5'-P, all of them being competitive inhibitors of the enzyme, supported the assumption that an essential histidine residue is bound to the phosphate group in the position 3' of the ribose ring. The circular dichroism measurements of native and modified RNAase Sa and of its complex with Cuo-3'-P showed that the modification of the essential histidine residue resulted in alteration of binding of RNAase Sa to Guo-3'-P; histidine thus may play a key role in the formation of such a complex.

Key words: Active site — Circular dichroism — Ethoxyformylation — Guanyloribonuclease Sa — Histidine residue

Introduction

Ribonuclease Sa (RNAase Sa, EC 3.1.27.3) isolated from the cultivation medium of *Streptomyces aureofaciens* (Bačová et al. 1971) is a guanylspecific extracellular endonuclease (Zelinková et al. 1971). It specifically cleaves 3'-5'-phosphodiester bonds in RNA and oligonucleotides at the guanine nucleotide through 2', 3'-cyclic phosphate intermediate. Due to the low molecular weight of RNAase Sa which was found to be $10,600 \pm 350$ (Ševčík et al. 1982), and a high stability, RNAase Sa is a suitable model for a detailed study of the relationship between the enzyme structure and function.

It is generally accepted that in enzyme reactions catalyzed by guanyloribonuc-

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leases, two histidine residues participate in the active site (Bezborodova and Bezborodov 1979). Two histidine residues in the active site of guanyloribonuclease probably mediate the proton transfer in the acido basic catalysis of the transphosphorylation step, similarly as in the mechanism of RNA splitting by RNAase A (Findlay et al. 1961). Hashimoto and Takahashi (1974), Grishenko et al. (1975) and Sakharovskij et al. (1976) concluded that two histidine residues are present in the active sites of guanyloribonucleases U_1, C_2 and Pch_1, respectively. Takahashi (1973) on the basis of chemical modification experiments, Osterman and Walz (1978) from kinetic studies, Rüterjans and Pongs (1971) based on NMR measurements suggested the participation of two histidine residues in the active site of RNAase T_1 which is the most studied guanyloribonuclease. Recently, two exceptions from the above finding have been found. Karpeisky et al. (1981) showed that RNAase Bi from *Bacillus intermedius 7P* has only one histidine residue in its enzyme molecule, while RNAase St from *Streptomyces erythreus* has two histidines in its enzyme molecule similarly as RNAase Sa, but only one of them is located in the active site as shown by Nakamura et al. (1982) by X-ray analysis.

Only few studies have so far dealt with the active site and the mechanism of action of RNAase Sa. Chemical modification experiments with phenylglyoxal and diketene showed that neither arginine nor lysine are located in the active site of the enzyme (Both et al. 1982). NMR study of RNAase Sa and its complex with Guo-3′-P suggested that only one of two histidine residues of RNAase Sa is accessible to the solvent and probably participates in the enzyme catalysis (Both et al. 1983).

The aim of the present work was to support the assumption concerning the participation of one histidine residue in the active site of RNAase Sa by experiments in which histidines were selectively blocked with ethoxyformic anhydride. Moreover, we shall try to characterize the role of the histidine residue in the catalysis by RNAase Sa.

Materials and Methods

Ribonuclease Sa was isolated and purified by the slightly modified method of Gašperík et al. 1982. The homogeneity was checked by G-50 chromatography and polyacrylamide gel electrophoresis. The activity of the enzyme was determined according to Egami et al. 1964. The protein content was estimated by the method of Kalb and Bernlohr (1977). In our experiments, lyophilized enzyme sample with specific activity of about 350,000 units . mg^{-1} was used.

RNA was purchased from Koch-Light. Low molecular fragments of oligonucleotides were removed by ethanol precipitation followed with dialysis.

Ethoxyformic anhydride (EFA) was obtained from Serva ; it was dissolved in absolute ethanol to make a stock solution. The concentration of EFA was determined spectrophotometrically at 230 nm by the reaction with 1 mmol . l^{-1} imidazole at pH 7.5. According to Sato and Uchida (1975) the change in ε_{230} for N-ethoxyformylimidazole is 3×10^6 cm^2 . mol^{-1}.

