The Specific Alkylation by Iodoacetamide of Histidine-12 in the Active Site of Ribonuclease*

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SUMMARY

Alkylation of ribonuclease A by iodoacetamide at pH 5.5 yields two products: inactive 3-carboxamidomethylhistidine-12-ribonuclease and fully active S-carboxamidomethylmethionine ribonuclease. No derivative substituted at histidine-119 was detected. This is in marked contrast to the products obtained on alkylation by iodoacetate at pH 5.5. The rate of alkylation of histidine in ribonuclease by iodoacetamide is about 1% that of iodoacetate, but the reaction is nevertheless facilitated since it is 10 to 100 times greater than the rate of alkylation of free histidine. The rate of alkylation of histidine-12 by iodoacetamide reaches a maximum at pH 5 to 5.5 and then decreases as the pH is increased. The rate is depressed by the presence of sodium chloride below about pH 5.5 but is unaffected above this pH. The dependence upon pH of this rate of alkylation of histidine-12 indicated that groups ionizing with apparent pK values of 3.5 to 4.3 and 6.2 ± 0.2 are involved in maintaining the reactive form of the enzyme.

One explanation of the facilitated reaction is an interaction between the dipole of the amide group of the reagent and a dipole on the protein surface. This explanation is supported by the observation that acetamide inhibits the alkylation of histidine-12 by iodoacetamide.

It is consistent with previous interpretations of the facilitated reactions of halo acids at either histidine-12 or at histidine-119 to suggest that it is a dipolar orientation of iodoacetamide by histidine-119 and an adjacent carboxyl group which is responsible for the unexpected specificity of iodoacetamide for histidine-12.

The rate of alkylation of methionine in ribonuclease by iodoacetamide at 24° was found to be approximately constant between pH 2.3 and 7.0, and independent of sodium chloride and acetamide concentrations. This fact indicates that only minor or very localized conformational changes can be occurring in the region of the methionine residue that has undergone alkylation.

The inactivation of bovine pancreatic ribonuclease by haloacetate ions has been well documented (2-6). Inactivation proceeds rapidly at pH 5.5 and involves alkylation of either imidazole N1 of histidine-119 or imidazole-N4 of histidine-12 (5, 6).

Increasing the chain length of the halo acid (7) changes the relative rates of alkylation of the 2 residues, but the rates still remain considerably higher than the rates of alkylation of free histidine. This highly specific, facilitated reaction with a halogenated carboxylic acid has been explained by an interaction of positively charged groups on the surface of the enzyme with the negatively charged carboxylate ion. Part of the evidence for this conclusion lay in the observation of Stark, Moore, and Stein (4) that the neutral reagent iodoacetamide did not inactivate ribonuclease at more than 1% of the rate of the inactivation by iodoacetate. In experiments with iodoacetate, it was observed that a side reaction, alkylation at methionine, could occur (5, 6) without inactivation of the enzyme (8, 9).

The experiments described in the present paper were initiated to determine whether iodoacetamide also would alkylate ribonuclease A at methionine without inactivation. However, the chromatographic method used to analyze the reaction mixture showed that two products were formed at similar rates. In one product, a single methionine residue had reacted without inactivation. The other, an inactive derivative, had occurred at histidine instead of at methionine.

The reaction by iodoacetamide at histidine, although much slower than that of iodoacetate, was nevertheless higher than the rate with free histidine, and the preference of the reaction for histidine-12 instead of histidine-119 was notable.

Since iodoacetamide is a small uncharged reagent, it was thought that the specificity of the reaction might reflect only the pK of the histidine-12 residue. Therefore a detailed study of the dependence of the rate of alkylation upon pH was undertaken. The results are more complex than expected on this basis and...
led to the proposal of a specific amide-binding site to explain the observed specificity.

**EXPERIMENTAL PROCEDURE**

**Materials**—Ribonuclease A purified by chromatography on sulfoethyl-Sephadex (8) was obtained in solution (11 mg per ml in 0.1 M phosphate buffer, pH 6.47) from Worthington. Iodoacetamide (K and K Laboratories) was recrystallized from glass distilled water. Acetamide (analytical grade) was obtained from Fisher and was used without further purification.

