INHIBITION OF ENZYMICALLY ACTIVE *N*-ACETYL-HOMOCYSTEINYL-RIBONUCLEASE BY SILVER IONS

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1. Introduction

Bovine pancreatic ribonuclease A (EC 2.7.7.16) is completely inactivated by reaction with *N*-acetylhomocysteine thiolactone in the presence of silver catalyst under carefully controlled conditions. Chromatography of the reaction products yields two monosubstituted ribonuclease derivatives, one of which is enzymically active and the other enzymically inactive. The enzymically active derivative is inhibited by silver ions, in contrast to ribonuclease A which is not affected by silver ions [1].

We have shown that enzymically active *N*-acetylhomocysteinyl-ribonuclease, free from phosphate ions, can be obtained from enzymically active *N*-acetylhomocysteinyl-ribonuclease-*S*-*p*-mercuribenzoate. This method avoids the difficulties previously encountered in the purification of the free thiol derivative, which were attributed to oxidation of the free thiol group. Pure, phosphate free, *N*-acetylhomocysteinyl-ribonuclease was used to show that the pH dependences of the kinetic parameters, K_m and k_{+2} of ribonuclease A are not altered by introduction of the free thiol group into the molecule. The pH dependence of the inhibition constant (K_i) for silver inhibition of enzymically active *N*-acetylhomocysteinyl-ribonuclease indicates that the interaction of silver ions with a histidine residue in the protein is responsible for the observed loss of enzymic activity.

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2. Methods

Ribonuclease A was prepared from bovine pancreatic ribonuclease (Armour Pharmaceuticals Limited., Eastbourne, England) by column chromatography on Amberlite CG 50, using 0.2 M phosphate buffer pH 6.42 as eluent [2]. After dialysis, it was deionised by passage through a small column of Amberlite MB 3, a mixed-bed-ion-exchange resin which removes all remaining phosphate ions. The deionised ribonuclease solution was neutralised with nitric acid and stored at –20°.

Ribonuclease activity was assayed spectrophotometrically, using cytidine-2′,3′-cyclic monophosphate as substrate [3]. All measurements were made at constant temperature (25°) and ionic strength ($I = 0.2$). Linear initial rates of hydrolysis were used to calculate the kinetic parameters K_m and k_{+2} by a computer method [4], using experimentally determined weighting factors.

Deionised ribonuclease A (30 μmole) was reacted with *N*-acetyl-homocysteine thiolactone (69 μmole) in the presence of silver catalyst (175 μmole) at pH 7.4, using the method of Shall and Barnard [1]. To prevent oxidation of the newly introduced thiol groups during purification, the product was treated with *p*-chloromercuribenzoate (75 μmole) in glycylglycine buffer pH 8.0 for one hour at 20°. After dialysis overnight against deionised water, *N*-acetylhomocysteinyl-ribonuclease-*S*-*p*-mercuribenzoate was purified by chromatography on Amberlite CG 50, using 0.17 M phosphate buffer pH 5.95 as eluent. Two major products, one enzymically active and

the other enzymically inactive, were obtained. The absorption spectrum of the enzymically active derivative showed that one mole of *p*-mercuribenzoate had been incorporated per mole of derivative [5].

Amino-acid analysis confirmed that reaction of ribonuclease A with *N*-acetylhomocysteine thiolactone leads to monosubstituted products. A preparation of *N*-acetylhomocysteinyl-ribonuclease was reacted with iodoacetate at pH 8.5 for 12 min. After dialysis, *S*-carboxymethyl-*N*-acetylhomocysteinyl-ribonuclease was purified by chromatography on Amberlite CG 50, yielding an enzymically active derivative and an enzymically inactive derivative. Acid hydrolysis, followed by amino-acid analysis of the hydrolysate showed that each derivative contained one mole of *S*-carboxymethylhomocysteine per mole of protein. This experiment demonstrates that the initial reaction with *N*-acetylhomocysteine thiolactone leads to monosubstituted products and confirms that enzymically active *N*-acetylhomocysteinyl-ribonuclease-*S*-*p*-mercuribenzoate is also monosubstituted.

