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 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 N,N of histidine-119 or imidazole-N,N 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. In the other, an inactive derivative, alkylation 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

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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 6.47, as described by Hirs, 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 × 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 solution 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 201 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 250 μm. 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 × 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 (15). The products of the cleavage were separated by gel filtration on a column (2 × 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 × 25 cm) of Sephadex G-25 and their activity toward 2',3'-cyclic cytidylate was determined according to the method described by Frueh and Crestfield (8).

To determine whether the additional carboxamidomethyl group affected the reaction with iodoacetate, samples of 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 about 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 TT11a) 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 mg of iodoacetamide per ml. This mixture was maintained at 25°C ± 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. The introduction of oxygen into the hydrolysis tube had led to the pattern, was observable in this case, 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. 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.

**RESULTS**

Identification of Products of Alkylation 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 has 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 sulfonium 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-carboxymethylmethionine (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-carboxamidomethylhistidine (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 (13), except that 1 residue of 3-carboxamidomethylhistidine was present instead of unmodified histidine. The residual protein moieties were 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 chromatogram 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 course of alkylation to that for

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**Fig. 1.** Separation by chromatography on the IRC-50:0.2 M phosphate system of the products of alkylation of ribonuclease A by iodoacetamid at pH 5.5. Column, 0.7 X 25 cm; flow rate, 30 ml per hour; load, 0.1 mg of protein.
ribonuclease A suggests that the methionine residue alkylation by iodoacetamide is remote from the active site, as is true for the methionine residue alkylation by iodoacetate (8, 0).

Properties of 3-CAMHis-12-RNase—A sample of 3-CAMHis-12-RNase was rechromatographed on sulfoethyl-Sephadex in 0.115 M phosphate buffer, pH 6.47. The highly purified material obtained was then assayed against 2',3'-cyclic cytidylyl 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 iodoacetate ion is not caused solely by electrostatic repulsion between the iodoacetate 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 concentration of the monosubstituted products to about 10 to 15% of the total protein. These data indicate that the alkylation 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 with respect to this alkylation agent. A second order rate constant calculated from the extent of reaction 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 in Fig. 2 drawn through the data obtained in 0.003 M NaCl is the sum of two calculated curves. One is calculated according to the equation

\[
   -\frac{k}{1 + \frac{[\text{H}^+]}{K_1} + \frac{[\text{H}^+]^2}{K_2}}
\]

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 \(k_2\) 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 \(1.0 \pm 0.2 \times 10^{-4} \text{ M}^{-1} \text{sec}^{-1}\) 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 alkylation 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.

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 temperature at which the two series of experiments were carried out.
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 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 permeable 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.
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 interaction between the carboxylate ion of the reagent and a positively charged histidine-12 orients the reagent for alkylation at histidine-119, whereas a positively charged histidine-12 orients for alkylation at histidine-12. If, as we suggest now, there is a carboxylate ion adjacent to histidine-119, then 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.3 As mentioned in the introductory section, when this work was initiated it was thought that a small uncharged alkylating 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 haloamines substituted on the amide nitrogen or the α-carbon to define further aspects of the architecture of the active site.

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