Carbon-Fluorine Bond Cleavage

II. STUDIES ON THE MECHANISM OF THE DEFLUORINATION OF FLUOROACETATE

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SUMMARY

Studies on the mechanism of the reaction

$$XCH_2COO^- + OH^- \rightarrow X^- + HOCH_2COO^-$$

(where X = F, Cl, and I) catalyzed by the enzyme haloacetate halidohydrolase indicate that the hydroxyl group of glycolate is derived from water. No evidence can be obtained for the reversibility of the dehalogenation. In addition, the exchanges between glycolate and $H_2^{18}O$ and between chloroacetate and $^{36}Cl^-$ are not catalyzed by the enzyme.

The action of horseradish peroxidase on $p$-fluoroaniline was the first enzyme-dependent cleavage of the carbon-fluorine bond to be described (1). Other apparently adventitious cleavages of this relatively stable chemical bond are catalyzed by several mixed function oxidases. These include the action of a phenylalanine-hydroxylating system on $p$-fluorophenylalanine (2) and the action of an aniline-hydroxylating system on $p$-fluorophenol (3). It is not clear whether a similar mechanism is responsible for the defluorination of 4-fluoroproline that occurs in collagen biosynthesis (4).

Recently pseudomonads which can utilize fluoroacetate as a sole carbon source have been isolated from soil (5-7). Such organisms have yielded an enzyme which carries out the stoichiometric conversion of fluoroacetate to glycolate and fluoride (7). The cleavage of this carbon-fluorine bond differs from those above in two respects: the fluoride is released from an aliphatic carbon atom rather than from a carbon atom in an aromatic ring, and the hydroxyl group of the glycolate apparently is derived from water rather than from reduced atmospheric oxygen.

In this paper evidence is presented that the hydroxyl group replacing the fluoride in fluoroacetate is indeed derived from water. In addition, experiments are described suggesting that the dehalogenation reaction is irreversible. On the basis of the data now available, the name haloacetate halidohydrolase is proposed for the enzyme catalyzing the reaction

$$XCH_2COO^- + OH^- \rightarrow X^- + HOCH_2COO^-$$

(where X = F, Cl, and I).

METHODS

The assay of the enzyme by measurement of the release of fluoride from fluoroacetate and its 30-fold purification by precipitation with protamine and ammonium sulfate, followed by chromatography on DEAE-cellulose, have been described (7). The 30-fold purified enzyme was used in the following experiments. Boiled enzyme was prepared by immersion for 5 min in boiling water and was shown to be inactive in the defluorination reaction.

The separation of glycolate from fluoroacetate on Celite 535 by a modification (7) of the method of Swim and Utter (8) was further extended to separate chloroacetate from these compounds and $Cl^-$. In this method the acidified sample was added to the column, which then was eluted with chloroform that had been equilibrated against 0.2 N $H_2SO_4$. Eight-milliliter fractions were collected, and the chloroacetate which appeared in tubes 7 through 9 was titrated to a bromthymol blue end point with 0.01 N $NaOH$ in ethanol. To assay for $^{36}Cl^-$, an aliquot of each neutralized sample was evaporated to dryness in a planchet and the radioactivity was determined in a Nuclear-Chicago, low background, end window, gas flow counter.

Mass spectra were recorded on an Associated Electrical Industries MS-9 double focusing mass spectrometer, with an ionizing voltage of 70 e.v. and an ion source temperature of 80° ± 20°. Accurate mass to charge ratios were measured by peak matching with $Na^+$ as a reference below $m/e$ 50 and perfluorotributylamine above $m/e$ 50. Sodium glycolate, isolated from the reaction mixture by chromatography on Celite 535, was prepared for mass spectrometry by dissolving it in 1.0 N $HCl$.

RESULTS

Source of Hydroxyl Group of Glycolate—The displacement of fluoride by an oxygenase suggests that the hydroxyl group in tyrosine formed from $p$-fluorophenylalanine contains an oxygen atom derived from atmospheric oxygen (9, 6). However, in the defluorination of fluoroacetate, the hydroxyl group of glycolate is derived from water as is shown by experiments with $H_2^{18}O$ that are described in Fig. 1. Crystalline glycolic acid gave the mass spectrum in Fig. 1A, which was identical with that given

1 Reference 7 is Paper I of this series.
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Fig. 1. Incubation with H$_2^{18}$O. Incubation mixtures contained 40 pmoles of 2-amino-2-methyl-1,3-propanediol-HCl buffer at pH 9.1 and 3.6 units of enzyme, and were 30% enriched for H$_2^{18}$O. The following additions were also made: B, 20 pmoles of FCH$_2$COONa; C, 20 pmoles of HOCH$_2$COONa; in another experiment, not shown because the spectrum was identical with that of C, 20 pmoles of HOCH$_2$COONa and 20 pmoles of NaF were added. The total volume was 0.32 ml, and the incubation, conducted at 30° for 80 min, was terminated by bringing the reaction mixture to 0.2 N H$_2$SO$_4$. Sodium glycolate was then isolated by chromatography on Celite and the mass spectra were measured. A is the spectrum of authentic HOCH$_2$COOH; B and C are the spectra of glycolate isolated from the corresponding reaction mixtures described above.

by a solution of sodium glycolate in 1 N HCl except that in the latter case ions from H$_2$O (m/e 17 and 18) and HCl (m/e 35, 36, 37, and 38) are observed. These are omitted from all the spectra in Fig. 1. The base peak of the spectrum of glycolic acid is at m/e 31 and is due to the stabilized ion CH$_2$OH$^+$. The remainder of the spectrum consists of a small molecular ion at m/e 76 (HOCH$_2$COOH$^+$) and slightly more abundant ions at m/e 44 (COO$^-$) and 45 (COOH$^+$).

