The Binding of Cupric Ions to 1-Carboxymethylhistidine-119-ribonuclease

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Binding of Cu^{2+} by 1-carboxymethylhistidine-119-ribonuclease was investigated by using diligand metal ion buffers. A single Cu^{2+} -binding site was found over the Cu^{2+} concentration range studied. The binding constants for this site were $8.33 \times 10^5 (\pm 2\%) \text{ M}^{-1}$ and $1.57 \times 10^4 (\pm 6\%) \text{ M}^{-1}$ at pH 7.0 and 6.1 respectively. An estimate of the pH-independent Cu^{2+} -binding constant suggests that the most avid Cu^{2+} -binding site has disappeared after carboxymethylation. This is consistent with an earlier report that binding of Cu^{2+} at the most avid site is associated with the loss of enzymic activity.

We have previously investigated the Cu^{2+} -binding properties of ox pancreatic ribonuclease by using diligand metal ion buffers (Saundry & Stein, 1967). The values of the binding constants of the two most avid Cu^{2+} -binding sites and the pH-dependence of these values led to the conclusion that the binding sites were at least bidentate and that one of the ligand groups at the most avid binding site was probably an imidazole residue. It was further demonstrated that the inhibition of the enzyme by Cu^{2+} was paralleled by binding at the most avid Cu^{2+} -binding site. Since it is believed that His-12 and His-119 are present at the enzyme active centre (Crestfield, Stein & Moore, 1963*a,b*) the possibility existed that either one or both of these two histidine residues is present at the observed Cu^{2+} -binding sites. In this paper is reported an investigation of the Cu^{2+} -binding properties of 1-CM-His-119-ribonuclease* in which the imidazole ring of His-119 is blocked by carboxymethylation.

EXPERIMENTAL

Materials

All reagents were as specified by Saundry & Stein (1967) with the following exceptions.

The ribonuclease was here supplied by Koch-Light Laboratories Ltd. (Colnbrook, Bucks.) as batch no. 30056.

Unlabelled iodoacetic acid (AnalaR grade; British Drug Houses Ltd., Poole, Dorset) was purified by recrystallization five times from *n*-hexane and stored cold in the dark under *n*-hexane. The iodoacetic acid was air-dried just before use.

Iodo[2- ^{14}C]acetic acid (batch no. CFA 269 no. 8, reported

specific radioactivity 6.5 mc/m-mole; The Radiochemical Centre, Amersham, Bucks.) was used without further purification.

Methods

1-CM-His-119-ribonuclease (Crestfield *et al.* 1963*a,b*) was prepared by a modification of the method of Yang & Hummel (1964). A 486 mg. sample of ribonuclease, dissolved in 7.5 ml. of water and adjusted to pH 5.5 with *N*-HCl, was added to 7.5 ml. of a solution of unlabelled iodoacetic acid in water (668 mg./100 ml.), the pH being again adjusted to 5.5, and immediately 25 μC of labelled iodoacetic acid was added. After incubation at 25° for 8½ hr. with the pH maintained at 5.5, the reaction mixture was freeze-dried and the powder was dissolved in 5 ml. of 0.2 M-sodium phosphate buffer and adjusted to pH 6.47 with *N*-NaOH. The solution was then loaded on a column (4.5 cm. $\times$ 36 cm.) of Amberlite IRC-50 and eluted with 0.2 M-sodium phosphate buffer, pH 6.47, with a flow rate of 72 ml./hr. The fractions corresponding to the carboxymethylated derivative were pooled, concentrated to about 20 ml. by evaporation under reduced pressure and then desalted by repeated gel filtration on Sephadex G-25 with 0.2 M-acetic acid as eluent. The acetate was removed by gel filtration in distilled water.

The freeze-dried material was free from phosphate when analysed for phosphate by the method of Loring & Pierce (1944), and was enzymically inactive against both purified yeast RNA (with the assay procedure of Dickman, Aroskar & Kropf, 1956) and 2',3'-(cyclic)-CMP (with the assay procedure of Crook, Mathias & Rabin, 1960). Samples of the material were hydrolysed and passed through a Technicon amino acid analyser. The result of such an analysis is given in Table 1. There were found to be 16 instead of the expected 15 serine residues, but the serine peak here was only partially separated from the threonine peak and the derived value is within experimental error. Seven of nine valine residues and less than two of three isoleucine residues were recovered, presumably because ribonuclease contains the hydrolysis-resistant sequence Ile-Ile-Val. The low recovery of cysteine was probably a

* Abbreviation: 1-CM-His-119-ribonuclease, 1-carboxymethylhistidine-119-ribonuclease.

Table 1. *Amino acid composition of 1-CM-His-119-ribonuclease*

Alanine was taken to be 12.00 residues/mol. and the values for the other amino acids were adjusted accordingly.

