Number of Formyl Groups in the Formic Acid Inactivated Protein Enzymes

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It has been clearly demonstrated that the formic acid induced reversible inactivation of protein enzymes is accompanied by reversible formylation.[1-5] From these studies it was also suggested that the formylation occurred in the hydroxyamino acid residues even though controversial conclusions were made about the nature of the reaction. However, attempts to demonstrate the number of formyl groups involved in the reaction by using radioactive formic acid-^{14}C were unsuccessful.[2] The large amount of unbound formic acid and ionically bound formate observed to be attracted to the proteins after the formic acid treatment made any conclusion about the relation between the formate carbon and the content of hydroxyamino acid residues impossible. Gel-filtration [6] removes, however, this interfering formate without any accompanying reactivation of the enzymes, and the number of formyl groups involved in the reversible inactivation of ribonuclease and lysozyme has now been determined.

Inactivation of the enzymes (egg lysozyme hydrochloride and bovine pancreatic ribonuclease, formerly characterized with respect to purity [7]) was carried out by the same procedure as previously described,[7] but by using ^{14}C-labelled formic acid. This was prepared by the addition of sodium formate-^{14}C (Amersham, England) direct to freshly distilled anhydrous formic acid. The specific activity of the radioactive formic acid was 940 counts/min/μmole. The inactivated, ethyl ether precipitated enzyme was dissolved (10 mg/ml) in a weakly acidic (pH 5.0), 0.05 M sodium chloride solution and filtered through a 30×1.2 cm column of Sephadex G-25, medium (Pharmacia, Uppsala) which had been previously equilibrated with 0.05 M sodium chloride solution at pH 5.0. The sample was eluted with the same salt solution and the effluent was collected in 2 ml fractions. Samples from each fraction were withdrawn for protein analysis [8] and radioactivity assays, made by using copper planchets with a thin end-window Geiger-Müller tube on samples of infinite thinness. Before being assayed for radioactivity, the pH of the samples was adjusted to pH 11 with 0.1 N sodium hydroxide. Samples withdrawn from the protein containing fractions were also assayed for radioactivity without pH-adjustment, yet no difference was found in the values.

The fractions containing protein were collected. The solution was adjusted to pH 7.5 with 0.05 N sodium hydroxide and the subsequent alkali utpake was measured with the aid of a pH-stat [9] under the same conditions as described previously.[10] After completion of the reaction, the solution was adjusted to pH 5.0 with 0.1 N hydrochloric acid. The gel-filtration procedure was then repeated and the collected fractions assayed for protein and radioactivity. The enzymatic activity was checked before and after the autotitration by the usual methods.[7] The results are presented in Table 1.

The value obtained for the radioactivity in ribonuclease after gel-filtration is in good agreement with the number óf hydroxyamino acid residues of the protein reported to be 25.[11] The decrease found in the value after the autotitration also coincides well with the number of equivalents of base consumed. The results thus strongly support the previous conclusion that the reversible inactivation of ribonuclease is due to a likewise reversible formylation at the hydroxyamino acid residues.

The results with lysozyme are more difficult to interpret since the radioactivity retained after the gel-filtration corresponds to about three times that required for formylation of the hydroxyamino acid residues, *i.e.* 17,[11,12] and the release during the reactivation is about twice the number of equivalents of base consumed. The possibility that all this radioactive formate is bound to lysozyme by covalent bonds seems unlikely because other tentatively sites as tyrosine residues are more numerous in ribonuclease. It is more likely that some formate carbon is attracted to the protein, too strongly to be eliminated by the gel-filtration, for example at the guanidine group of the arginine residues. The more basic character of lysozyme compared with ribonuclease may thus partly account for the observed difference. However, the previous observations that lysozyme, in contrast to ribonuclease, showed partial unfolding as well as an increase in its amino-nitrogen content after

Table 1. Radioactivity recovered from formic acid-^{14}C treated enzymes.

Samples	Counts/min[a] per mg protein	Mole formyl groups per mole of protein	Per cent[b] formylation	Mole base consumed at pH 7.5	Per cent of the total reactivation
Ribonuclease					
a) after gel filtration	730	29	118		0
b) after gel filtration, autotitration at pH 7.5 and repeated gel filtration	250	10	41	16	62
Lysozyme					
a) after gel filtration	1 120	49	294		0
b) after gel filtration, autotitration at pH 7.5 and repeated gel filtration	590	26	155	11	98

[a] Standard deviation ± 15 %.
[b] Based on the number of hydroxyamino acid residues.

the formic acid treatment [7] indicate that formic acid reacts differently with the two proteins. This conclusion is supported also by the present results.

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