Mechanisms of Carboxymethylation of Bovine Pancreatic Nucleases by Haloacetates and Tosylglycolate*

BRYCE V. PLAPP‡

From The Rockefeller University, New York, New York 10021, and the Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242

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SUMMARY

Certain histidine residues in bovine pancreatic ribonuclease and deoxyribonuclease react with iodoacetate about 1000 times faster than free histidine reacts. The mechanisms of these facilitated, active-site-directed reactions were investigated by study of the kinetics of carboxymethylation with reagents with varied leaving groups: chloride, bromide, iodide, and tosylate. With ribonuclease, the rate of reaction gave hyperbolic dependence on the concentration of reagent. The data fit a Michaelis-Menten mechanism, with dissociation constants of 8 to 24 mM for the various reagents. The similar magnitudes of the constants lead to the conclusion that the reagents bind (reversibly) in a common way to the enzyme. The pseudo bimolecular rate constants for reaction (after correction for the differing inherent reactivities of the reagents) were also similar, indicating that the leaving group is not very important in the mechanism. These studies support the proposal that the reaction of haloacetates with ribonuclease is facilitated by ionic attraction of the carboxylate ion by a protonated imidazole while an unprotonated imidazole displaces the halide ion.

In contrast, the kinetics of carboxymethylation of the Cu²⁺-Tris complex of deoxyribonuclease showed that the reagents bound loosely, with dissociation constants of 130 ± 25 mM for the three reagents. The larger groups may be more sterically hindered in the reaction; alternatively, the smaller groups may be more strongly attracted to the electrophilic Cu²⁺. In either case, during carboxymethylation, both the carboxylate ion and the leaving group interact with DNase. While the carboxylate ion by a protonated imidazole while an unprotonated imidazole displaces the halide ion.

The noncovalent, complex has not been directly detected, nor has its dissociation constant (K = k₂/k₁) been determined. We have studied the kinetics of carboxymethylation in order to confirm the mechanism and to determine the kinetic constants. Iodo-, bromo-, and chloroacetates and tosylglycolate (carboxy-

The mechanisms proposed for the carboxymethylation of the nucleases presume Michaelis-Menten behavior, in which the noncovalent complex of enzyme and iodoacetate forms and is converted to the irreversibly modified enzyme:

\[ E + I \overset{k₃}{\rightarrow} E \cdot I \overset{k₄}{\rightarrow} E + I \]

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methyl tosylate) were used to study the effect of the leaving group (halide ion or tosylate) on the reaction.

The p-toluenesulfonyl group (tosyl) is used widely in synthetic and physical organic chemistry as a leaving group; it is displaced by nucleophiles more easily than iodide (11). We thought that tosylglycolate might have some affinity for the active sites of RNase and DNase or might have some special reactivity because of the similarity of the leaving anion to the phosphodiester substrates. Examination of a three-dimensional model of RNase S (9) showed that the tosyl group could fit into the cleft that holds the pyrimidine base of the substrate, near phenylalanine 120 and valine 43, while the carboxylate was attracted to lysine 7 or 41 and the methylene carbon was attacked by N-1 of histidine 119.

EXPERIMENTAL PROCEDURE

Materials—Chloroacetic acid (zone-refined), bromoacetic acid, and iodoacetic acid were obtained from Aldrich. Tosylglycolate was prepared by the two-step procedure of Lichtenberger and Faure (12):

\[ p-\text{CH}_2\text{CH}_2\text{SO}_2\text{Cl} + \text{NaCN} + \text{HCHO} \quad \rightarrow \quad p-\text{CH}_2\text{CH}_2\text{SO}_2\text{OCH}_2\text{CN} + \text{NaCl} \]

\[ p-\text{CH}_2\text{CH}_2\text{SO}_2\text{OCH}_2\text{CN} + \text{NaOH} + \text{H}_2\text{O} \quad \rightarrow \quad p-\text{CH}_2\text{CH}_2\text{SO}_2\text{OCH}_2\text{COONa} + \text{NH}_3 \]