**Separation of Reaction Products**—In these experiments the course of the alkylation reaction was monitored by chromatography on Amberlite IRC-50 in 0.2 M phosphate buffer, pH 5.5, as described by Hirn, Moore, and Stein (10) for the purification of ribonuclease A. A fraction of resin sedimenting at 10 to 15 cm per hour was used in the column (0.7 x 25 cm) which was operated at a rate of 25 to 40 ml per hour. The setup of pump, filter column, sample injection coil, analytical column, and flow cell in Zeiss spectrophotometer and automatic recorder was the same as that described in recent papers (6, 7, 11). The phosphate buffer contained no phenol and, to diminish microbial growths, was prepared freshly by mixing 76 ml of 1 M disodium phosphate buffer with 124 ml of 1 M monosodium phosphate solution, and diluting the mixture to 1 liter with water. In analytical chromatograms, the spectrophotometer was operated at 210 nm to take advantage of the high sensitivity for detection of proteins, while 280 nm was used for preparative scale work where a low sensitivity was needed. This chromatographic system is referred to herein as the IRC-50:0.2 M phosphate system.

**Preparation and Identification of Reaction Products**—In preliminary experiments, about 4 to 5 mg of ribonuclease A in 0.5 ml of 0.02 M sodium acetate buffer, pH 5.5, were mixed with a solution of 10 mg of iodoacetamide in 0.5 ml of water. The reaction mixture was maintained at room temperature in the dark. Samples (100 µl) were removed periodically, diluted with 1 ml of phosphate buffer, and chromatographed on the IRC-50:0.2 M phosphate system. The two major products of the alkylation were eluted at about 35 ml and at about 82 ml, as shown in Fig. 1.

For preparative work, a solution of about 50 mg of ribonuclease A in 2 ml of 0.05 M sodium acetate buffer, pH 5.5, was mixed with 20 mg of iodoacetamide and the reaction was allowed to proceed for 25 hours at 25°C. The reaction mixture was fractionated on the same IRC-50 column used for the analytical work; the effluent was monitored continuously at 280 nm. Unreacted monomer and the two major products were collected in separate fractions as they emerged from the column.

Samples of the two main products were desalted by passage through a column (0.5 x 20 cm) of Sephadex G-25 equilibrated with 5% acetic acid. One portion containing about 0.05 µmole of protein was hydrolyzed and submitted to amino acid analysis (9). Another portion of 0.05 µmole of each derivative was oxidized with performic acid according to the method of Moore (12) prior to acid hydrolysis.

The analysis of the product eluted at 82 ml showed that 1 histidine residue was missing and that 1 residue of 3-carboxymethylhistidine was present. Approximately 1.5 mg of this derivative was desalted and cleaved with cyanogen bromide according to the method of Gross and Witkop (13). The products of the cleavage were separated by gel filtration on a column (2 x 235 cm) of Sephadex G-25 and subjected to acid hydrolysis and amino acid analysis.

To determine whether the derivatives were enzymically active, samples of each of the products eluted from the IRC-50 column were transferred to 0.02 M Tris-0.06 M NaCl buffer, pH 7.45, by passage through a column (0.5 x 25 cm) of Sephadex G-25 and their activity toward 2',3'-cyclic cAMP was determined according to the method described by Fruehler and Crestfield (8).

To determine whether the additional carboxamidomethyl group affected the reaction with iodoacetate, the derivatives were transferred to 0.1 M sodium acetate buffer, pH 5.5, and reacted with 0.037 M iodoacetate as described previously (7). The course of this reaction was followed by analysis of portions of the reaction mixture by chromatography on the IRC-50:0.2 M phosphate system or on the IRC-50:0.266 M NaCl system described previously (7).

**Effect of pH on Reaction of Iodoacetamide with Ribonuclease**—A stock solution containing 7 mg of ribonuclease A per ml of 0.02 M NaCl was prepared and stored frozen. Samples (0.5 ml) of this solution were diluted with 1 ml of water or of 0.02 M NaCl, and the pH was adjusted to the desired value by the addition of traces of 0.01 M HCl or of 0.01 M NaOH that had been freshly prepared to minimize the presence of carbonate. A sample containing 0.1 mg of ribonuclease A was chromatographed on the IRC-50:0.2 M phosphate system. Solutions of iodoacetamide were prepared by dissolving 50 mg in 1 ml of water or of 0.02 M NaCl; the pH was adjusted to the desired value and the solution was made up to 2.5 ml with the appropriate solution. A sample (1 ml) of the protein solution was mixed with 1 ml of the iodoacetamide solution contained in a water jacketed vessel. A pH meter (Radiometer, type TTT1a) equipped with a microassembly (conversion kit, type J452) and the Radiometer micro-electrodes, K 4112 and G 222 B, was used to determine the pH during the course of the reaction. The temperature of the vessel was maintained at 24 ± 0.2°C by water from a Haake circulating water bath. The reaction vessel was shielded from the light.