Chemical modification of RNAase Sa with EFA. To 495 µl of the modification reaction mixture which

containing the enzyme, 0.1 mol . l^{-1} citrate-phosphate buffer, pH 6.0 adjusted to 0.2 mol . l^{-1} ionic strength by KCl, five μl of stock solution of EFA were added at 20 °C. The extent of the modification was determined from ultraviolet absorption at 242 nm change in ε_{242} is 3.2×10^6 cm^2 . mol^{-1}, according to Sato and uchida 1975. When ethoxyformylation was completed, the modified RNAase Sa was desalted on a Sephadex G-50 M column and subsequently lyophilized. To determine enzyme activity, 10 μl aliquots were taken from the reaction mixture and immediately closed into polyethylene test tubes. The reaction was stopped by cooling to −60°.

Determination of the enzyme activity after ethoxyformylation. Frozen samples stored in polyethylene test tubes were transferred into an ice bath and diluted 100-fold. Then, 10 μl of diluted enzyme samples were added to 990 μl of RNA solution (0.11 mg . ml^{-1} of RNA in 0.1 mol . l^{-1} phosphate buffer, pH 7.0 with an ionic strength of 0.2 mol . l^{-1}). After mixing, increase of absorption at 280 nm was measured which is proportional to RNA splitting. The enzyme concentration used for the determination of the enzyme activity was about 3.5 nmol . l^{-1}.

Reactivation of ethoxyformylated RNAase Sa by hydroxylamine. Ethoxyformylated RNAase Sa (50 μl of 50 μmol . l^{-1}) was diluted by adding of 300 μl of citrate-phosphate buffer, pH 6.0 and 250 μl of 0.7 mol . l^{-1} NH$_2$OH solution. The enzyme activity was determined as mentioned above.

Measurements of circular dichroism and absorption spectra. Circular dichroism spectra were measured at 25° on a Mark III Dichrograph Jouan Roussel in an 0.1 cm quartz cell in 0.02 mol . l^{-1} acetate buffer, pH 5.6 (+)-10-Camphorosulfonic acid was used for dichrograph calibration. Differential molar ellipticity was obtained according to Oshima and Imahori (1971)

$$\Delta[\theta]_\lambda = (A - E - N + N . m/n) . 3300/m \qquad [\text{deg} . \text{cm}^2 . \text{dmol}^{-1}]$$

where A, E and N represent the magnitudes of the circular dichroism effects of the mixture, the enzyme and the nucleotide measured at the wavelength λ, respectively; n and m denote the molar concentration of the nucleotide and the enzyme inhibitor complex formed in the mixture, respectively. CD measurements of the complexes of both, non-modified and modified enzyme with Guo-3′-P were
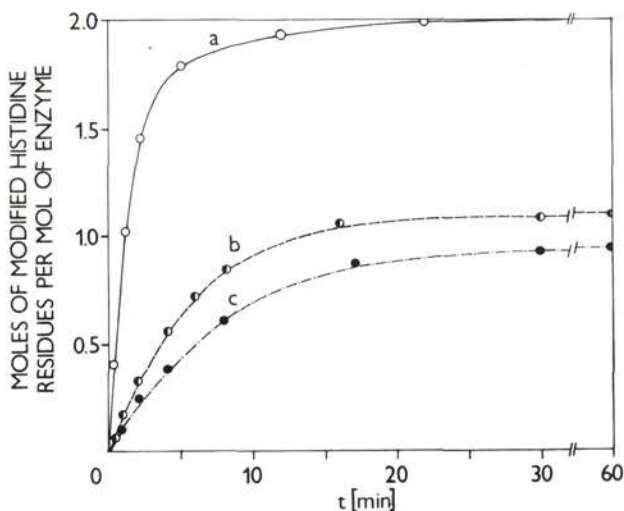

Fig. 1. Effect of EFA on histidine modification of RNAase Sa. The molar concentration ratios of EFA versus RNAase Sa were 2,700 (*a*), 170 (*b*), and 40 (*c*).

performed at about 4-fold molar excess of the nucleotide. Under these conditions, $m \approx e_o$ and $n \approx n_o$; e_o and n_o are molar concentrations of the enzyme and the nucleotide in the mixture, respectively.

Absorption spectra of the enzyme shown in Fig. 4 were measured before and after ethoxyformylation in 0.2 mol . 1^{-1} citrate-phosphate-KCl buffer, pH 6.0 at 20° using a 1 cm quartz cell.