NaCN (1.96 g, 0.04 mole) was dissolved in 5 ml of H_2O and cooled to 0°. Formaldehyde (3.05 ml of 37% CH_2O, 0.04 mole) was added dropwise with stirring over a period of 15 min. Recrystallized p-toluenesulfonyl chloride (5.7 g, 0.03 mole) was added over 20 min and the cloudy white mixture was stirred at 0° for 1 hour and allowed to warm up to room temperature for 30 min. The white crystals were collected by filtration, washed with water, and dried in vacuo for 2 hours; yield was 5.7 g. The crystals were dissolved in 50 ml of diethyl ether and the solution was dried over CaCl_2, filtered, and evaporated to a small volume under vacuum. The crystals that formed (2.3 g) sintered at less than 35°, m.p. 45-62°. A second crop of crystals was collected upon further concentration: 2.2 g, m.p. < 35°. The crude tosylglycunitrile (second crop) was dissolved in 55 ml of 0.2 M NaOH. The mixture was stirred for 50 min at room temperature and filtered, and the filtrate was acidified to pH 1.2 with concentrated HCl. The product that formed (0.45 g) was collected by filtration and recrystallized by dissolving it in 25 ml of 0.2 M NaOH and acidifying the solution again to pH 1.2 with concentrated HCl. The crystals that formed were dried over CaSO_4 in vacuo, yield 0.3 g (4%); m.p. 132-135° (literature 131°).

C_6H_7O_2S (230.2)

Calculated: C 46.95, H 4.38, N 0
Found: C 46.85, H 4.41, N 0.26

In the synthesis described, ^14C can be introduced into the carboxyl group of the reagent. Nonradioactive reagent is prepared in better yields from commercial cyanomethyl p-toluenesulfonate (Aldrich). The reagent has been stored for over 2 years in a desiccator without change in melting point or reactivity.

Bovine pancreatic RNase A (phosphate-free) and DNase (DP grade), yeast RNA, and calf thymus DNA were purchased from Worthington. The A form of DNase (13) was used in the following studies.

Methods—RNase was assayed by the procedure of Kunitz (14) and DNase by the modified procedure (15) of Kunitz (16).

Carboxymethylated RNase was hydrolyzed (17) and analyzed for amino acids (18) with accelerated systems (19) on columns of Beckman-Spinco AA-15 (0.9 x 55 cm) and AA-27 (0.9 x 11 cm). The color value of 1-carboxymethylhistidinamide was taken as that of glycine (2). The color value of cysteine was taken as 0.15 times that of leucine (20), and that of S-carboxymethylcysteine as 0.93 times that of aspartic acid (18). Alkaline hydrolysis and ninhydrin analysis were also performed (21, 22).

The kinetics of inactivation of RNase by various carboxymethylation reagents were studied at 37° with 0.2 M sodium 2-[(N-morpholino)ethyl]sulfonate buffer, pH 5.30. This buffer was chosen because it did not affect the rate of inhibition by iodoacetic acid; sodium acetate buffers were strongly inhibitory. Reactions with iodoacetate were protected from light. The data were fitted to the equation for a hyperbola, by means of a least squares method and on the assumption of equal variance of the velocities (23).

The carboxymethylation of DNase was studied with reaction mixtures of 0.4 ml final volume prepared as follows. To 0.1 ml of 4 mg per ml of dialyzed and lyophilized DNase A suspended in water was added 0.1 ml of 16 mM CuCl_2 in 0.1 M Tris-HCl buffer, pH 7.2, that was readjusted to pH 7.2 with 1 M NaOH; 0 to 0.2 ml of 0.05 M Tris-HCl buffer, pH 7.2, was added, and the reaction was initiated by the addition of 0 to 0.2 ml of 0.2 M reagent dissolved in 0.2 M NaOH and 0.05 M Tris-HCl buffer, pH 7.2. The final pH was 7.2 and the temperature of the solutions and reaction mixtures was 25°.

The relative chemical reactivity of tosylglycolate and the haloacetates was determined by the reaction with 4-[(p-nitrobenzyl)pyridine (24) according to the procedure of Baker and Jordan (25).

RESULTS

Reactivity of Tosylglycolate—To show that tosylglycolate was a carboxymethylating reagent, we studied its reaction with cysteine. Cysteine-HCl (8.75 mg, 50 μmole) and tosylglycolate (12.6 mg, 55 μmole) were dissolved in 5 ml of H_2O and brought to pH 8 with 0.11 ml of 1 M NaOH. The pH was maintained between 8 and 10 for 2 hours at room temperature, and the reaction was stopped by acidification to pH 1.2 with HCl. The solution was made up to 10 ml with H_2O and diluted 20-fold with pH 2.2 sample buffer. A 45% yield of carboxymethylcysteine was obtained, as well as 25% cysteine and 27% half-cysteine. A 3% yield of an unknown compound at the threonine position was also seen, but this could have been a contaminant in the cysteine.