After 1 to 2 hours of reaction, about 0.5 mg of protein from the reaction mixture was analyzed on the IRC-50:0.2 M phosphate system and the yields of the products of the reaction were estimated from the areas of the peaks. After 4 to 6 hours, a sample of 0.1 mg of protein was analyzed and the decrease in the amount of ribonuclease A was estimated.

When the reactions were carried out above pH 6.5, CO₂-free nitrogen was passed slowly over the surface of the reaction mixture to prevent access of atmospheric carbon dioxide.

**Inhibition by Acetamide**—A stock solution containing 12.3 mg of ribonuclease A per ml in 0.02 M sodium acetate buffer, pH 5.3, was prepared and stored frozen. Samples (0.25 ml) of this solution were mixed with 0.25 ml of a solution containing a known concentration of acetamide (0 to 4 M) in the same buffer and with 0.5 ml of a solution containing 20 µg of iodoacetamide per ml. This mixture was maintained at 25 ± 1°C for 90 min, then mixed with 1 ml of 0.2 M phosphate buffer (pH 6.47), and applied to the IRC-50 column for analysis.

**Model Reactions**—Histidine monohydrochloride monohydrate (419 mg) was dissolved in 0.02 M NaCl, the pH was adjusted to 5.3 with 0.01 M NaOH, and the solution was made up to 10 ml with 0.02 M NaCl, pH 5.3. A portion (2 ml) of this solution was mixed with 2 ml of a 2% solution of iodoacetamide of the same...
Sephadex G-25, a tridecapeptide was obtained in 78% yield that cleaved by oyanogen bromide and the products were separated on conversion of cystine to oysteio acid.)

Because the inadvertent obscured by the cystine peak in the usual amino acid analyzer ribonuclease A. (The 3-oarboxymethylhistidine peak, which is after performic acid oxidation, which indicated that methionine residues of methionine sulfone were recovered from the material after prolonged reaction. In no case did the areas of these peaks exceed 10% of the area of the 3-CAMHis-12-RNase peak. As mentioned above, minor peaks are observed in the chromatograms of alkylation mixtures after prolonged reaction. In no case did the areas of these peaks exceed 10% of the area of the 3-CAMHis-12-RNase peak. However, as a result of the inaccuracy in measuring the small amounts of the products formed, the possibility cannot be excluded that histidine-119 is alkylated at a rate less than 10% of the rate of reaction at histidine-12.

NOTES

Identification of Products of Rlkylation of Ribonuclease by Iodoacetamide—Fig. 1 shows the separation by chromatography on the IRC-50:0.2 m phosphate system of the products of alkylation of ribonuclease by iodoacetamide after about 10% of the ribonuclease had reacted. Later in the reaction minor peaks appear at about 43 and 74 ml.

The component eluted at 35 ml in Fig. 1 was identified as an S-carboxamidomethylmethionine ribonuclease by the general procedure of Gundlach, Stein, and Moore (14). In this method, advantage is taken of the fact that S-carboxymethylmethionine is not oxidized by performic acid, whereas methionine is oxidized to methionine sulfone by this reagent. Thus, as Neumann, Moore, and Stein (15) have shown, the content of sulfonyl salt in a derivative of ribonuclease may be determined indirectly from the difference between the methionine sulfone content of oxidized ribonuclease A and that of the oxidized, alkylated protein. Amino acid analyses of the material eluted from the IRC-50 column at about 35 ml (Fig. 1) were performed before and after performic acid oxidation according to the procedure of Moore (12). The analysis of the unoxidized material showed that acid hydrolysis released 3.98 residues of unmodified histidine and 2.61 residues of methionine, while minor amounts of S-carboxymethylhomocysteine, homoserine, and homoserine lactone were obtained corresponding to 0.84 residue of S-carboxymethylhomocysteine (15). Analysis after performic acid oxidation yielded 3.00 residues of methionine sulfone. Unalkylated ribonuclease A yields 4 residues of methionine sulfone under the same conditions, so the results show that the derivative contains 1 residue of alkylmethionine.