Results

The aim of the chemical modification of RNAase Sa was to block selectively its histidine residue(s) by EFA. The extent and the time course of histidine residues modification strongly depended on EFA concentration (Fig. 1). At 40- or 170-fold molar concentration excess of EFA versus RNAase Sa, only one histidine residue was modified within 60 min, while at 2,700 fold excess of EFA, both histidine residues of the enzyme were found to be modified. At the highest EFA concentration the enzyme activity was almost completely lost within the first two minutes of ethoxyformylation.
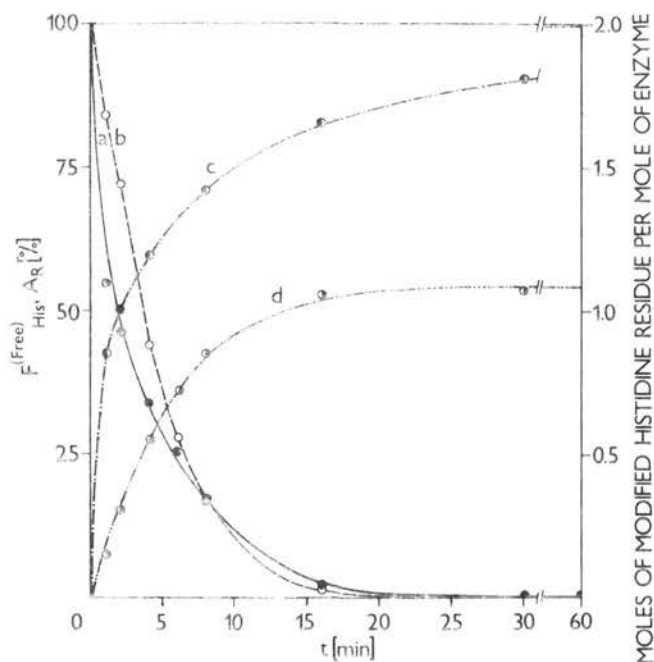

Fig. 2. Time course of RNAase Sa modification with EFA and reactivation of modified RNAase Sa with hydroxylamine. The concentrations of reagents for modification were: $c_{Sa} = 50.1 \ \mu\text{mol} . 1^{-1}$, $c_{EFA} = 2.0 \ \text{mmol} . 1^{-1}$; those for reactivation were: $c_{Sa} = 4.75 \ \mu\text{mol} . 1^{-1}$; $c_{NH_2OH} = 0.3 \ \text{mol} . 1^{-1}$. (a) relative enzyme activity A_R in %; (b) fraction of non-modified histidine residues $F(\text{Free})_{His}$ in %; (c) relative enzyme activity in % in the course of reactivation of RNAase Sa; (d) moles of modified histidine residues per mole of enzyme.

Then, the activity of RNAase Sa was studied in the course of ethoxyformylation and the relative enzyme activity A_R was calculated with respect to its initial activity. Using the initial enzyme concentration and the concentration of the enzyme with the modified histidine residue, the relative fraction of non-modified histidine residues $F(Free)_{His}$ was also determined (Fig. 2). The complete modification of one histidine residue per mole of the enzyme was taken 100 %. Both, A_R and $F(Free)_{His}$ corresponded to each other quite well. At the same time, the modification of only one histidine residue per mole of enzyme was observed, and an almost total recovery of the relative enzyme activity after the addition of hydroxylamine was recorded.

For quantitative comparison of the effects of different modification conditions, the use of the logarithm of the relative enzyme activity in the course of chemical modification has been recommended by Ray and Koshland (1961). Fig. 3 shows plots of A_R logarithms of RNAase Sa without and with its competitive inhibitors, such as Guo-3'-P, Guo-5'-P and dGuo-5'-P. Since all the four plots gave straight lines except for the initial stages of the reaction, it can be assumed that there are no differences in the mechanism of RNAase Sa modification in the presence or absence of these competitive inhibitors.