RNase was slowly inactivated by tosylglycolate. The site of alkylation was determined by chromatographic analysis of the products of the reaction (Fig. 1) and by amino acid analysis. The major product from the tosylglycolate reaction corresponded in elution position to the major product from the iodoacetate reaction, that is 1-carboxymethylhistidine 119-RNase (26). A small amount of 3-carboxymethylhistidine 119-RNase (eluted at about 185 ml) was also present. (Some RNase A and some minor, active peaks eluted before the inactive derivatives) The ratio of histidine 119 to histidine 120 products was about 7:1, which agrees with the ratio found previously at 25° (21). Thus tosylglycolate reacts with somewhat more preference for histidine 119 than does iodoacetate.

The major product from the tosylglycolate reaction was desalted by gel filtration on a column of Sephadex G-25 (medium)
Fig. 1. Separation and identification of products of reaction of RNase with tosylglycolate or iodoacetate. A, RNase A (phosphate free), 20 mg, in 2 ml of 0.1 M sodium acetate buffer, pH 5.5, was treated with sodium tosylglycolate (36 pmoles) for 10 hours at room temperature and 12 hours at 37°, after which time the enzyme had lost 80% of its activity. The products were chromatographed on a column (0.9 × 57 cm) of sulfoethyl-Sephadex C-25 (fine (2.0 meq per g) equilibrated with 0.1 M sodium phosphate buffer containing 0.055% phenol, pH 6.60, at 25° with a flow rate of 10 ml per hour (26). B, RNase A, 11 mg, in 1.0 ml 0.1 M sodium acetate buffer, pH 5.5, was treated with sodium iodoacetate (36 pmoles) at 25° for 195 min, at which time the enzyme had lost 60% of its activity, and the products were chromatographed as in A. The peaks were located by analysis with ninhydrin and identified from their elution positions (26).

Fig. 2. Rates of inhibition of RNase by tosylglycolate. RNase A (1 mg per ml) in 0.2 M sodium 2-(N-morpholino)ethanesulfonate buffer, pH 5.50, was allowed to react at 37° with sodium tosylglycolate at the indicated concentrations. (2 × 40 cm) equilibrated with 5% acetic acid, lyophilized, and hydrolyzed to the amino acids. As compared to the composition of RNase A, there was one less histidine, and 0.9 residue of I-carboxymethylhistidine was found.

Kinetics of Carboxymethylation—When RNase was treated with more than a 100-fold molar excess of tosylglycolate, the inactivation followed pseudo first order kinetics (Fig. 2). Similarly, inactivation by bromoacetate, iodoacetate, and chloroacetate was pseudo first order. With all four reagents, however, as the concentration of reagent was increased the rate of inactivation increased, but not with first order dependence. The increase was hyperbolic, which is readily apparent from the double reciprocal plots (Fig. 3). The kinetic constants derived from such data are given in Table I. The relatively small standard errors leave little doubt that the data fit hyperbolic rather than first order kinetics.

The inactivation of DNase by carboxymethylating reagents was pseudo first order with respect to DNase activity, as shown previously (10). As with RNase, the rate of inactivation of DNase by tosylglycolate, bromoacetate, and iodoacetate gave hyperbolic dependence on the concentration of reagent, as shown by the double reciprocal plots (Fig. 4). The inactivation by chloroacetate, however, was first order with respect to its concentration (Fig. 4B). The kinetic constants for carboxymethylation of DNase are given in Table I.

In an experiment to test the effect of another metal ion, the enzyme was treated with 25 mM iodoacetate in the presence of 10 or 50 mM CdCl₂, at 25° and pH 7.2. The calculated second order rate constant was 0.14 M⁻¹ min⁻¹.

