On the action of cyanide on ribonuclease

The enzyme ribonuclease does not contain any thiol groups[1,2] and the presence of eight cysteine residues per molecule suggests four disulfide linkages per molecule[1,3], these results having been obtained with Armour[1,2] and with Worthington[1] ribonuclease*. Contrary to these observations, it has been suggested[4,5,6,7] on the basis of activity measurements in the presence of *p*-chloromercuri-benzoate and cyanide and on other grounds that ribonuclease (Worthington and General Biochemicals) is an –SH enzyme, albeit the –SH groups are sluggish[8].

To explore this contradiction, ribonuclease (Armour) has been subjected to the action of cyanide at several pH's and the course of the reaction has been followed by simultaneous determination of enzyme activity and thiol groups.

The experiments were carried out at pH 10.14, 8.03 and 5.00. In a typical run at pH 10.14, 200 mg of ribonuclease (crystalline, salt-free) was dissolved in 14.88 ml of water in a glass-stoppered flask and to this solution 1.12 ml of 0.1 *M* tris (trihydroxymethyl-amino-methane) and 4.00 ml of 0.5 *M* sodium cyanide were added, the final volume being 20.0 ml. For the runs at pH 8.03 and 5.00, the ribonuclease was dissolved in a smaller amount of water and after the addition of 0.1 *M* tris, the requisite amount of 1.0 *M* hydrochloric acid to bring the final pH of the reaction mixture to the indicated value was added.

Enzyme activity was determined by removing at appropriate times a 0.10 ml sample from the reaction mixture, diluting this 1000 times with 0.1 *M* acetate buffer, pH 5.0, and assaying a 1.00 ml aliquot by the method of ANFINSEN *et al.*[1]. This technique greatly diminishes the contribution of the components of the reaction mixture to the ionic strength of the assay medium, the constancy of which is important to the accuracy of the assay[9].

Thiol groups were determined by the ferricyanide method[10] or by titration with N-ethyl-maleimide[11] on the protein precipitated from 1.00 ml samples of the reaction mixture by the addition of 2.00 ml of 2.0 *M* trichloracetic acid.

Control experiments at the several pH's and temperatures employed showed remarkably little loss in enzyme activity nor did any thiol groups appear.

The results of the several runs in the presence of cyanide are best interpreted as due to nucleophilic displacement of cyanide ion on a sulfur atom of the disulfide bond,

$$CN^- + P\begin{matrix}-S\\-S\end{matrix} \rightleftharpoons P\begin{matrix}-S^-\\-SCN\end{matrix}$$

followed by changes in the protein resulting in loss of enzyme activity.

Thus at pH 5.00 and 50° (data not recorded in the figures) where cyanide ion concentration is extremely small, the pK_A of hydrocyanic acid being[12] $4 \cdot 10^{-10}$, no thiol groups appear and enzyme activity remains unchanged for twenty-three hours, the duration of the experiment. At pH 8.03 and 50° (Fig. 1, curves IIE and IISH) where the cyanide concentration is of the order of $5 \cdot 10^{-3}$ *M*, thiol groups appear, albeit slowly and only to the maximal extent of 0.3 moles per molecular weight[3] of 14,000. Following this appearance of thiol groups, enzyme activity declines. At pH 10.14 and 45° (Fig. 1, curves IE and ISH) where the cyanide ion concentration is some $85 \cdot 10^{-3}$ *M*, the rate and extent of thiol formation are increased and enzyme activity drops off almost simultaneously with the appearance of thiol groups.

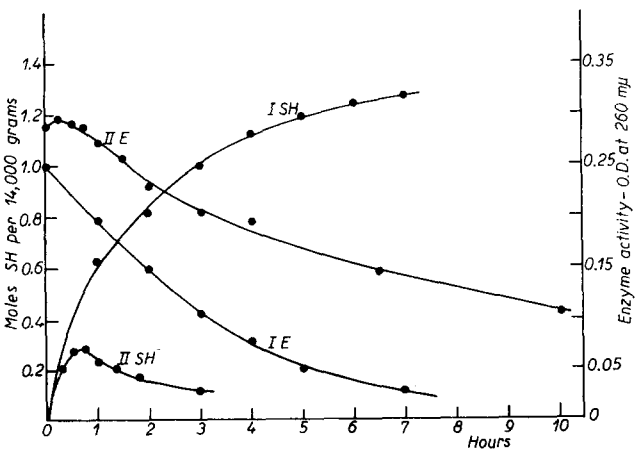

Fig. 1. Influence of cyanide on ribonuclease activity and thiol content. Curves I SH and I E, thiol content and enzyme activity, respectively, at pH 10.14 and 45°. Curves II SH and II E, the same at pH 8.03 and 50°. Thiol appearance followed by the ferricyanide method.

It might be argued from the data at pH 8.03 and 50° that initial activity is augmented by appearance of thiol groups and

* We have indicated the source since it has been reported[8] that similar biological effects are not obtained with ribonuclease from different sources.

that dropping off of activity is due to disappearance of –SH groups. The latter point is disproved by the data at pH 10.14 and 45° where activity drops off as thiol groups appear. The first point was not further investigated since it is clear from the data that opening one or several disulfide groups makes the relatively rigid single polypeptide chain with its four cyclic disulfide bonds[1] lose activity under the conditions of the experiments. The initial data at pH 8.03 suggest that opening of a disulfide bond, *per se*, is not responsible for loss in activity but that a subsequent structural alteration, in the denaturation sense, is responsible for loss in activity. At pH 10.14 it would seem that opening of disulfide bonds is immediately followed by denaturation.

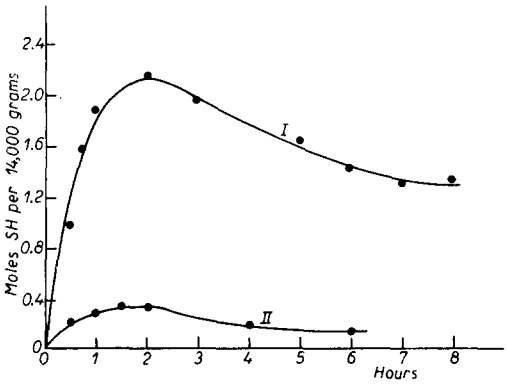

Fig. 2. Influence of cyanide on thiol content of ribonuclease. Thiol appearance followed by titration with N-ethyl-maleimide. Curve I at pH 10.14 and 50°. Curve II at pH 8.03 and 50°.

The disappearance of thiol groups with time (Fig. 1, IISH) is of interest. Of several possible explanations, including lanthionine formation[13], only thiazoline formation[14] followed by a Bacitracin to Bacitracin F type of conversion[15] was experimentally considered since it was noted that a rather flat absorption band appeared in the 295 mμ region as the experiment progressed. Preliminary investigation indicated the absorption to be due to cyanide since a cyanide control in the absence of ribonuclease showed similar changes.

Fig. 2 presents data on several runs in which the appearance of thiol groups was followed by titration with N-ethyl-maleimide. At pH 8.03 and 50° the curve is in agreement with that obtained under the same conditions by the ferricyanide method. At pH 10.14 and 50° the maximum thiol value is almost twice that obtained by the ferricyanide method at the same pH but at 45°.

In summary, it would seem, in agreement with others[1,2], that ribonuclease (Armour) is not an –SH enzyme and indeed, that appearance of enzyme –SH groups diminishes the stability of the enzyme.

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