In the next step, UV and CD spectra of native and modified RNAase Sa and its complex with Guo-3'-P were determined. As it is clear from Fig. 4, no changes in absorption spectra in the region of tyrosine chromophores absorption were observed. Comparing CD spectra of ethoxyformylated RNAase Sa with those of
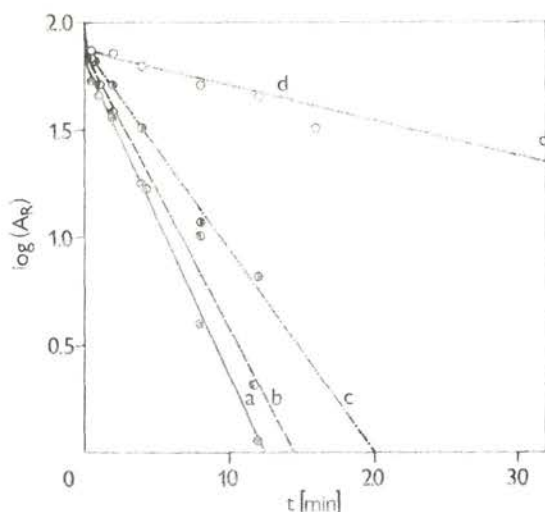

Fig. 3. Ethoxyformylation of RNAase Sa in the absence (*a*) and in the presence of 74-fold excess of competitive inhibitors dGuo-5'-P (*b*); Guo-5'-P (*c*); and Guo-3'-P (*d*). The concentrations of RNAase Sa, EFA and inhibitors were 8.75 μmol . l^{-1}, 3.27 mmol . l^{-1} and 0.64 mmol . l^{-1}, respectively.

the non-modified enzyme, only slight changes were recorded around 240—280 nm; more important changes were visible around 285 nm. The decreases in differential molar ellipticity of the modified RNAase Sa in complex with Guo-3′-P around 250 and 285 nm were found to be smaller than those of the nonmodified enzyme complex with the same inhibitor (Fig. 5).
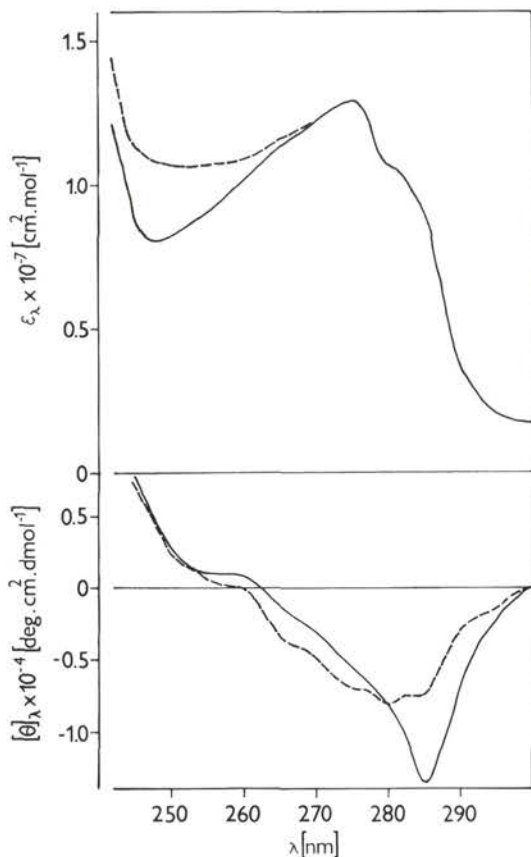

Fig. 4. Absorption and CD spectra of native RNAase Sa (full lines) and RNAase Sa with one histidine residue modified (broken lines).

Discussion

Miles (1976) and Sato and Uchida (1975) suggested that higher EFA concentrations may induced conformational changes within the enzyme molecule. It is quite probable that the second histidine residue His B of RNAase Sa which was not modified at low EFA concentrations, might be accessible to the modification

reagent at high EFA concentrations due to conformational changes of the enzyme. This histidine residue may be the same which is not accessible to the solvent in the native enzyme (see Introduction).

According to Ray and Koshland (1961), the enzyme activity in the course of chemical modification is proportional to the fraction of enzyme molecules in which no essential amino acid residue is modified. The fact that the time curve of A_R closely follows the time curve of $F(free)_{His}$ (Fig. 2) supports the assumption on the participation of only one of the two histidine residues (His A) in the active site of the enzyme; this was also suggested by NMR studies (Both et al. 1983). The addition of NH_2OH into the diluted solution of ethoxyformylated RNAase Sa resulted in the recovery of almost all the initial activity. This suggests that during ethoxyformylation of RNAase Sa at lower EFA concentrations, only a slight irreversibile denaturation of the enzyme molecule occurred.

