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STUDIES ON THE INACTIVATION OF WHALE PANCREATIC RIBONUCLEASE W_1 WITH IODOACETATE

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SUMMARY

The influence of iodoacetate on the whale pancreatic ribonuclease (ribonuclease W_1) (EC 2 7 7 16) was studied The inactivation of ribonuclease W_1 by action of iodoacetate occurred most rapidly at pH 5 0–5 7 The presence of 3 molar equivalents of uridine 2′(3′)-phosphate and cytidine 2′(3′)-phosphate to ribonuclease W_1, largely inhibited the inactivation, but the inhibition was very slight when purine nucleoside 2′(3′)-phosphates instead of pyrimidine nucleoside 2′(3′)-phosphate were present

The products of the reaction of iodoacetate with ribonuclease W_1 at pH 5 0 were separated by column chromatography using SE-Sephadex The main product seemed to be a modified ribonuclease W_1, which was produced by introduction of one mole of carboxymethyl group per one mole of the protein

The products obtained by the similar reaction performed at pH 8 5 were separated into at least nine peaks of the fractions, among which only one peak had no enzymatic activity

INTRODUCTION

The catalytic sites of several enzymes so far investigated generally contain some chemically unique or unusually reactive amino acid residues Therefore, comparative studies of chemical modifications of several enzymes from various sources, should offer valuable information on the relations between structures and functions of those enzymes

Many papers have reported on the chemical modification with various reagents[1] of bovine pancreatic ribonuclease Among those, the carboxymethylation with α-halogenoacetic acids[2–9] provided the most fruitful information about the active center of this enzyme, that is, α-halogenoacetic acids such as α-iodo- or α-bromoacetic acid specifically reacted with histidine residues at the 12 or 119 position of the ribonuclease A at about pH 5 5 to produce a modified ribonuclease A having 1-carboxymethyl histidyl or 3-carboxymethyl histidyl[7,8] residues As these products have no enzymatic activities, it became clear that these histidine residues were involved in the active sites of ribonuclease A

Recently TAKAHASHI *et al* [10] reported that the reaction of α-bromoacetate with ribonuclease T_1 (EC 2 7 7 26) produced a modified enzyme which had no enzymatic activity and that in the modified product γ-carboxy group of the glutamic acid at the position 58 was carboxymethylated, thus it was considered that this glutamic acid residue should be involved in the active site of the ribonuclease T_1 (refs 10, 11)

These observations that α-halogenoacetate modified different kinds of amino acid residue, each of which was likely involved in the active sites of two kinds of ribonucleases having different substrate specificities suggested the possible significance of these amino acid residues in the substrate specificities of the corresponding ribonuclease

We have reported the purification and properties of pancreatic ribonucleases from various mammalian species including human[12], whale[13], horse[14] and porcine[15] The substrate specificity of all these enzymes was similar to that of bovine pancreatic ribonuclease A which cleaves the internucleotide phosphate linkages between pyrimidine ribonucleoside 3′-phosphates and 5′-hydroxyl groups of the neighboring nucleotides The whale pancreatic ribonuclease (ribonuclease W_1), however, had considerably different amino acid composition from that of the bovine ribonuclease A so that it should be interesting to know the behavior of the ribonuclease W_1 towards the chemical modification with α-halogenoacetate, which has been known to inactivate the ribonuclease A by modifying the enzyme at the specific amino acid residues involved in the active center[9] This report deals with the results of the study on the reaction of α-iodoacetate with ribonuclease W_1

MATERIALS AND METHODS

Enzymes Whale pancreatic ribonuclease (ribonuclease W_1) was obtained from pancreas of *Belaenoptera physalus* by the procedure of IRIE *et al* [13] and was further purified on a SE-Sephadex C-25 column

The pure ribonuclease A was kindly prepared from bovine pancreas by Mr E Sukegawa of this laboratory

Substrates RNA used as substrate was prepared by exhaustive dialysis of commercial yeast RNA against distilled water and subsequent lyophilization Cytidine 2′,3′-cyclic phosphate was prepared according to the method of SMITH *et al* [16]