DISCUSSION

The hyperbolic dependence of the rate of inactivation of RNase on the concentration of carboxymethylating reagent supports the proposal (4) that the reagents bind reversibly to the enzyme before reacting chemically. Such kinetic behavior is expected for active-site-directed reagents (27, 28). The affinity of the reagent for the active site has been used to explain the faster rate of reaction of amino acid residues in the enzyme as compared to free amino acids. Since RNase binds the reagents relatively weakly, the precise orientation of the bound reagent apparently facilitates the chemical reaction. Dafforn and Koshland (29) have predicted that a properly oriented intramolecular (or en-
The relative reactivities of the reagents with 4-(p-nitrobenzyl)pyridine (NBP) in 75% 2-methoxyethanol at pH 4.2 and 37°C were also determined, and the pseudo bimolecular rate constants \( k/K \) were corrected for comparative purposes.

\[
k_{\text{obs}} = k_2/(K + I)
\]

with calculation of the standard errors. The relative reactivities of the reagents with 4-(p-nitrobenzyl)pyridine (NBP) in 75% 2-methoxyethanol at pH 4.2 and 37°C were also determined, and the pseudo bimolecular rate constants \( k/K \) were corrected for comparative purposes.

| Reagent | \( K \) | \( k_2/K \) | \( k/K \) | NBP |
|---------|--------|-------------|-------------|-----|
| RNase   |        |             |             |     |
| ClAc    | 19 ± 0.3 | 3.4 ± 0.02 | 0.17 ± 0.002 | 14  |
| BrAc    | 23 ± 2  | 120 ± 6    | 5.1 ± 0.03   | 8.4 |
| IAc     | 8.2 ± 0.6 | 25 ± 1  | 3.0 ± 0.1   | (3.0) |
| TG      | 24 ± 2  | 12 ± 0.6   | 0.50 ± 0.03  | 4.2 |
| DNase   |        |             |             |     |
| ClAc    | -.      | -.          | 0.51 ± 0.02  | 42  |
| BrAc    | 140 ± 10 | 970 ± 60  | 7.0 ± 0.2   | 11  |
| IAc     | 150 ± 30 | 110 ± 20  | 0.85 ± 0.06  | (0.85) |
| TG      | 150 ± 40 | 3.4 ± 0.6  | 0.022 ± 0.002 | 0.19 |

* XAc represents the halacetate; Tg is tosylglycolate.

† Magnitudes were too large to determine; \( k_2/K \) is second order rate constant.

As with RNase, the reaction of a histidine residue in DNase with iodoacetate is greatly facilitated. In contrast to RNase, DNase binds the reagents loosely, with dissociation constants larger than 0.1 M. If the affinity of the reactants actually facilitates the reaction, the orientation of reactants in the DNase-inhibitor complex must be more favorable for reaction than in the RNase-inhibitor complex. However, with DNase, the magnitude of the facilitation \( k/K \) (Table I) increases as the size of the leaving group decreases. Steric hindrance could decrease the rates of reaction of tosylglyclolate and iodoacetate as compared to chloro- and bromoacetates. On the other hand, DNase may have a catalytic group that facilitates the formation of the transition state by interacting with the leaving group. Since a divalent metal ion is required for carboxymethylation, the ion could act as an electrophilic catalyst. The relative rates of reaction, chloro > bromo > iodo, and Cu^{2+} > Cd^{2+} > Mn^{2+} > Ca^{2+} (10), are consistent with the stabilities expected for complexes of the metal halides (31).

It should be noted that a variety of divalent metal ions, including Cu^{1+}, activate DNase for hydrolysis of DNA (32, 33). The mechanism of carboxymethylation of DNase may involve ionic attraction of the carboxylate ion and electrophilic catalysis by Cu^{2+}. Comparison of the carboxymethylation of RNase and DNase indicates that
these enzymes facilitate (catalyze) active-site-directed reactions by orienting the reactants or by using catalytic groups or both.

Tosylglycolate carboxymethylates sulphydryl and imidazole groups and is about one-eighth as reactive as iodoacetate with a nucleophilic nitrogen base. Tosylglycolate is not specially reactive with RNase and has no more affinity for the active site than do haloacetates. It reacts with histidines 119 and 12 with a ratio of about 17:1, and thus is more selective for histidine 119 than are the haloacetates, but less selective than some long chain α-bromo acids (3). The reagent may be used to carboxymethylate proteins that react too fast with iodo- or bromoacetate and too slowly with chloroacetate, or where the halide ions interfere with reaction. Alternatively, it may be used where the affinity of the reagent for the active site may be a significant factor in obtaining a specific reaction. While this work was in progress, Nakagawa and Bender (34) used methyl p-nitrobenzenesulfonate to methylate histidine 57 in chymotrypsin. Since tosylates are readily prepared from alcohols, active-site-directed reagents of this type are available.

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