Identification of Histidine-119 as the Target in the Site-specific Inactivation of Ribonuclease A by Ferrate Ion

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Ferrate ion, a powerful oxidant which is an analog of orthophosphate ion, has previously shown some promise as a site-specific probe of enzymes which interact with phosphate compounds. In order to explore the general applicability of this reagent, it has been tested against ribonuclease A, an enzyme whose structure and active center have been well described. Treatment with a molar ratio of ferrate to enzyme of less than 20 leads to a loss of 87% of the activity. The known competitive inhibitors, 2'-cytidylic acid, inorganic pyrophosphate, and histidine-119, whose essential role at the active center has been established (2), directed the phosphate binding site. Further work from Benisek's laboratory has established tyrosine-75 as the amino acid which is oxidized (2).

Rajababu and Axelrod (3) found that all phosphatases of a large number tested are inactivated by ferrate ion in a site-specific manner. Those enzymes tested which do not utilize phosphate were not appreciably affected by the reagent.

The present study was undertaken to locate precisely the region modified by the reagent. Pancreatic RNase was an obvious choice due to the detailed information on its structure and function and its ready availability. Despite the ephemeral existence of the ferrate at the pH range utilized, conditions had to be defined to minimize the possibility of overoxidation of the enzyme and hence of irrelevant nonspecific destruction of amino acid residues. The results implicate a single amino acid residue, histidine-119.

MATERIALS AND METHODS

Chemicals—Bovine pancreatic RNase A, type II-A; bovine pancreatic RNase S-protein, type XII-A; bovine pancreatic RNase S-peptide, type XII-S; Protease type VIII (subtilopeptidase A) and 2'-CMP were all products of Sigma. Soluble RNase from yeast was obtained from General Biochemicals. Tosylamidophenylthiolsulfomethyl ketone (TPCK)-treated trypsin was purchased from Worthington. Staphylococcus aureus V8 protease was from Miles Laboratories. Potassium ferrate was synthesized according to the method of Thompson et al. (5). All other chemicals were of reagent grade.

Enzyme Assays—RNase A was assayed essentially as described by Kalnitsky et al. (6) in the following manner: 2.6 ml of sodium cacodylate buffer, 25 mM, pH 7.0, and 0.3 ml of 0.1% RNA were placed in a 1.0-cm quartz cuvette and mixed. The reaction was initiated by adding the enzyme as an 0.1-ml aliquot and mixing with a plastic paddle. The decrease in absorption at 290 nm was followed in a Perkin-Elmer Spectrophotometer model 575 at 25°C.

Amino Acid Analysis—Protein samples were hydrolyzed in 6 n HCl at 110°C for 24, 48, or 72 h. After removing the HCl at 70°C in vacuo, the samples were analyzed on a Durrum model D-500 amino acid analyzer. Cysteine residues were determined as free cysteine or as the carboxymethylated product using the method of Crestfield et al. (7). Correction factors for cysteine and tyrosine following acid hydrolysis were 1.23 and 1.14 following the experience of Cha and Scheraga (8).

Preparation of RNase S—RNase S was prepared by the method of Doscher and Hirs (9). The maximum yield of RNase S was obtained after 6 to 10 h as determined by trypsin digestibility (10). Fractionation of S-protein and S-peptide was done with trichloroacetic acid by the method of Richards and Vithayathil (4). Additional purification of the S-protein was accomplished by the procedure of Bradbury et al. (11).

Preparation of Ferrate-inactivated RNase A—RNase A was inactivated with potassium ferrate in 0.1 M sodium acetate buffer, pH 5.0. In a typical large scale preparation, 50 to 100 mg of RNase was dissolved in a sufficient volume of the above buffer to ensure that the added ferrate caused no significant change in pH. A stock solution of ferrate (6.0 mM) was prepared in cold 10⁻⁴ M NaOH. The ferrate was added to the RNase in aliquots until the residual activity was about 10% or less of the original activity. For preparation of ferrate-inactivated RNase S, the enzyme was first cleaved with subtilisin. Ferric hydroxide was generally separated from ferrate-inactivated RNase by chromatography on a Sephadex G-10 column (3.5 × 35 cm) which was equilibrated and eluted with 9% formic acid.