Chromatographically homogeneous, enzymically active *N*-acetylhomocysteinyl-ribonuclease-*S*-*p*-mercuribenzoate was used to prepare the corresponding pure, free thiol derivative. After deionisation on a column of Amberlite MB 3, the *p*-mercuribenzoate derivative (3.0 μmole) was treated with redistilled 2-mercaptoethanol (2.0 mmole) for one hour at room temperature, and the product dialysed against deionised water. Comparison of the spectra of the protein solution before and after reaction with 2-mercaptoethanol showed that the *p*-mercuribenzoate is completely removed by this treatment. The product, enzymically active monosubstituted *N*-acetylhomocysteinyl-ribonuclease, had an activity of 75% compared to ribonuclease A. To minimise oxidation of the free thiol group, the derivative was always stored in acid solution (pH 4), tightly stoppered, at −20°.

3. Results

The steady state kinetic parameters K_m and k_{+2} were determined at a range of pH values for ribonuclease A and enzymically active *N*-acetylhomocysteinyl-ribonuclease. Plots of pK_m and log k_{+2} against pH (fig. 1) show that the pH dependence of each of these parameters remains unchanged as a result of modification. The pH dependence curves (the solid lines) were constructed as described by Herries et al. [6], using the pK_a values for the ionising groups at the active site which they found. The absolute magnitude of $1/K_m$ and of k_{+2} are both lowered in the derivative, compared to ribonuclease A. The activity that is present however, has the same pH dependent character as that in ribonuclease A.

The steady state kinetic parameters were then redetermined under the same conditions, with the addition of silver nitrate (11 or 55 μM) to each assay. The results showed that the value of K_m is not sig-

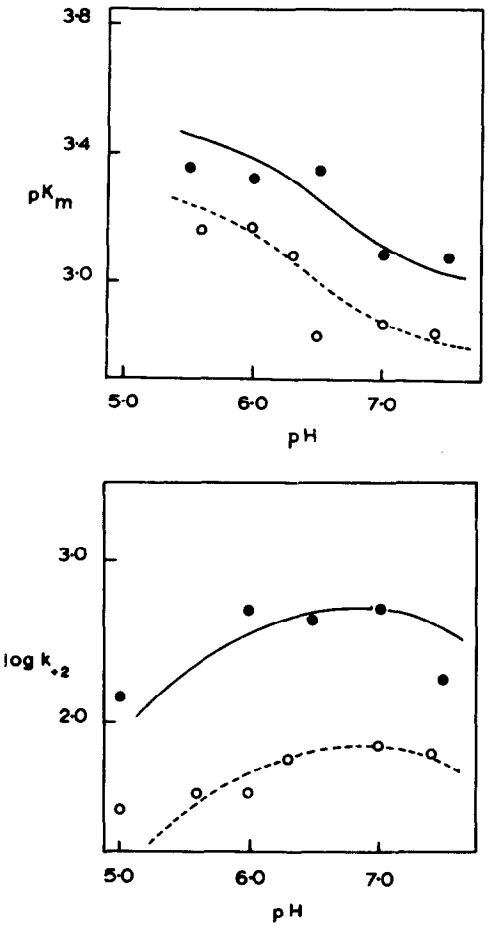

Fig. 1. The kinetic parameters of ribonuclease A (•) and *N*-acetylhomocysteinyl-ribonuclease A (○) determined at different pH values using 2′,3′-cyclic cytidylic acid as substrate. The solid lines are calculated using the pK_a values of Herries et al. [6], the points are experimental values.