Amino acid	Composition of 1-CM-His-119-ribonuclease used in this work	Theoretical composition of 1-CM-His-119-ribonuclease
Asp	15.1	16
Thr	9.6	10
Ser	15.9	15
Gln	12.4	12
Pro	—	5
Gly	3.0	3
Ala	12.00	12
Val	6.8	9
Cys	5.9	8
Met	3.8	4
Ile	1.4	3
Leu	2.1	2
Tyr	5.5	6
Phe	3.0	3
Lys	10.0	10
His	2.9	3
Arg	3.7	4

result of partial oxidation to cysteic acid. A ninhydrin-positive radioactive peak was eluted almost at the same place as proline, so that the concentration of proline could not be separately evaluated. The data are, however, clearly consistent with the amino acid composition for a ribonuclease molecule in which one histidine residue has been carboxymethylated.

The effluent from the amino acid analyser was passed through the flow cell of a Packard Tri-Carb model 3003 liquid-scintillation counter. The ninhydrin-positive radioactive peak eluted from the analyser column near the position expected for proline had the published chromatographic properties of 1-CM-histidine (Crestfield *et al.* 1963*a,b*).

The final yield of 1-CM-His-119-ribonuclease was 112 mg. and its specific radioactivity was 24.7 $\mu\text{C}/\text{m.mole}$.

Determination of copper. Copper was determined by the oxalyldihydrazide method of Stark & Dawson (1958).

Cu²⁺-binding measurements. Measurements of Cu²⁺-binding were performed as previously described (Saundry & Stein, 1967). Since the carboxymethyl derivative possessed no enzymic activity its concentration was measured from its radioactivity. The Cu²⁺ ion buffers used were identical with those used in the investigation of Cu²⁺-binding properties of ribonuclease. Cu²⁺-binding was investigated at pH 7.0 and 6.1.

RESULTS

The parameter R , the number of g.ions of Cu²⁺ bound/mole of protein, was determined and plotted as a function of the free Cu²⁺ concentration, M , according to the procedure of Hughes & Klotz (1956) as described by Saundry & Stein (1967). A least-square analysis of the data gave the following

results (the parameters being given as means $\pm$ S.E.M.):

For pH 7.0 (20 determinations):

$$\frac{1}{R} = \frac{(1.05 \pm 0.03) \times 10^6}{M} + (1.32 \pm 0.27)$$

For pH 6.1 (ten determinations):

$$\frac{1}{R} = \frac{(0.56 \pm 0.05) \times 10^4}{M} + (1.23 \pm 0.27)$$

The limiting value of $1/R$, when $1/M$ is zero, does not differ significantly when these two pH values are compared and does not differ significantly from unity. The limiting value of $1/R$ is significantly different ($P < 0.01$) from 0.5. Thus the number of Cu²⁺-binding sites/mol. is less than two and is probably one.

DISCUSSION

The Hughes-Klotz plots for Cu²⁺-binding by 1-CM-His-119-ribonuclease at both pH 7.0 and 6.1 indicate that only one Cu²⁺-binding site operates over the Cu²⁺ concentration range studied. Straight lines were drawn through all the points so as to cut the ordinate at unity in accordance with the Hughes-Klotz plot for a single binding site. The slope of the derived line gives the reciprocal of the binding constant at the given pH. The Cu²⁺-binding constants for 1-CM-His-119-ribonuclease are $8.33 \times 10^5 (\pm 2\%) \text{M}^{-1}$ and $1.57 \times 10^4 (\pm 6\%) \text{M}^{-1}$ at pH 7.0 and 6.1 respectively. The binding constants are somewhat smaller than for either of the two most avid Cu²⁺-binding sites of native ribonuclease at the corresponding pH values (Table 2).

The ratio of the Cu²⁺-binding constants for 1-CM-His-119-ribonuclease at pH 7.0 and 6.1 is 54 for a 0.9 rise in pH. We have previously argued (Saundry & Stein, 1967) that this is inconsistent with the single binding site's being monodentate.

If we assume that this binding site is bidentate, and further (merely for ease of computation) that the two ligand groups are identical (Saundry & Stein, 1967), a comparison with the titration curve of a monobasic acid gives an intrinsic $\text{p}K_a$ value of 8.0 for the two pH-dependent ligand groups at the Cu²⁺-binding site and a value of $10^{8.0}$ for the pH-independent Cu²⁺-binding constant. Alternatively, if we assume that the two ligand groups are not identical, and that the $\text{p}K_a$ of one of them is so high (i.e. 9.0) that at the pH range studied the effect of change of pH on this group is maximal, then by comparison with the titration curve for a monobasic acid (Saundry & Stein, 1967) the $\text{p}K_a$ of the other ligand group will be 7.7.