Determination of ribonuclease activity Ribonuclease activity was determined by the following two methods using RNA (a) and cytidine 2′,3′-cyclic phosphate (b) as substrates

(a) Assay of ribonuclease activity using RNA as substrate To 1 0 ml of 1% RNA solution in 0 1 M Tris–HCl (pH 8 0), or sodium acetate (pH 5 0), 20–50 μl of enzyme solution were added, and the mixture was incubated at 37° for 5 min The reaction was terminated by addition of 0 5 ml of 25% $HClO_4$ containing 0 75% uranyl acetate After standing for 15 min at room temperature, the precipitate was centrifuged and 0 1 ml of the supernatant solution was taken and diluted with 3 0 ml of water The change of absorbance at 260 mμ was determined against a proper blank using a Beckman DU spectrophotometer

(b) Assay of ribonuclease activity using cytidine 2′,3′-cyclic phosphate as substrate This was carried out by practically the same procedure as described by HERRIES *et al* [17] at pH 4 0 using sodium cytidine 2′,3′-cyclic phosphate as substrate

Column chromatography on SE Sephadex C-25 SE Sephadex C-25 (Pharmacia, 2.3 ± 0.3 mequiv/g) was buffered with 0.1 M sodium phosphate (pH 6.5). After charging ribonuclease W_1 on the column (0.9 × 45 cm), elution was started with a linear gradient system from 0.1 M sodium phosphate (pH 6.5) to 0.3 M of the same buffer. Ribonuclease W_1 was eluted at the position corresponding to the buffer concentration of 0.2 M. The fractions containing ribonuclease W_1 were combined and desalted by passing through a column of Amberlite MB-3, and the effluent was lyophilized.

Reaction of iodoacetate with ribonuclease W_1 The reaction of iodoacetate with ribonuclease W_1 was performed in three different conditions. Condition (a) was used to know the behavior of iodoacetate on the enzymatic activity of ribonuclease W_1 and conditions (b) and (c) were employed for the large-scale preparation of the reaction products. (a) Ribonuclease W_1 (0.1%) was reacted with iodoacetate (0.1%) at appropriate pH's at 37°. Aliquots (20 μl) were taken and diluted with 1.0 ml of water to determine the ribonuclease activity. (b) Reaction at pH 5.0. Ribonuclease W_1 was dissolved in water in a concentration of 0.67% and to this solution was added iodoacetic acid in an amount half the weight as the enzyme. The solution was immediately adjusted to pH 5.0 with 1 M NaOH and incubated at 37°. The mixture was passed through an Amberlite MB-3 column to terminate the reaction, and the effluent was lyophilized. (c) Reaction at pH 8.5. Ribonuclease W_1 (100 mg) was dissolved in 15 ml of 0.05 M Tris–HCl (pH 8.5), and to this solution was added 10 mg of iodoacetic acid and the pH was adjusted to 8.5 with 1 M Tris. The reaction was carried out at 37° for 15 h. Termination of the reaction was performed as mentioned above.

Determination of the protein concentration Determination of the protein concentration was performed by measuring the absorbance at 280 mμ of the test solution in a Beckman DN spectrophotometer. The absorbance at 280 mμ of 0.1% solution of ribonuclease W_1 was 0.530.

Counting of the radioactivity Counting of the radioactivity was carried out in a Packard Tri-Carb liquid scintillation spectrophotometer. The scintillator solution contained 10 g of 2,5-diphenyloxazole, 0.25 g of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene and 100 g of naphtalene in 1 l of dioxane[18]. The efficiency of counting for ^{14}C was 67%.

RESULTS

Column chromatography of ribonuclease W_1 *on SE Sephadex C-25*

In Fig. 1, a typical pattern in the purification of ribonuclease W_1 by column chromatography (Step 6 given in the report of IRIE *et al.*[13]) is shown. The elution was performed using a linear density gradient system from 0.1 to 0.3 M of sodium phosphate buffer.

In some preparations, just before the major peak of ribonuclease W_1, a minor peak having a ribonuclease activity appeared. All ribonuclease W_1 used in this research was obtained by this SE-Sephadex fractionation.