Ligand Binding of RNase A—Binding of 2'-CMP to RNase was determined by the gel filtration method of Hummel and Dreyer (12). The Sephadex G-25 column (0.5 × 115 cm) was equilibrated with 0.1 M sodium acetate buffer, pH 5.0, containing 80 μM 2'-CMP, until the absorbance at 285 nm was constant. RNase A, either native or ferrate-inactivated (10% residual activity), was dissolved in 100 μl of the same buffer and loaded on the column. Elution was at a rate of 0.9 ml/min and fractions of 0.3 ml were collected. The absorbance at 285 nm was determined for each fraction.

Cloning of Ferrate-inactivated RNase A with Cyanogen Bro-

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1 RNase S is the noncovalent complex of the two fragments obtained by the subtilisin-catalyzed hydrolysis of the peptide bond between amino acid residues 20 and 21. The smaller fragment is the "S-peptide." The larger is the "S-protein" (4).
Ferrate Inactivation of Ribonuclease

3255

Ferrate—Ferrate-inactivated RNase A (100 mg) was dissolved in 5.0 ml of 70% formic acid and 0.35 g of CNBr was added. The solution was kept in the dark for 24 h, after which it was diluted 10-fold with water and immediately lyophilized. The lyophilized digest was reduced and carboxymethylated by the method of Crestfield et al. (7). The peptides were separated on a Sephadex G-50 (superfine) column (2.5 x 100 cm) which was equilibrated and eluted with 9% formic acid.

Digestion of the Cyanogen Bromide Peptide 80-124 with Trypsin—Seven milligrams of CNBr peptide 80-124 was dissolved in 2.0 ml of 50 mM NH4HCO3, pH 8.0. Approximately 70 µg of TPCK-treated trypsin was added in 150 µl of H2O. The mixture was incubated at 37°C for 4 h and then lyophilized. The lyophilized peptides were dissolved in a minimal amount of 9% formic acid and separated on a Sephadex G-25 column (1.0 x 100 cm) by elution with the same solvent. Fractions of 50 drops were collected and the absorbance at 280 nm was measured. The A280 absorbing material that eluted at the void volume was pooled and lyophilized. This material was shown by automated amino acid sequence analyses to be peptide 105-124 in a state of approximately 75% purity. This peptide was used for sequencing without further purification.

Sequential Analysis of Ferrate-inactivated RNase A—All sequence analyses of RNase were done by the method of Hermodson et al. (13) in a Beckman 890 C sequenator.

Digestion of Ferrate-oxidized RNase A with S. aureus Protease V8—Thirty milligrams of ferrate-oxidized RNase (75% residual activity) was dissolved in a minimal volume of 50 mM acetic acid and brought to pH 4.0 with NaOH. Protease V8 (0.3 mg) was added and the mixture was allowed to incubate for 20 h at 37°C (14). The incubation mixture was loaded directly on a Sephadex G-50 column (2.5 x 100 cm) and eluted with 9% formic acid. Fractions containing small peptides were pooled and lyophilized.

Table I

| Enzyme species       | Enzymatic activity |
|----------------------|--------------------|
| RNase A              | 100                |
| RNase A (treated)    | 2                  |
| RNase S              | 97                 |
| RNase S (treated)    | 2                  |
| RNase S (treated)    | 10                 |
| RNase S (treated) + S-peptide | 50            |
| S-Peptide + S-protein | 2               |
| S-Peptide (treated) + S-protein | 100          |
| S-Peptide + S-protein (treated) | 92            |
| S-Peptide + S-protein (treated) | 10             |

The abbreviation used is: TPCK, tosylamidophenylethylchloromethyl ketone.