Table 2. *Binding constants of Cu²⁺ on ribonuclease and CM-ribonuclease and computed proton dissociation constants of the ligand groups*

The pH-dependent constants are derived from the slopes of the Hughes-Klotz plots described in the text (for CM-ribonuclease) or recalculated from Saundry & Stein (1967) (for native ribonuclease) and are reported as derived means ($\pm$ s.e.m. as a percentage of the mean). The hypothetical pH-independent binding constants and the pK_a values of the ligands are computed from the data at pH 7.0 and 6.1 on the bidentate model as described in the text.

Binding constants (M^{-1})	Native ribonuclease		1-CM-His-119-ribonuclease
	First binding site	Second binding site	
At pH 7.0	$2.97 \times 10^7 (\pm 3\%)$	$5.27 \times 10^6 (\pm 3\%)$	$8.33 \times 10^5 (\pm 2\%)$
At pH 6.1	$7.97 \times 10^5 (\pm 32\%)$	$1.71 \times 10^5 (\pm 17\%)$	$1.57 \times 10^4 (\pm 6\%)$
At pH 5.2	$1.25 \times 10^4 (\pm 8\%)$		
pH-independent constant (assuming two identical ligand sites)	$10^{8.5}$	$10^{7.6}$	$10^{8.0}$
Intrinsic pK_a (two identical ligands)	7.4	7.3	8.0
Lowest possible pK_a of more acidic ligand (two unequal ligands)	7.1	6.9	7.7
True $K \times K_{HZ}$	6.4	0.90	0.48

We cannot now obtain an absolute value for the pH-independent binding constant ('true K '; Saundry & Stein, 1967), but obtain rather a composite term, the product of true K and the dissociation constant of the more basic site on the ligand K_{HZ} . The computed value of the product true $K \times K_{HZ}$ for CM-ribonuclease is 0.48 whereas for the most avid and less avid sites of ribonuclease this quantity has the values 6.4 and 0.9 respectively. For CM-ribonuclease, if K_{HZ} is less than 10^{-9} (i.e. if the more basic ligand has pK greater than 9), the true Cu²⁺-binding constant is greater than $2.1 \times 10^9 M^{-1}$. If therefore we assume the Cu²⁺-binding site of CM-ribonuclease to be bidentate, the pK_a of the more acidic site on the ligand has a minimum value of 7.7 and a maximum value of 8.0, whereas the pH-independent Cu²⁺-binding constant has a minimum of $10^{8.0}$ and a maximum value of 2.1 times the reciprocal of the dissociation constant of the more basic ligand. For comparison, Table 2 gives the values computed for the most avid and less avid binding sites of native ribonuclease on these limiting assumptions from the results obtained at pH 7.0 and 6.1. From Table 2 it can be seen that the magnitude of the term (true $K \times K_{HZ}$) for CM-ribonuclease approximates to the value of this term for the less avid Cu²⁺-binding site of ribonuclease and is an order of magnitude less than the value for the most avid Cu²⁺-binding site. The pK of the acidic ligand is, however, over 0.5 pH unit higher in CM-ribonuclease than in native ribonuclease. This might well arise directly from the introduction of a carboxymethyl group into the molecule in the neighbourhood of the remaining Cu²⁺-binding site.

It therefore appears that on blocking the imidazole ring of His-119, when enzymic activity

is lost, the most avid Cu²⁺-binding site of ribonuclease has disappeared. We have already shown (Saundry & Stein, 1967) that Cu²⁺-binding at this site inhibits the activity of the enzyme. Thus the active-centre histidine residue in position 119 forms part of the most avid Cu²⁺-binding site. It is not possible to decide, however, whether the imidazole ring of His-12 acts as a ligand group at the second most avid Cu²⁺-binding site in ribonuclease.

However, the lower limits placed on the pK_a values for the ligand groups at the binding sites are at least 1 pH unit higher than the values for the pK_a values of the two imidazoles believed to be present at the enzyme active centre (Deavin, Mathias & Rabin, 1966*a,b*; Lambden, Mathias & Rabin, 1962; Bradbury & Scheraga, 1966; Meadows, Markley, Cohen & Jardetzky, 1967).

The binding of Cu²⁺ is consistent with the thesis that the two most avid Cu²⁺-binding sites are bidentate, but it is possible that both these sites are tridentate. If this is so, the average pK_a values found may result from the combination of an imidazole with a low pK_a and two further ligand groups with higher pK_a values. The possibility that the Cu²⁺-binding sites may be tridentate does not, however, invalidate our conclusion that one ligand involved in the most avid binding site of the native enzyme is His-119.

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