Time-course of inactivation of ribonuclease W_1 *with iodoacetate*

Time course of inactivation of ribonuclease W_1 with iodoacetate is shown in

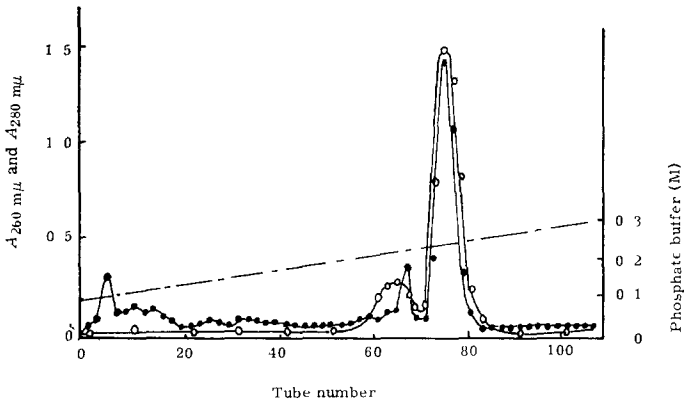

Fig 1 Column chromatographic pattern of ribonuclease W_1 on SE-Sephadex C-25 Approx 80 mg of ribonuclease W_1 (Step 6 (ref 13)) were loaded The chromatography was operated at room temperature at a flow rate of 5 ml/h and each 10-ml fraction was collected Ribonuclease activity was determined using RNA as substrate at pH 5 0 by the procedure given in MATERIALS AND METHODS ●—●, protein (absorbance at 280 mμ), ○—○, ribonuclease activity (absorbance at 260 mμ)

Fig 2 The inactivation was estimated using RNA and cytidine 2′,3′-cyclic phosphate as substrates The rates of inactivation against the both substrates were the same

The results showed that the activities in both transesterification and hydrolysis of cyclic phosphate of this enzyme were inhibited by the modification of some amino acids with iodoacetate, which must be involved in the active site and essential for these enzyme activities The rate of inactivation by iodoacetate of ribonuclease W_1

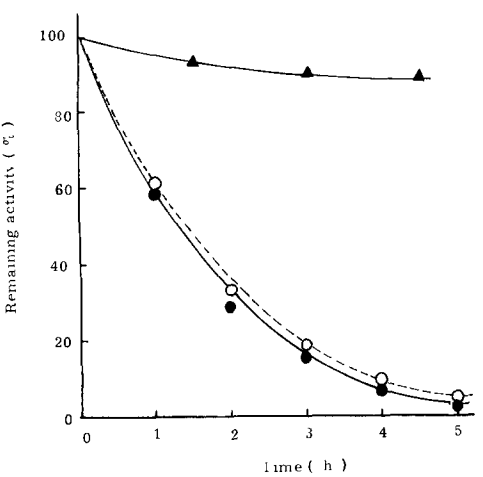

Fig 2 Rates of inactivation of ribonuclease W_1 by iodoacetate and iodoacetamide at pH 5 0 and 37° 1 ml of reaction mixture contained 1 mg of ribonuclease W_1, 1 mg of iodoacetic acid or 1 5 mg of iodoacetamide and 50 μmoles of sodium acetate (pH 5 0) Ribonuclease activity was determined against RNA and cytidine 2′,3′-cyclic phosphate as substrates ●—●, iodoacetate, RNA as substrate, ○—○, iodoacetate cytidine 2′,3′-cyclic phosphate as substrate, ▲—▲, iodoacetamide, RNA as substrate

at pH 5 0 was comparable to the case of ribonuclease A As was reported for ribonuclease A (ref 5) and ribonuclease T_1 (ref 10) iodoacetamide inactivated ribonuclease W_1 only very slowly and this result indicated that the carboxyl group of iodeacetate should be important for the inactivation probably by its interaction with some cationic group(s) in the ribonuclease W_1 molecule

The pH dependence of the inactivation

The pH optimum in the inactivation of ribonuclease W_1 by iodoacetate was estimated and the result is given in Fig 3