Amino acid analyses of the material eluted from the IRC-50 column at about 82 ml (Fig. 1) suggest that this derivative is 3-carboxamidomethylhistidine ribonuclease. Nearly 4 (3.83) residues of methionine sulfone were recovered from the material after performic acid oxidation, which indicated that methionine had not been alkylated. From the unoxidized material 2.95 residues of histidine, 0.93 residue of 3-carboxymethylhistidine (presumably from a residue of 3-carboxamidomethylhistidine), and no 1-carboxamidomethylhistidine were recovered, in contrast to the 4 residues of unmodified histidine recovered from unalkylated ribonuclease A. (The 3-carboxamidomethylhistidine peak, which is obscured by the cystine peak in the usual amino acid analyzer pattern, was observable in this case, because the inadvertent introduction of oxygen into the hydrolysis tube had led to the conversion of cystine to cysteic acid.)

When the 3-carboxamidomethylhistidine ribonuclease was cleaved by cyanogen bromide and the products were separated on Sephadex G-25, a tridecapeptide was obtained in 78% yield that corresponded in amino acid composition to the NH2-terminal tridecapeptide obtained from ribonuclease A after this treatment (12), except that 1 residue of 3-carboxymethylhistidine was present instead of unmodified histidine. The residual protein moiety was identical with that derived from ribonuclease A. These data identify this second derivative of the parent protein as 3-carboxamidomethylhistidine-12-ribonuclease.

The two products described above were the only ones produced in sufficient amounts for analysis. A sample of ribonuclease A eluted from the IRC-50:0.2 m phosphate system during an analysis of a 5-hour alkylation mixture was rechromatographed on the IRC-50:NaCl system. Only a single peak of ribonuclease A was obtained, indicating that additional carboxamidomethyl derivatives that moved as ribonuclease A in the presence of phosphate ions were not formed. As mentioned above, minor peaks are observed in the chromatograms of alkylation mixtures after prolonged reaction. In no case did the areas of these peaks exceed 10% of the area of the 3-CAMHis-12-RNase peak. However, as a result of the inaccuracy in measuring the small amounts of the products formed, the possibility cannot be excluded that histidine-119 is alkylated at a rate less than 10% of the rate of reaction at histidine-12.

Properties of S-CAMMet-RNase—When assayed against 2',3'-cyclic cytidylate the S-CAMMet-RNase showed 100% of the activity of ribonuclease A. When reacted with iodoacetate at pH 5.5, the rate of disappearance of this derivative was identical with the rate of disappearance of ribonuclease A under the same conditions. During the alkylation of S-CAMMet-RNase by iodoacetate two new peaks appeared in the IRC-50:0.2 m phosphate chromatograms; by analogy with the reaction with unsubstituted ribonuclease the positions of the peaks were those to be expected for S-CAMMet-1-CMHis-119-RNase (at 54 ml) and S-CAMMet-3-CMHis-12-RNase (at 90 ml). The molar ratio of the presumed 119 derivative to the presumed 12 derivative was 7:1. The similarity of this source of alkylation to that for

1 The derivatives of ribonuclease carboxamidomethylated (CAM) or carboxymethylated (CM) on given residues are abbreviated as follows: 3-CAMHis-12-RNase; S-CAMMet-RNase; S-CAMMet-1-CMHis-119-RNase; S-CAMMet-3-CMHis-12-RNase.
Ribonuclease A suggests that the methionine residue alkylated by iodoacetamide is remote from the active site, as is true for the methionine residue alkylated by iodoacetate (8, 0).

Properties of 3-CAMHis-12-RNase—A sample of 3-CAMHis-12-RNase was rechromatographed on sulfonethyl-Sephadex in 0.115 M phosphate buffer, pH 6.47. The highly purified material obtained was then assayed against 2',3'-cyclic cytidylylade and was found to possess 1.3 ± 0.5% of the specific activity of ribonuclease A. When alkylated at pH 5.5 by iodoacetate, the 3-CAMHis-12-RNase reacted at a rate about 14-fold lower than the rate of alkylation of ribonuclease A under comparable conditions. Thus the carboxamido group when present on nitrogen 3 of histidine-12 can block alkylation at histidine-119, as does a carboxymethyl group in this position. This shows that the observed lack of a second alkylation on histidine by iodoacetamide ion is not caused solely by electrostatic repulsion between the iodoacetamide anion and a previously incorporated carboxymethyl group.