The role of His A in enzyme reaction catalyzed by RNAase Sa was investigated by ethoxyformylation in the presence of a great excess of Guo-3'-P, Guo-5'-P and dGuo-5'-P, functioning as competitive inhibitors. The rate constants
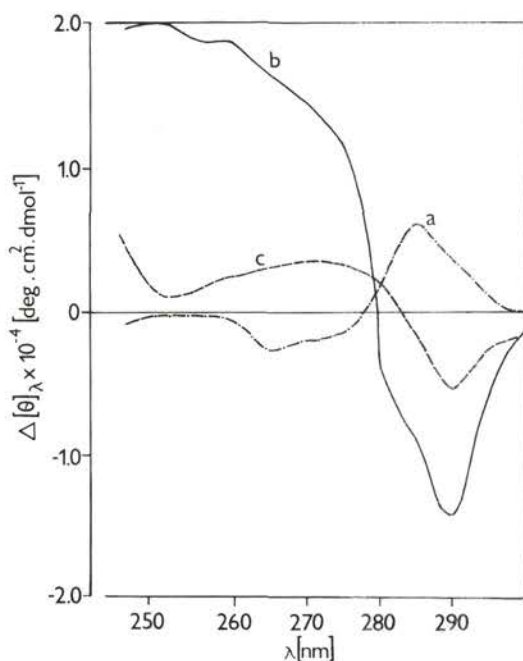

Fig. 5. Differential CD spectra of non-modified RNAase Sa and RNAase Sa with one histidine modified (*a*), non-modified RNAase Sa in the complex with Guo-3'-P (*b*), and RNAase Sa with one modified histidine residue in the complex with Guo-3'-P (*c*).

of RNAase Sa modification in the presence of 74-gold excess of Guo-5'-P
(0.106 min^{-1}) and dGuo-5'-P (0.115 min^{-1}) are comparable with the rate constant
of the enzyme modification in the absence of the inhibitors (0.15 min^{-1}). On the
other hand, the rate constant of RNAase Sa modification in the presence of an
equal excess of Guo-3'-P (0.017 min^{-1}) was by nearly one order smaller than that
for the free enzyme. All the above facts support the assumption that His A of
RNAase Sa binds to the phosphate group in position 3' of the nucleotide ribose
ring. Another important assumption is that concerning the function of the modified
histidine residue. This assumption is based on CD measurements of complex of
native and modified RNAase Sa with Guo-3'-P. The decrease in $\Delta[\Theta]_\lambda$ of the
complex after ethoxyformylation of RNAase Sa suggested that when the essential
His A is modified the formation of the RNAase Sa-Guo-3'-P complex is very
restricted. The data taken together indicated that His A may play a key role in the
formation of the enzyme-inhibitor complex.

It is known that EFA, in addition to the reaction with the imidazole ring of the
histidine residues, can also react with the OH group of the tyrosine residue
(Burstein et al. 1974). This reaction is accompanied with a decrease in UV enzyme
absorbancy at 278 nm. As for the RNAase Sa, no decrease in absorbancy in this
region was observed, even after ethoxyformylation (Fig. 4); consequently, no
detectable simultaneous modification of the tyrosine residue could occur.

Hashizume et al. 1967 attributed the CD peak recorded at 280 nm of several
proteins to tyrosine residues. In comparing this peak with the CD pattern of
poly-L-tyrosine it was concluded that the 280 nm band is mainly due to tyrosine
residues buried inside the protein molecule. Owing to this fact, their freedom is
restricted by interactions with neighboring groups, and their ellipticity is thus
strongly increased.

On the contrary, when tyrosine residues are exposed outside the enzyme
molecule, their free rotation around $C_a - C_\beta$ and $C_\beta - C_\gamma$ bonds is less tight and
a decrease in the ellipticity around 280 nm should be observed. Ethoxyformylation
of the histidine residue in RNAase Sa resulted in a decrease in molar ellipticity
around 285 nm. According to the above arguments, this fact may be interpreted as
being due to conformational changes within the enzyme molecule, which can result
in an increase in the mobility of the tyrosine residue inside the enzyme molecule.
Also, His A may be in close proximity to some of the tyrosine residue (s).

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