The figure indicates that the optimum pH for this inactivation is 5 0–5 7 This pH value is also the optimum in the similar inactivation for ribonuclease A

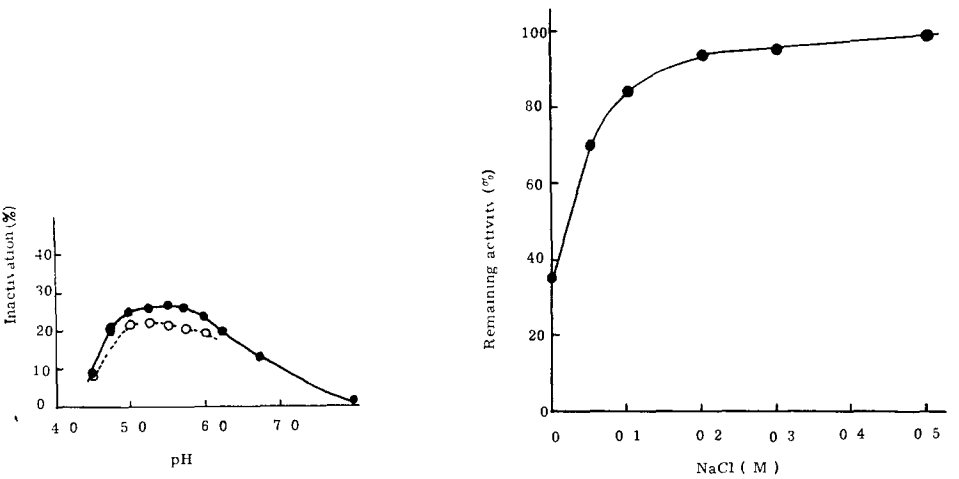

Fig 3 pH dependence of the inactivation of ribonuclease W_1 by iodoacetate at 37° 1 ml of the reaction mixture contained 1 mg of ribonuclease W_1, 1 mg of iodoacetate, 25 μmoles of buffer and 50 μmoles of NaCl Ribonuclease activity was determined against RNA as substrate at pH 8 0 Buffers used were sodium acetate buffer for pH 4 0–6 75 and Tris-HCl buffer for pH 8 0 ●—●, ribonuclease W_1, ○—○, ribonuclease A

Fig 4 Effect of NaCl on the inactivation of ribonuclease W_1 by iodoacetate at pH 5 0 1 ml of the reaction mixture contained 1 mg of ribonuclease W_1, 1 mg of iodoacetate (pH 5 0) and given amount of NaCl Reaction was carried out for 1 h at 37° Ribonuclease activity was determined against RNA as substrate

At alkaline pH region (more than pH 6.0), the inactivation became gradually low and at pH 8 5 nearly complete ribonuclease activity remained even after 2-h reaction with iodoacetate This does not mean that the carboxymethylation does not occur on the enzyme at all As is shown in the last figure (Fig 7), most of the reaction products at pH 8 5 had the ribonuclease activity, so at this pH value we could not follow the rate of the carboxymethylation by assaying the ribonuclease activity

Effect of ionic strength on the inactivation of ribonuclease W_1 by iodoacetate

The reaction of iodoacetate with ribonuclease W_1 was much influenced by the ionic strength of the reaction mixture As is shown in Fig 4, the inactivation by

iodoacetate hardly occurred when the concentration of NaCl in the reaction mixture was over 0.2 M.

This result indicates that ionic interaction is an important factor for the reaction of ribonuclease W_1 and iodoacetate.

Effects of mononucleotides on the inactivation

It is well known that the inactivation of ribonuclease A by α-halogenoacetate was protected by the presence of pyrimidine nucleoside 2'(3')-phosphate in the reaction mixture[3]. The similar protection only by guanosine 2'(3')-phosphate among four major ribonucleoside 2'(3')-phosphates was observed against the inactivation of ribonuclease T_1 by α-halogenoacetate[10,11].