nificantly altered in the presence of silver ions, while at each pH value studied the apparent value of k_{+2} is decreased when silver ions are added. Inhibition of enzymically active *N*-acetylhomocysteinyl-ribonuclease by silver ions is therefore of the non-competitive type. The inhibition constant (K_i) was calculated at each pH value from the k_{+2} values determined in the presence and absence of inhibitor (k^1_{+2} and k_{+2} respectively), using the equation:

$$k_{+2} = k^1_{+2}(1 + i/K_i) \quad (1)$$

The pH dependence of pK_i is shown in fig. 2. The experimental points are joined by a curve constructed according to Dixon's method [7]. As indicated by the intersection point of the projected straight lines, an ionising group with pK_a = 6.2 is responsible for the observed pH dependence. The values of K_i all lie within the range 0.79–7.67 × 10^{-4} M. The data can be fitted to the following equation:

$$K_i = \overline{K_i}\,(1 + K_a/\mathrm{H}) \quad (2)$$

where K_i is the pH dependent inhibition constant, $\overline{K_i}$ is the pH independent inhibition constant and K_a is the dissociation constant of the ionising group. This equation is analogous to that derived by Waley [8].

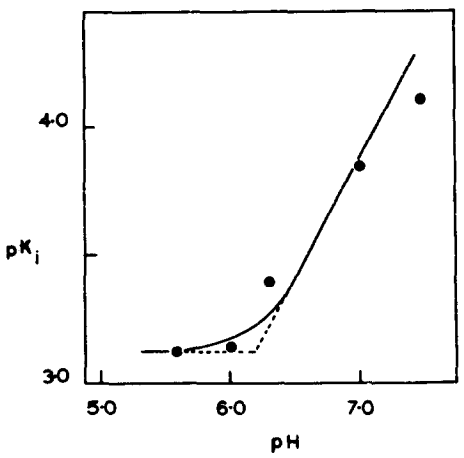

Fig. 2. The pH dependence of the silver inhibition of the enzymic activity of *N*-acetylhomocysteinyl-ribonuclease. At each pH the inhibition constant was measured at two concentrations of silver nitrate. 2′,3′-Cyclic cytidylic acid was the substrate.

The dissociation constants (K_{diss}) for a series of amino-acid-silver complexes were measured by Myrbäck and Willstaedt [9]. They obtained the following results:

methionine-Ag^+	K_{diss} = 3.6 × 10^{-4} M
histidine-Ag^+	K_{diss} = 2.0 × 10^{-4} M.
cysteine-Ag^+	K_{diss} immeasurably small.

From the magnitude of the inhibition constants obtained for the silver ion inhibition of enzymically active *N*-acetylhomocysteinyl-ribonuclease, we can conclude that formation of a cysteine-silver complex is not directly responsible for the loss of enzymic activity. This is confirmed by the observation that the free thiol group in this derivative can be alkylated with either iodoacetate or *p*-chloromercuribenzoate without effect on its enzymic activity. Methionine is probably not the reacting group, since the inhibition is pH dependent and so must involve an ionising group. However, the free thiol group must be involved in some way in the inhibition process, because the enzymic activity of ribonuclease A was shown to be unaffected by silver ions at the concentration used in these experiments.

Both the magnitude of the observed inhibition constant and its pH dependence indicate that interaction of a silver ion with a histidine residue in the protein is responsible for the observed inhibition of enzymically active *N*-acetylhomocysteinyl-ribonuclease. It is evident that the silver ion must also be bound to the newly introduced thiol group in the molecule.

The pK_a values of the four histidine residues of ribonuclease have been determined by NMR spectroscopy [10,11]. The results indicated that, of the two active site histidines (His-12 and His-119), His-119 has the lower pK_a value and so is more likely to be present at the active site in the non-protonated form.

It is therefore proposed that silver inhibition of enzymically active *N*-acetylhomocysteinyl-ribonuclease is due to the interaction of a silver ion with a basic histidine residue at the active site, presumed to be His-119. This conclusion can be experimentally tested by examining the NMR spectrum of the inactive silver complex. This will also show whether formation of the silver complex affects the pK_a values of more than one histidine residue.

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