Kinetics of Alkylation Reaction—The products shown in Fig. 1 account completely for the decrease in the ribonuclease peak until about 20% of the initial ribonuclease has disappeared. The absence of more rapid further alkylation of the derivatives indicates that there is no major conformational difference between ribonuclease A and the two derivatives. As the concentration of these two monosubstituted products increases, secondary alkylations occur, forming polysubstituted products which are not eluted from the column. This limits the yield of each of the monosubstituted products to about 10 to 15% of the total protein. A plot of the logarithm of the ribonuclease concentration over the range 0.25 to 2.5% iodoacetamide concentration, the rate of alkylation is proportional to the reaction is first order with respect to protein. At a given protein concentration, the rate of alkylation is proportional to the iodoacetamide concentration over the range 0.25 to 2.5% iodoacetamide, which indicates that the reaction is also first order as estimated from chromatograms such as that shown in Fig. 1, provided that samples are taken during the initial stages of the reaction.

Effect of pH on Rates of Alkylation of Ribonuclease A by Iodoacetamide—Fig. 2 shows the dependence upon pH of the second order rate constant for the formation of 3-CAMHis-12-RNase in two different concentrations of sodium chloride. The errors in the data are quite large because of the low yield of the derivative and because of instrumental limitations. Above pH 6.5, a further error is present because the sum of the two products shown in Fig. 1 is less than the loss of ribonuclease A. This difference increases with increasing pH above 6.5 and may be due to the alkylation of the ε-amino group of lysine-41 (cf. Heinrikson (16)). The pH of the alkylation mixture remained within ±0.1 pH unit during the course of the reaction except above pH 7.0, where a decrease of about 0.1 pH unit per hour was observed.

The continuous curve drawn through the data obtained in 0.003 M NaCl is the sum of two calculated curves. One is calculated according to the equation

\[
\frac{k_t}{k_c} = \frac{k}{K_1 + [H^+]/K_2}
\]

where \(K_1\) and \(K_2\) are the acid dissociation constants of ionizing groups of pK 6.2 and 3.8, \(k\) is the pH-independent rate constant, and \(k_t\) is the pH-dependent rate constant. The other calculated curve is derived from the Henderson-Hasselbalch equation and is the rate of alkylation of free histidine assuming a pK of 3.8 and a maximum rate of alkylation of \(6.8 \times 10^{-4} \text{ M}^{-1} \text{sec}^{-1}\). This latter value was that calculated from the observed rate at one pH for the maximum rate of alkylation by iodoacetamide of the imidazole ring of free histidine, when the ring is uncharged. This value is shown in Fig. 2 by the dotted line.

The dashed line through the data obtained in 0.02 M NaCl was not calculated. Comparison of the data in 0.003 M NaCl and in 0.02 M NaCl indicates that, below the pH for the maximum rate, sodium chloride inhibits the alkylation of histidine-12, whereas it has little effect above the pH maximum. At pH 3.7, the second order rate constants in 0.003, 0.01, and 0.02 M NaCl are 0.55 ± 0.07, 0.24 ± 0.07, and 0.30 ± 0.07 \(\times 10^{-4} \text{ M}^{-1} \text{sec}^{-1}\), respectively; that is, the NaCl decreases the rate significantly up to 0.01 M NaCl, but not between 0.01 and 0.02 M NaCl.

The second order rate constant for the alkylation of methionine by iodoacetamide was approximately constant between pH 2.3 and 7.3. A value of \(1.0 ± 0.2 \times 10^{-4} \text{ M}^{-1} \text{sec}^{-1}\) was obtained at all pH values in both 0.003 M NaCl and 0.02 M NaCl. This value is about the same as the rate for the alkylation of methionine in ribonuclease by iodoacetate at pH 5.5 (1.5 ± 0.5 \(\times 10^{-4} \text{ M}^{-1} \text{sec}^{-1}\)) (7).