We investigated the effect of various nucleotides on the inactivation of ribonuclease W_1 by iodoacetate. The Table I indicates that 71% and 70% of the inactivation of ribonuclease W_1 by iodoacetate were protected by uridine and cytidine 2'(3')-phosphates, respectively, which were added to the reaction mixture in a molar ratio of the nucleotides to the enzyme being 3:1. On the other hand, the protection by adenosine and guanosine 2'(3')-phosphates were only 23% and 22%, respectively. The Table I involved results of the similar experiments for ribonuclease A, which entirely resembled the case of ribonuclease W_1 with respect to the base specificity of nucleotide used.

TABLE I

EFFECTS OF NUCLEOSIDE 2'(3')-PHOSPHATE ON THE INACTIVATION OF RIBONUCLEASE W_1 WITH IODOACETATE

1 ml of the incubation mixture contained 1 mg of ribonuclease W_1, 1 mg of iodoacetate, 3 mole equivalents of nucleoside 2'(3')-phosphate (assuming that the ribonuclease W_1 has the molecular weight of 15 000) and 50 μmoles of sodium acetate (pH 5.0). The control mixture did not contain nucleotides. The mixture was incubated at 37° for 3 h. Ribonuclease activity was estimated with RNA as substrate at pH 8.0.

Nucleoside 2'(3')-phosphate	*Protection (%)*	
	Ribonuclease W_1	*Ribonuclease A*
None	0	0
Up	70.6	83.0
Cp	69.4	78.7
Ap	23.5	21.4
Gp	22.4	17.0

From the results that the inactivation of the ribonuclease W_1 by iodoacetate at pH around 5 was protected more effectively by pyrimidine nucleotides than by purine nucleotides, it may be concluded that the carboxymethylation occurred at the active site of ribonuclease W_1.

Column chromatography of the products obtained by the reaction at pH 5.0

Column chromatographic patterns of reaction products which were obtained after various reaction times are shown in Figs. 5a, 5b and 5c.

The chromatographic pattern, using SE-Sephadex C-25 of the mixture obtained

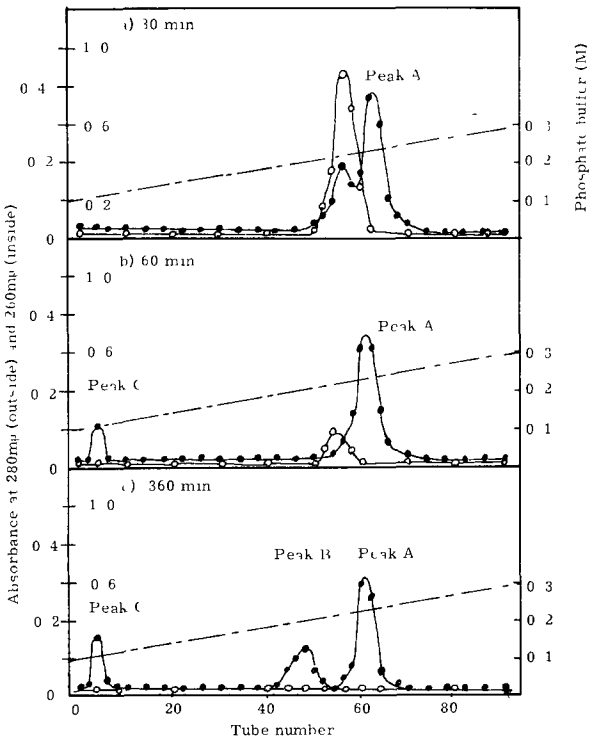

Fig 5 Column chromatographic patterns of the products obtained by reaction of iodoacetate with ribonuclease W_1 at various reaction times Ribonuclease W_1 (90 mg) was dissolved in 15 ml of 0 02 M acetate buffer (pH 5 0) and 45 mg of iodoacetic acid were added to the solution The solution was immediately adjusted to pH 5 0 with conc NaOH solution Reaction was carried out at 37° for time intervals of (a) 30 min, (b) 60 min and (c) 360 min At the end of each interval, 5-ml aliquots were withdrawn and the reaction was terminated by passing the mixture through a small column (0 9 cm × 10 cm) of Amberlite MB-3 to remove salts and unreacted iodoacetate The column was washed with 10 ml of water and the effluents were combined and lyophilized The lyophilisate was dissolved in 4 ml of 0 1 M sodium phosphate buffer (pH 6 5) and applied on a SE-Sephadex C-25 column The chromatography was operated at room temperature at a flow rate of 10 ml/h and 4-ml fractions were collected ●—●, protein (absorbance at 280 mμ), ○—○, ribonuclease activity, determined against RNA as substrate at pH 5 0 (absorbance at 260 mμ)