RESULTS

Inactivation of RNase A and RNase S by Ferrate Ion—Ferrate has been shown to be a site-specific inactivator of phosphate-binding enzymes (1-3, 15). RNase A, which has a substrate phosphate binding site, is also inactivated by ferrate. Molar ratios of ferrate to RNase of less than 20 are sufficient to inactivate RNase to 13% of the original activity (Fig. 1).

RNase S is also inactivated by ferrate (Table I). Assays of the S-protein and S-peptide from ferrate-treated RNase S shows that the modification is in the S-protein.

The pH of the incubation mixture appears to be critical to the ferrate inactivation. Because the oxidation potential of ferrate increases as the pH decreases (16), care must be taken to utilize the optimal pH conditions for the inactivation procedure. An advantage to using ferrate as a site-specific oxidation probe is that at lower pH ferrate is reduced by H2O with the release of O2 (17) and thus there is no residual ferrate ion to oxidize amino acid residues randomly. The importance of pH control in preventing overoxidation became evident when preliminary inactivations were carried out at pH 7.0. Amino acid analyses of ferrate-inactivated RNase A at this pH revealed that approximately 2 tyrosines, 2 lysines, and 1 histidine were destroyed. When the inactivation was performed at pH 5.0 only 1 histidine was destroyed.

Protection from Ferrate Inactivation by Competitive Inhibitors—If ferrate is indeed a phosphate site-specific reagent, then inhibitors known to bind at the phosphate site should protect against inactivation. Lee and Benisek (1) found that 5'-AMP, 2'-AMP, 3'-AMP, and 5'-IMP all provided substantial protection to ferrate inactivation of phosphorylase b. Rajababu and Axehod (3) later showed that competitive inhibitors protected different phosphatases from ferrate inactivation, and Walker and Axehod (15) obtained similar results with lactate dehydrogenase. RNase A was shown to be protected by competitive inhibitors from ferrate inactivation (Table II). 2'-CMP, pyrophosphate, and orthophosphate all afforded protection from ferrate inactivation as was expected.

Binding of 2'-CMP by Ferrate-inactivated RNase A—Walker and Axehod (15) demonstrated that ferrate inactivation of lactate dehydrogenase resulted in loss of NADH binding, although it did not affect the ability of the enzyme to bind to Cibacron Blue F3G-A. Phosphorylase b lost the ability to bind 5'-AMP following ferrate inactivation (1). To examine the binding of 2'-CMP to ferrate-inactivated RNase A the gel filtration method of Hummel and Dreyer (12) was employed. As may be seen in Fig. 2, the ferrate-treated enzyme, unlike the untreated enzyme did not retard the elution of 2'-CMP. This result suggests that ferrate is acting at or near the active site of ribonuclease in a site-specific manner.

Amino Acid and Sequential Analysis of Ferrate-inactivated RNase—In an effort to locate the amino acid residue(s) attacked by ferrate, the amino acid composition of ferrate-inactivated RNase was determined. Histidine and tyrosine were approximately 1 residue lower than the control values and lysine and methionine were fractionally lower (Table III). Due to the inherent errors of this technique the analyses were...
TABLE II

Protections to ferrate inactivation of RNase A

| Substance tested | Native RNase | Ferrate-oxy. RNase A | Ferrate-oxy. CNBr peptide 50-124 | Ferrate-oxy. peptide 1-2, 3-9, and 112-124 |
|------------------|-------------|---------------------|----------------------------------|-------------------------------------------|
|                  | m.w.        | %                   | %                               | %                                         |
| 2’-CMP           | 0.10        | 85                  | 85                              |                                            |
| 2’-CMP           | 0.01        | 31                  | 35                              |                                            |
| Pyrophosphate    | 0.40        | 50                  | 72                              |                                            |
| Pyrophosphate    | 0.10        | 28                  | 39                              |                                            |
| Orthophosphate   | 40.0        | 51                  | 83                              |                                            |
| Orthophosphate   | 10.0        | 20                  | 54                              |                                            |

not definitive in determining which residue(s) were affected by the ferrate. In order to see if tyrosine was oxidized by ferrate, peptide fragments which contained all 6 tyrosines in the original RNase were isolated from the ferrate-treated RNase. These peptides were sequenced and were found to contain all of the tyrosines in the expected amounts.