Inhibition of Alkylation at Histidine-12 by Acetamide—In Fig. 3 is shown the decrease in the yield of 3-CAMHis-12-RNase under fixed conditions of pH and temperature when ribonuclease A is alkylated in the presence of different concentrations of acetamide. The dashed line indicates that the yield of CAMMet-RNase is not affected by these concentrations of acetamide.

\[7\] A comparison in an earlier paper (8) with the model experiments of Stark and Stein (17) was not valid owing to the difference in the temperatures at which the two series of experiments were carried out.
Amide at pH 5.3 is 1.2 times greater than that of iodoacetate, and histidine-12 is less than 7 times lower with the haloamide than with the halotetrate. Hence at pH 5.3, the rate of alkylation of methionine in ribonuclease A by iodoacetamide was immeasurably low compared with the rapid rate of alkylation of free histidine (7, 18). The possibility that the inhibition of the histidine reaction results from traces of metal ions or other impurities present in the acetamide cannot be excluded.

**Discussion**

As mentioned in the introductory section, Stark et al. (4) observed that the rate of inactivation of ribonuclease A by iodoacetamide was immeasurably low compared with the rapid rate of inactivation by iodoacetate. Our results show that the rate of alkylation of histidine-12 by iodoacetamide at pH 5.3 in 0.02 M NaCl is 1.1 ± 0.1 × 10^{-4} M^{-1} sec^{-1}. According to Heinrikson et al. (7) the rates of alkylation of histidine-119 and histidine-12 by iodoacetate at pH 5.5 are 5.1 × 10^{-4} and 7.3 × 10^{-4} M^{-1} sec^{-1}, respectively. Thus the over-all rate of inactivation by iodoacetamide is only 1 to 2% of that of iodoacetate, and hence would not have been observed under the conditions employed by Stark et al. (4). However, the rate of alkylation at histidine-12 is less than 7 times lower with the haloamine than with the haloacetate.

One might think, at first, that the observed low rate for the reaction of histidine-12 with iodoacetamide might merely be that of an exposed and freely accessible imidazole group. However, the model experiments show that the over-all rate of alkylation of the imidazole ring of free histidine at N$_1$ and N$_2$ by iodoacetamide at pH 5.3 is 1.2 × 10^{-4} M^{-1} sec^{-1}. Assuming a pK of 5.9, the maximum rate of alkylation that would be found when the imidazole ring is completely unprotonated may be calculated to be 6.8 × 10^{-4} M^{-1} sec^{-1}. Hence at pH 5.3, the rate of alkylation of imidazole N$_1$ of histidine-12 is 10- to 100-fold higher than the rate of alkylation of free histidine. For comparison, at pH 5.5, the rate of alkylation of histidine-12 by iodoacetate is 30 to 150 times the rate of alkylation of free histidine (7, 18).

The facilitation of the reaction with the protein imidazole must result from the character of the groups surrounding histidine-12. One clue to the nature of these groups lies in the dependence of the alkylation by iodoacetamide upon pH. The continuous line in Fig. 2 through the data obtained in 0.003 M NaCl was calculated, as described earlier, on the assumption that groups with apparent pK values of 3.8 and 6.2 determine the rate of alkylation of histidine-12. However when the concentration of sodium chloride in the alkylation mixture is raised from 0.003 M to 0.02 M, the lower limb of the curve occurs about 0.5 pH unit higher. The maximum rate of alkylation remains approximately the same, which suggests that the additional ions (presumably the chloride anions) bind specifically to the protonated form of the enzyme that is present below pH 5.3. If the ions were bound to the species that is alkylated, then the maximum rate as well as both upper and lower limbs of the curve would be affected (cf. Alberty (19)). Since additional sodium chloride raises the apparent pK of the more acidic group involved, it is likely that in the complete absence of sodium chloride the apparent pK of the group would be less than 3.8. The intrinsic pK might be as low as 3.5.

It was observed that the rate of alkylation at pH 3.7 was the same in 0.01 M NaCl as in 0.02 M NaCl, which suggests that chloride ion binding at this site is already maximal at 0.01 M. This is consistent with the results of Loeb and Saroff (20) who used permselective electrode membranes to determine the extent of binding of chloride ions to ribonuclease. They reported that at pH 4.5 chloride ion binding did not change much between 0.01 and 0.1 M chloride ion, although it decreased significantly below the former value.

In attempting to explain the specificity of the alkylation of ribonuclease by iodoacetamide, the following observations must be accounted for: First, alkylation occurs specifically at imidazole nitrogen 3 of histidine-12 at a rate at least 10- to 20-fold higher than the maximum rate of alkylation of free histidine. Second, the reactive form of the enzyme is lost either upon dissociation of groups of pK 3.5 to 4.3 or 6.2 ± 0.2. The facilitation of the reaction may be envisaged either as the result of an increased rate of nucleophilic displacement of iodide ion by histidine-12 or of an increase in the effective concentration of iodoacetamide at the active site due to a specific binding of the amide residue.