after 30 min of reaction (Fig 5a) indicated the appearance of a new protein peak (Peak A) just after the original peak of ribonuclease W_1 It was found that the fraction corresponding to this new peak did not show any ribonuclease activity, thus it was assumed that this inactive protein was a ribonuclease W_1 modified with iodoacetate

After 60 min of reaction, almost all ribonuclease W_1 was converted into the product corresponding to Peak A The Fig 5c, the pattern obtained after 360 min of reaction shows that the compound contained in Peak A (Fig 5b) was further converted into products (Peaks B and C) Both of these new products did not show any ribonuclease activity

In further research on the modification of ribonuclease W_1 with iodoacetate, the reaction with [^{14}C]iodoacetate was performed at pH 5 0 In order to obtain the ^{14}C-labeled product corresponding to Peak A in the Fig 5a, the reaction was carried

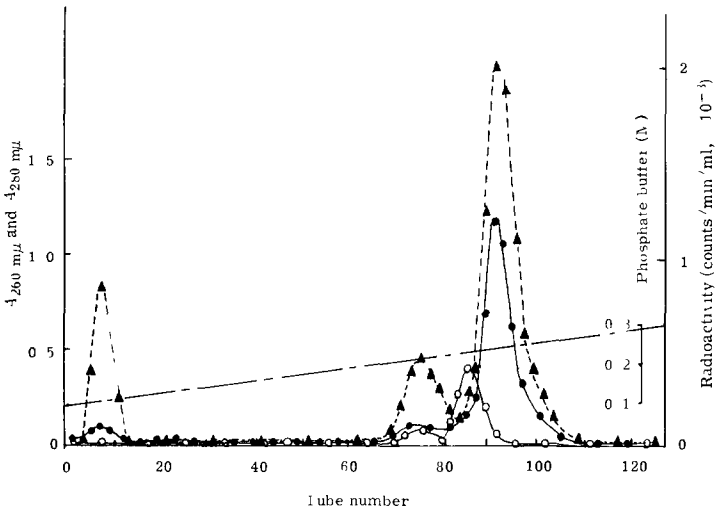

Fig 6 Column chromatography of ^{14}C-carboxymethylated ribonuclease W_1 Ribonuclease (240 mg) was dissolved in 36 ml of water and 120 mg of [^{14}C]iodoacetate (0 033 μC/mg) were added to this solution The solution was adjusted immediately to pH 5 0 with 2 M NaOH and incubated at 37° for 90 min The reaction was terminated by passing the solution through an Amberlite MB-3 column (1 2 cm × 15 cm) The effluent was lyophilized and the lyophilisate was dissolved in 10 ml of 0 1 M sodium phosphate buffer (pH 6 5) The solution was applied on a SE-Sephadex C-25 column (2 5 cm × 35 cm) as described in MATERIALS AND METHODS The elution was performed by the same procedure as shown in Fig 5 Flow rate was 25 ml/h, and 10-ml fractions were collected Ribonuclease activity was determined against RNA as substrate at pH 5 0 ●—●, protein (absorbance at 280 mμ), ○—○, ribonuclease activity determined against RNA as substrate (absorbance at 260 mμ) ▲—▲, radioactivity (counts/min per ml)

out for 1 5 h according to Condition (b) (see MATERIALS AND METHODS), which gave Peak A as a major product In this case, at the end of the reaction time more than 95% of the original enzymatic activity disappeared

As is seen in Fig 6, one major and two minor radioactive components could be separated by chromatography using SE-Sephadex C-25 column the peaks corresponding to the Peak A, B and C in Fig 5