Ribonuclease A contains 4 histidine residues at positions 12, 48, 105, and 119 (18). Two of these histidine residues, 12 and 119, have been shown to be at the active site and appear to be involved in the enzymatic catalysis (18). The cyanogen bromide peptides of RNase were separated and their amino acid composition determined in an effort to locate the histidine which had been oxidized by ferrate. Fragment 1-13 yielded 1 residue of histidine, as did Fragment 31-79, thus eliminating His-12 and -48 as oxidation sites. Fragment 80-124 yielded 1.25 residues of histidine instead of 2 (Table III), eliminating His-105 and -119 (18). Two of these histidine residues, 12 and 119, have been shown to be situated at the active site and appear to be involved in the enzymatic catalysis (18). The cyanogen bromide peptides of RNase were separated and their amino acid composition determined in an effort to locate the histidine which had been oxidized by ferrate. Fragment 1-13 yielded 1 residue of histidine, as did Fragment 31-79, thus eliminating His-12 and -48 as oxidation sites. Fragment 80-124 yielded 1.25 residues of histidine instead of 2 (Table III), implicating either His-105 or His-119 as the residue oxidized by ferrate.

To establish the identity of the oxidized residue sequential analysis was performed on the tryptic peptide 105-124 which was obtained from the CNBr Fragment 80-124. The sequence analysis was precisely as expected except that His-119 did not appear. Histidine-105 was recovered quantitatively. Thus, it may be concluded that histidine-119 is the amino acid residue which is oxidized by ferrate.

To substantiate this finding, ferrate-oxidized RNase A was hydrolyzed with S. aureus V8 protease in acetate buffer pH 4.0. Under these conditions the enzyme is specific for peptide bonds on the carboxyl side of glutamic acid residues (14). Since the RNase was not subjected to disulfide reduction, one can expect three peptides, 1-2, 3-9, and 112-124, which are readily resolved by gel filtration on Sephadex G-50 from the larger peptide 10-111. The individual small peptides which were poorly separated were pooled and analyzed. The amino acid analysis corresponded to the expected mixture. Only 0.22 residues of histidine was found, confirming the above results since the only histidine present in the intact enzyme in Fragments 1-2, 3-9, and 112-124 is at 119.

Effect of Ferrate Treatment on the Histidine Content of RNase as Determined with Diethylpyrocarbonate—Diethylpyrocarbonate is a fairly specific modifying reagent for histidine and is analytically useful in its determination owing to the increase in absorption of the ethoxy-formylated histidine at 240 nm (19). Melchior and Fahrney have used this 14C-labeled reagent on RNase A and found that only 3 of the 4 residues are modified (20). They attribute their results to the inaccessibility of one of the histidines. Roosemont (21) has recently shown that the inaccessible histidine residues of

TABLE III

Amino acid composition of native and ferrate-inactivated RNase

Ferrate treatment of RNase and the CNBr and proteolytic degradations with S. aureus protease are described under "Materials and Methods." Values in parentheses indicate expected values of the amino acid residues per molecules of RNase based on the literature (18).