The mass spectrum of glycolic acid derived from fluoroacetate in a medium containing 30% H$_2^{18}$O is shown in Fig. 1B. The base peak at m/e 31 is in this case accompanied by a peak of about 31% of the intensity at m/e 32. Similarly, a new molecular ion at m/e 78 has appeared. These new peaks, which are absent in the spectrum of normal glycolic acid, have their accurate mass to charge ratios given in Table I. The results clearly establish that there has been a 30 ± 5% incorporation of $^{18}$O at the C-2 hydroxyl of glycolic acid.

When glycolate is substituted for fluoroacetate in the incubation mixture, the glycolate isolated from the system gives the mass spectrum shown in Fig. 1C. This spectrum is identical with that of normal glycolate, indicating that $^{18}$O exchange does not occur between H$_2^{18}$O and glycolate. Similarly, no exchange with glycolate occurs when 0.01 M NaF is added to the reaction mixture. This may be considered as evidence for the irreversibility of the over-all enzymatic conversion of fluoroacetate to glycolate.

Further Studies on Reversibility of Reaction—The inhibition of the enzyme by sulphydryl-binding reagents and its pH optimum above 9 suggested that a mercaptide ion on the enzyme acts to displace fluoride as well as other halides from halogen-substituted acetate. The following mechanism has been proposed (7).

\[
\text{Enz-S-} + \text{XCH$_2$COO}^- \rightarrow \text{Enz-S-CH$_2$COO}^- + \text{X}^- \\
\text{Enz-S-CH$_2$COO}^- + \text{OH}^- + \text{Enz-S-} + \text{HOCH$_2$COO}^- 
\]

(where X = Cl, F, and I). The first part of this mechanism is supported by the nonenzymatic removal of halides from iodo-, bromo-, and chloroacetic acid at slightly alkaline pH by glutathione to yield a thioether (10).

The action of the enzyme in liberating chloride from chloroacetate suggested an experiment to determine whether the first part of the proposed reaction mechanism is reversible, namely an examination for $^{18}$Cl exchange with chloroacetate. From

| TABLE I |
|---|---|---|---|
| m/e found | Formula | m/e calculated |
| 31.0189 | CH$_3^{18}$OH | 31.0184 |
| 33.0235 | CH$_2^{18}$OH | 33.0230 |
| 76.0170 | C$_4$H$_7$O$_2$ | 76.0160 |
| 78.0207 | C$_4$H$_7^{18}$O$_2$ | 78.0203 |

| TABLE II |
|---|---|---|---|
| Isolation of chloroacetate after incubation with Na$^{18}$Cl |
The reaction mixture contained 100 pmoles of sodium chloroacetate, 50 pmoles of Tris-HCl buffer at pH 9.1, 7 units of enzyme, and the amount of Na$^{18}$Cl specified. Incubation in a volume of 0.5 ml was conducted for 45 min at 30° and was terminated by the addition of 1 N H$_2$SO$_4$. Chloroacetate was then isolated and determined by titration of the eluent from a Celite column. The radioactive material in the fractions containing chloroacetate was assayed and the results are tabulated.

| Conditions | Na$^{18}$Cl added | Chloroacetate Isolated |
|---|---|---|---|
| | pmols | cpm | pmols | cpm |
| Experiment 1 | | | | |
| Enzymatic | 407 | 4,600,000 | 86 | 440 |
| Boiled enzyme | 407 | 4,600,000 | 100 | 472 |
| Experiment 2 | | | | |
| Enzymatic | 57 | 575,000 | 81 | 51 |
| Boiled enzyme | 57 | 575,000 | 101 | 50 |
Table II it can be seen that, as expected, the enzyme catalyzes the disappearance of chloroacetate. However, the $^{36}$Cl associated with the chloroacetate shows no difference from the boiled enzyme control. The small amount of radioactive material associated with the chloroacetate peak (about 0.01% of the starting material) did not chromatograph identically with chloroacetate and was not identified.

**DISCUSSION**

From the $^{18}$O experiments, it may be concluded that in the reaction $X\text{CH}_2\text{COO}^- + \text{OH}^- \rightarrow \text{HOCH}_2\text{COO}^- + X^-$ the oxygen of the hydroxyl group is derived from water. Furthermore, the over-all reaction does not appear to be reversible, as shown by the failure to detect $^{18}$O in the glycolate isolated after the incubation of glycolate and fluoride in $\text{H}_2^{18}\text{O}$.

The lack of reversibility in the over-all reaction appears to extend to both parts of the mechanism proposed above in that, if Part I were reversible, one would expect the exchange of isotope between $^{36}$Cl and chloroacetate, while, if Part II were reversible, one would expect $^{18}$O to exchange between $\text{H}_2^{18}\text{O}$ and glycolate.

**REFERENCES**