The major component, the combined fractions in Tubes 89–95 revealed approx 75% of the total absorbance at 280 mμ of the original sample loaded and has no enzymatic activity when tested with RNA as substrate The elution position of peak of radioactivity and that of protein ($A_{280\ m\mu}$) were entirely the same and each fraction in Tubes 89–95 had the same specific radioactivity 965 ± 32 counts/min per mg of protein The analysis indicated that the radioactive product in Peak A contained 0 9 mole carboxymethyl group per 1 mole of ribonuclease W_1 based on the assumed molecular weight of 15 000 of the enzyme

As was described above, the two peaks each corresponding to Tubes 5–9 and 69–81, respectively, were thought to be the secondary reaction products The peak corresponding to Tubes 69–81 had 2-fold specific activity of that of the major peak This indicated that the product in this peak contained 2 moles of carboxymethyl group incorporated to 1 mole of ribonuclease W_1 The first peak corresponding to Tubes 5–9 showed a 3 8-fold specific activity of the major peak, and it was shown

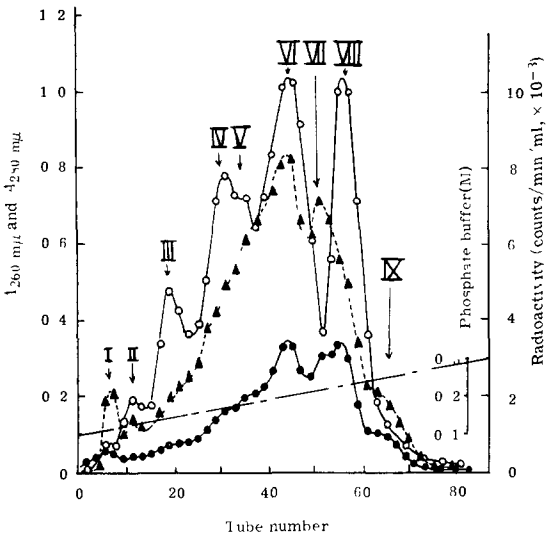

Fig 7 Column chromatography of the reaction products of ribonuclease W_1 with iodoacetate at pH 8 5 Reaction was carried out using the Condition (c) in MATERIALS AND METHODS Specific activity of [^{14}C]iodoacetate was 0 2 μC/mg Procedures for terminating the reaction and column chromatography were as given in Fig 5 ●—●, protein (absorbance at 280 mμ), ○—○, ribonuclease activity determined against RNA as substrate (absorbance at 260 mμ), ▲—▲, radioactivity (counts/min per ml)

that the product in this fraction had 4 moles of carboxymethyl group incorporated into 1 mole of ribonuclease W_1, on an average

Column chromatographic separation of the products obtained after reaction at pH 8 5

The ribonuclease W_1 dissolved in 0 05 M Tris–HCl (pH 8 5) at 0 67% concentration was reacted with iodoacetate, which was added to the solution to 0 067% concentration, at 37° for 15 h

The reaction condition was arranged to involve a low concentration of the reagent and a longer interval of the reaction time in order to minimize the over-carboxymethylation Fig 7 shows the pattern of chromatographic separation on SE-Sephadex C-25 of the products obtained after reaction at pH 8 5 The chromatogram in Fig 7 differs markedly from that of Fig 6, indicating that the reaction of iodoacetate at pH 8 5 is quite complicated in contrast to that at pH 5 0 At least seven peaks (I–VI and VIII) having enzymatic activity and five radioactive peaks (I, II, VI, VII and IX) were separable by this chromatography

Peak VIII seemed to lack the radioactivity and its elution position was identical with that of ribonuclease W_1 which had been observed in a separate experiment using the same column Therefore, Peak VIII must be that of the native ribonuclease W_1 Peak VII which was eluted just before ribonuclease W_1 had no or little, if any, enzymatic activity but a remarkable radioactivity

HEINRIKSON demonstrated that the major product of the reaction of ribonuclease A with bromoacetate at pH 8 5 was an inactive modified ribonuclease A, which was produced by carboxymethylation at the ε-amino group of lysine 41 (ref 9)

Therefore, in our case also, there is a possibility that Peak VII corresponded to a modified ribonuclease W_1 produced by carboxymethylation at pH 8.5 of some amino acids involved in the active site.