| Amino acid | Native RNase | Ferrate-oxy. RNase A | Ferrate-oxy. CNBr peptide 50-124 | Ferrate-oxy. peptide 1-2, 3-9, and 112-124 |
|------------|--------------|----------------------|----------------------------------|-------------------------------------------|
| Aspartic acid | 14.91 (15) | 14.94 (15) | 6.01 (5) | 2.52 (2) |
| Threonine | 9.96 (10) | 10.39 (10) | 3.65 (4) | 1.01 (1) |
| Serine | 15.89 (15) | 14.43 (15) | 3.47 (4) | 0.99 (1) |
| Glutamic acid | 12.00 (12) | 12.90 (12) | 4.00 (3) | 2.26 (2) |
| Proline | 4.57 (4) | 4.46 (4) | 3.06 (3) | 2.36 (2) |
| Glycine | 3.87 (3) | 3.32 (3) | 2.00 (3) | 1.34 (1) |
| Alanine | 12.35 (12) | 11.59 (12) | 4.10 (4) | 3.68 (4) |
| Valine | 8.86 (9) | 8.53 (9) | 3.83 (4) | 3.33 (3) |
| Methionine | 3.87 (4) | 3.51 (4) | 0.00 (0) | 0.08 (6) |
| Isoleucine | 2.67 (3) | 2.82 (3) | 2.44 (3) | 0.07 (0) |
| Leucine | 2.41 (2) | 2.16 (2) | 0.00 (0) | 0.11 (0) |
| Tyrosine | 6.50 (6) | 5.42 (6) | 2.85 (3) | 1.02 (1) |
| Phenylalanine | 3.09 (3) | 2.76 (3) | 0.90 (1) | 1.81 (2) |
| Histidine | 3.93 (4) | 2.90 (4) | 1.25 (2) | 0.22 (1) |
| Lysine | 10.30 (10) | 9.79 (10) | 3.51 (3) | 1.99 (2) |
| Arginine | 4.02 (4) | 3.83 (4) | 1.22 (2) | 0.02 (0) |
| Cysteine | 7.98 (8) | 7.87 (8) | 3.40 (3) | 0.00 (0) |

* Values given are the average of five independent ferrate inactivation and hydrolyses. Glutamic acid was taken as the basis for molar ratios.

** Values are time zero extrapolations from 24-, 28-, and 72-h hydrolysis.

† Corrected by 5% (Thr) and 12% (Ser) (8).

de Corrected as stated under "Materials and Methods."

& Determined as carboxymethylcysteine.

TABLE IV

Effect of ferrate treatment on the number of diethylpyrocarbonate-reactive histidine residues in RNase A

Histidine was determined spectrophotometrically by Roosemont's procedure (21). The determinations were made by adding 0.1 ml of an alcoholic solution of diethylpyrocarbonate to 2.9 ml of 100 mM acetate buffer, pH 5.0, containing 1.95 mg of the enzyme, and 6.7 M urea (when used), and incubating 20 min at room temperature. The ferrate-treated RNase (7% active) was prepared as described under "Materials and Methods." The action of diethylpyrocarbonate on histidine was followed by the change in optical density at 240 nm. The molar ratio of diethylpyrocarbonate to nominal histidine was 12 in the absence of urea and 120 in its presence.
native proteins can be made quantitatively reactive with the reagent by recourse to higher ratios of the reagent in the presence of denaturants such as urea or sodium dodecyl sulfate. We confirmed the results of Melchior and Fahney that native RNase has only 3 accessible histidine residues when tested with the normal concentration of the reagent. The ferrate-incubated enzyme, we found, contained only 2 sulfate. We confirmed the results of Melchior and Fahrney presence of denaturants such as urea or sodium dodecyl sulfate. We confirmed the results of Melchior and Fahrney a 10-fold increase in the diethylpyrocarbonate in the presence of urea, the native enzyme showed 4 reactive residues, while the ferrate-treated enzyme showed the expected 3 (Table IV).

DISCUSSION

The present results clearly demonstrate that the inactivation of RNase A by ferrate ion is specifically targeted at the active site. The only amino acid modified in this reaction, His-119, has long been implicated in the active center of this enzyme (see review by Richards and Wyckoff (18)). It is well known that iodoacetate can alkylate histidine-12 or histidine-119, although not both in the same enzyme molecule (16), indicating that the 2 histidines are very near each other. However amino acid analysis of the CNBr peptide 1-13 obtained from ferrate-treated RNase gave 1 residue of histidine, showing that only insignificant amounts of His-12 could have been oxidized. Recently, Antonov et al. (22) studying complexes of RNase A with 2'-deoxy-2'-fluororibose substrate analogues by NMR techniques have obtained results which are consistent with a close association between protonated His-119 and the phosphate group of the substrate. The isolation of a phosphohistidine moiety from rat liver microsomal glucose-6-phosphatase by Feldman and Butler (23), from rat liver phosphatase by Igarashi et al. (24), from wheat germ phosphatase by Hickey et al. (25) and from human prostate acid phosphatase by McTigue and Van Etten (26) provide evidence that histidine may be involved in the active centers of some phosphatases. On the other hand, the phosphate intermediate of alkaline phosphatase of Escherichia coli, yields phosphoserine (27); thus one cannot maintain without further evidence that all phosphatases act through essential histidines, although this possibility cannot be excluded. There are abundant examples of other enzymes which utilize phosphate substrates and which appear to act through phosphohistidine intermediates. Examples are nucleoside diphosphokinase (28, 29), ATP-citrate lyase (30), phosphoglycerate mutase (31) bacterial phosphotransferase systems (32, 33), phosphoenolpyruvate synthetase (34), and succinyl-CoA synthetase (35, 36).