An increased rate of nucleophilic displacement might result from the increased polarization of the carbon-iodine bond resulting from an appropriately positioned positive charge. Such a mechanism has been proposed for the alkylation of ficin (21). In such a case, the position of the polarizing positive group (possibly histidine 119 or lysine-41) would account for the specificity of the reaction.

An increase in the effective concentration of iodoacetamide at the active site might result from a dipole-dipole interaction between the amide group of iodoacetamide and a permanent or inducible dipole on the surface of the enzyme. The inhibition of the reaction by acetamide is consistent with the possibility of an amide-binding site. The concentration of acetamide used would not be expected to induce a general conformational change (cf. Gordon and Jencks (22)) and this is supported by the constancy of the rate of alkylation of methionine in ribonuclease in the presence of different concentrations of acetamide. However, the possibility does exist that a local conformational change might result from other interactions, and hence the inhibition data are not conclusive proof of an amide-binding site.

The dependence of the alkylation upon pH might reflect either the ionization of groups specifically involved in the facilitation or of groups that play a role in maintaining the crucial reactive
conformation. A pK of about 6.2 suggests an imidazole group, and is within the range of values suggested by other authors (23–26). Histidine-119 which is known to be in the vicinity of histidine-12 is a possible candidate. A pK of 3.5 to 4.3 suggests a carboxyl group, although it is conceivable that a histidine residue might have a pK as low as this if positioned in a highly positively charged site. Other authors (20, 27) have suggested that a carboxyl group is present at the active site. An orienting dipole on the enzyme surface might therefore consist of the positively charged imidazolium ion of histidine-119 and some carboxylate ion.

Ideally, this proposal should be compatible with previous explanations of reactions at the active site. In explaining the reaction of haloacetate ions with the histidine residues of ribonuclease, it has been proposed (4, 6, 7, 28) that the positively charged active site attracts the anionic reagent, while specific explanations of reactions at the active site. In explaining the carboxylate ion.

dipole on the enzyme surface might therefore consist of the that a carboxyl group is present at the active site. An orienting residue might have a pK as low as this if positioned in a highly

trostatic nature may be masked and replaced by stereospecific hindrances (7). Thus the suggestion that a carboxyl group is present near histidine-119 is not incompatible with the former orientation would be favored relative to the latter, and the alkylation would occur, as is observed, predominantly at histidine-119. However, if the halo acid has other structural features in the side chain, this primary orienting effect of electrostatic nature may be masked and replaced by stereospecific alkyl group hindrances (7). Thus the suggestion that a carboxyl group is present near histidine-119 is not incompatible with the observations and interpretations for the halo acid reactions. Further indication of a carboxyl group near histidine-119 is derived from the observation of Kenkare and Richards (29) that the cationic dye, methylene blue, facilitates the photooxidation of histidine-119 while histidine-12 seems to be abnormally unreactive.

As mentioned in the introductory section, when this work was initiated it was thought that a small uncharged alkylation agent might react in a manner that reflected only the pK of the histidine-12 residue. However, our results show that the reaction between iodoacetamide and ribonuclease is more complex than this and is dependent on the adjacent groups at the active site. Just as the use of other halo acids extended our understanding of the interaction of the negatively charged reagents with groups in the vicinity of the active site, so it may be possible in future work to use haloamides substituted on the amide nitrogen or the α-carbon to define further aspects of the architecture of the active site.

Acknowledgments—The authors are indebted to Drs. William H. Stein and Stanford Moore for their counsel and aid in the preparation of this manuscript.

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Since this manuscript was written, the x-ray data of Kartha, Bello, and Harker (30) and of Wyckoff et al. (31) have been announced. The molecular model of ribonuclease S constructed by Wyckoff et al. (32) shows that there are carboxyl groups in the neighborhood of histidine-119. Thus the existence of a dipole on the enzyme which could facilitate the reaction of iodoacetamide with histidine-12 is not incompatible with current information provided by x-ray crystallography.
The Specific Alkylation by Iodoacetamide of Histidine-12 in the Active Site of Ribonuclease
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