As is shown in Fig. 7, specific activity (counts/min per mg of protein) of the products corresponding to each peak was fairly constant, except for Peaks I and VIII. Furthermore, the specific activity of the peaks, except Peaks I and VIII, indicated that about 2 moles of carboxymethyl group were introduced into ribonuclease W_1.

DISCUSSION

The inactivation of bovine pancreatic ribonuclease by iodoacetate was first reported by ZITTLE[2]. At that time, as the absence of sulfhydryl groups in this enzyme had not yet been established, he misinterpreted that the iodoacetate reacted with the sulfhydryl groups in the ribonuclease. But the results of the more detailed studies[2–9] on the inactivation using purified ribonuclease A by α-halogenoacetate, which have been obtained in the past decade, indicated that the mode of the inactivation was influenced by the conditions under which the reaction was carried out. Thus, in an acidic medium, iodoacetate reacted exclusively with sulfur of the methionine residues of the enzyme, while at neutral or slightly acidic pH region, a rapid specific carboxymethylation on the imidazole groups of two histidine residues lead to the inactivation of the enzyme, but even under this condition a longer contact of the reagent resulted in the carboxymethylation of other amino acid residues such as methionine and lysine. At alkaline pH, an extensive modification occurred on several amino acid residues involving lysine and methionine.

It has been reported that the amino acid residues which were specifically carboxymethylated by reaction of α-halogenoacetate at pH approx. 5, *i.e.* 12th and 119th histidine residues in pyrimidine nucleotide specific ribonuclease A[7,8] and 58th glutamic acid residue in guanylic acid specific ribonuclease T_1 (ref. 10), were involved in the active sites of the respective enzymes because the previous addition of the substrate analogs, pyrimidine nucleoside 3′-phosphate and guanosine 3′-phosphate to ribonuclease A and ribonuclease T_1, respectively, inhibited these carboxymethylations.

These results suggested that the application of α-halogenoacetate under the similar specific conditions on other ribonucleases should provide some useful informations on the amino acid residues in the active sites of these enzymes.

The above-described chemical modification was applied to the whale pancreatic ribonuclease, ribonuclease W_1 (ref. 13). This ribonuclease seemed to be one of the suitable materials for this kind of research because the pure ribonuclease W_1 was easily obtained in good yield and found to be a simple protein which did not contain sugar moiety like porcine pancreatic ribonuclease[19]. Furthermore, there was another interest in ribonuclease W_1, that is, the amino acid composition of this enzyme is considerably different from that of ribonuclease A (T. YAMADA AND T. UKITA, unpublished data) though its substrate specificity is similar to ribonuclease A.

The optimal pH for the inactivation by iodoacetate of ribonuclease W_1 was at pH 5.0–5.7, which was similar to the cases of ribonuclease A and ribonuclease T_1. The main product (Peak A) of the reaction at pH 5.0, which was separated by chromatography on SE-Sephadex C-25 (Fig. 6), possessed one mole of carboxymethyl group per

one mole of the enzyme and no enzymatic activity This result should support that the carboxymethylated amino acid residue was involved in the active site of this enzyme The determination of the residue is now in progress

We investigated the reaction of iodoacetate with ribonuclease W_1 at pH 8 5 In this case, in order to avoid the over-carboxymethylation, the ratio in amount in weight of iodoacetate to ribonuclease W_1 was reduced to 1 10 and the reaction time was prolonged to 15 h As shown in Fig 7, the products were separated into at least eight peaks by SE-Sephadex C-25 column chromatography The radioactive Peak VII, which appeared just before the native ribonuclease W_1 (Peak VIII) revealed low enzymatic activity Therefore, it is interesting to determine the carboxymethylated amino acid residue in the product corresponding to Peak VII The further detailed study on the carboxymethylated ribonuclease W_1 which showed enzymatic activities in Fig 7 should give useful informations on the tertiary structure of ribonuclease W_1

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