In previous work in this laboratory, it was shown that all of a large number of phosphatases examined were inactivated by low concentrations of ferrate ion. In those cases examined, the inactivation was found to occur in a site-specific manner, although no effort was made to identify the modified amino acid residues. Recently, Lee and Benisek have reported that tyrosine-75 is oxidized when rabbit muscle phosphorylase b is inactivated by ferrate (2). In this instance the enzyme loses its activity because its capacity to interact with its activator, 5'-AMP, is lost. We have also found that dehydrogenases utilizing pyridine nucleotide were also inactivated in a site-specific manner (15). These results encourage the view that ferrate inactivation may be a useful tool in probing active centers of enzymes which utilize phosphates as substrates or effectors.

The utility of ferrate ion for such studies is contingent on the presence of an easily oxidizable amino acid closely positioned to the ferrate ion, which presumably binds at the phosphate site. It is known that the reagent readily oxidizes amines and alcohols (37). The potentially vulnerable amino acid residues include tyrosine, serine, threonine, histidine, tryptophan, methionine, lysine, cysteine, and possibly cystine. In preliminary studies, the three amino acids we have examined, lysine, histidine, and tyrosine, were found to be oxidized when their carboxyl and α amino groups are protected. Despite the fact that we found but one amino acid was modified in RNase, it must be recognized that had methionine been oxidized to a sulfoxide, its reaction would not have been detected in the amino acid analysis following HCl hydrolysis. Ray and Koshland (38) discovered that under those conditions 85% of the sulfoxide is converted back to methionine. It was possible, however, to rule out any substantial formation of the sulfoxide on the basis of the CNBr fragmentation pattern and sequence analysis. CNBr cannot cleave at a methionine sulf oxide (39).

Ferrate, unlike other site-specific reagents which consist of a chemically reactive group attached to a substrate-site-seeking group, is both the reactive group and binding group. The preferred site of action is presumably in the area where its effective concentration is high, namely in the binding area. However other vulnerable sites, which abound in proteins, might also be slowly attacked in a nonspecific manner. Fortunately the ferrate ion has only an ephemeral existence, and thus, its time-averaged concentration must be considerably less than the nominal value based on its initial concentration. Experience has shown us that at pH 5.0 nonspecific oxidation is at a minimum. The oxidation-reduction potential of ferrate ion is known to increase with $[H^+]$ (16), while the rate of reduction by water increases (17). As a consequence there is little residual ferrate to act nonspecifically on the sites not favored by the concentrating mechanisms of the substrate binding site. Earlier work at pH 7.0 resulted in the conversion of about 2 residues of lysine to lysine δ-semialdehyde. Nonspecific oxidation is minimized by working at pH 5.0 and titrating the enzyme by repeated addition of small amounts of ferrate to about 90 to 95% inactivation.

RNase is, of course, among the best described and understood enzymes. The present work was not undertaken to elucidate the behavior of this enzyme but to examine the action and ability of ferrate ion as a site-specific probe for the enzymes which utilize phosphate compounds. Our results encourage the belief that ferrate ion will prove generally useful for the exploration of many of the enzymes belonging to this class, as was suggested by the original work of Lee and Benisek (1).

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