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STRUCTURE OF CADYSTIN, THE UNIT-PEPTIDE OF CADMIUM-BINDING PEPTIDES INDUCED IN A FISSION YEAST, SCHIZOSACCHAROMYCES POMBE

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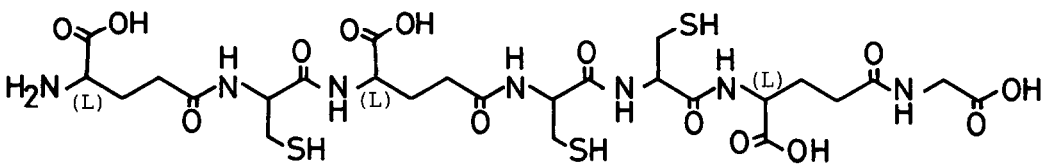
The unit-peptide, cadystin, of cadmium-binding peptides occurring in a fission yeast, S. pombe was determined to have structure H-γ-Glu-Cys-γ-Glu-Cys-Cys-γ-Glu-Gly-OH; all Glu and two Cys being L-form, whereas one of the Cys's D-form.

Metallothioneins, the heavy metal-binding proteins, have been found in various eucaryotic cells and considered to be present for detoxification of the metals, such as Cd, Zn, Hg and Ag.[1] In a primordial eucaryote, Schizosaccharomyces pombe (a fission yeast), two cadmium-binding peptides, Cd-BP1 and 2, were found to be induced by cultivating the yeast in the presence of Cd(II).[2] The molecular weights of these peptides, Cd-BP1 and 2, were determined to be ca 4,000 and 1,800, respectively, both of them being composed of cadmium ions and four and two molecules, respectively, of a common unit-peptide, that we named cadystin. Murasugi et al.[2] determined cadystin (**1**) to be composed of seven amino acids, namely Cys-3, Glu-3 and Gly-1, based on acid hydrolysis of some derivatives of Cd-BP1 and 2. Surprisingly, such a small molecule as cadystin has the ability similar to that of high-molecular metallothioneins[1] by molecular association.



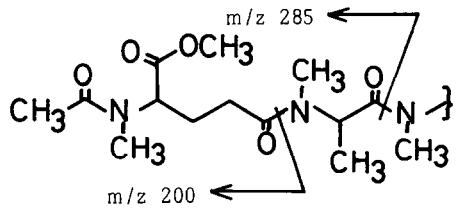
Cadystin (1)

Cadystin (**1**) did not undergo the usual Edman degradation, suggesting that it must be an unusual peptide. We herewith describe the determination of its structure. The molecular weight of cadystin (**1**) calculated from the amino acid composition [Glu(3)+ Cys(3)+ Gly(1)- 6 H_2O = 771] was confirmed by FAB-MS (Jeol DX-300) of aminoethylated cadystin (AE-**1**) prepared by treatment of **1** with ethylenimine;[2] a peak at m/z 901 corresponded to M+1 of AE-**1**. Cd-BP1 (115 μg) was

reduced with Raney Ni (W-2, wet ca 2 mg) in Tris-HCl buffer (50 µl, 0.05M, pH 7.6) at 50 °C for 12 hr. After evaporation of the solvent, the residue was dissolved in 6N HCl (ca 1 ml) below 30 °C, and the resulting solution was lyophilized. The residue was applied on a Bio-Gel P2 column (0.6 x 20 cm) with 3M AcOH, and peptidic components in the eluates were detected by the fluorescamine method.[3] Dethio-cadystin (DeS-**1**) thus obtained in 52% yield gave Glu(3.0 mol), Ala(2.5 mol) and Gly(0.9 mol) on acid hydrolysis;[4] no Cys being detected.

Dansylation of DeS-**1** (0.7 µg) followed by acid hydrolysis afforded DNS-Glu, but no PTH-Glu was produced by Edman degradation of DeS-**1**. Furthermore, after one-cycle-process of Edman degradation was carried out, attempted dansylation did not proceed. These facts suggests that the amino terminal is Glu which forms peptidic linkage with its γ-carboxyl group. EI-MS spectrum of the N-acetyl-permethylated DeS-**1** showed peaks at m/z 285 and 200 (Fig. 1), indicating the presence of H-Glu-Ala- moiety in DeS-**1**.

Fig.1.



Partial hydrolysis of DeS-**1** (3.5 µg) with 1.2N HCl at 95 °C for 1 hr[5] gave a mixture of Gly, Glu and peptides; only a trace amount of Ala being detected. Edman degradation of the mixture gave only PTH-Ala. Second Edman degradation of the residual mixture gave no PTH-amino acids, but dansylation followed by acid hydrolysis gave DNS-Glu, indicating -Ala-γ-Glu-.

During the course of the acid hydrolysis of DeS-**1** (0.4 µg) in 1.2N HCl at 100 °C, we found that 2nd and 3rd molecules of Ala were liberated slower than Glu and Gly; namely, Glu(3.0 mol), and Gly(1.0 mol) were liberated within 1 hr, whereas 1.6, 2.0, 2.2 and 2.5 mol of Ala were liberated, respectively, after 1, 2, 4 and 96 hr heating. These facts indicate the presence of a sequence of -Ala-Ala-. Digestion of DeS-**1** (0.7 µg) with thermolysin, which can hydrolyze the amino side of Ala, in 0.1M N-ethylmorpholinium acetate buffer (pH 8.0, 35 °C, 20 hr) gave Ala (0.4 mol). Since no Glu was afforded by thermolysin,[6] the liberated Ala should not be derived from a sequence of H-Glu-Ala-Ala-(Glu)-.

DeS-1 (0.07 µg and 0.7 µg) yielded Gly(1.0 mol) and Gly(0.5 mol), respectively, but no other amino acids by digesting with each of carboxypeptidase P and St. aureus V_8 protease; the former enzyme is an exopeptidase hydrolyzing peptides from the carboxyl end and the latter cleaves them specifically at the carbonyl side of Glu.[7] Thus, the carboxyl end of DeS-**1** must be -Glu-Gly-OH.

Combined analyses of the above results led to the structure A or B for DeS-**1**.

(A) H-γ-Glu-Ala-γ-Glu-Ala-Ala-(γ)-Glu-Gly-OH

(B) H-γ-Glu-Ala-Ala-Ala-γ-Glu-(γ)-Glu-Gly-OH

If B were the correct structure, specific hydrolysis at the second Ala-Ala bond must be assumed during the partial hydrolysis. It is very improbable since the first Ala-Ala bond should be also

hydrolyzed simultaneously, and since γ-Glu-Ala and γ-Glu-Gly bonds should be hydrolyzed faster than Ala-Ala bond as discussed above. Thus, DeS-1 must have structure A; and hence structure **1** (without absolute configuration) can be assigned for cadystin. That all of Glu are linked at their γ-carboxyl groups was determined[8] by reduction of the free α-carboxyl groups of AE-1 into the corresponding hydroxymethyl groups followed by acid hydrolysis to yield only 4-amino-5-hydroxypentanoic acid (**2**) but not 2-amino-5-hydroxy isomer (**3**). Thus, AE-1 (9 μg) was suspended in 100 μl of a mixture of MeOH, $HC(OCH_3)_3$ and $SOCl_2$ (80:20:5) and heated at 40 °C for 4 hr to give the tetramethyl ester, which was reduced with $LiBH_4$ in anhyd. THF (0.3 M, 50 μl) at 80 °C for 6 hr and then hydrolyzed with 6N HCl (200 μl) at 105 °C for 12 hr. The hydrolysate was analyzed with HPLC as shown in Fig. 2;[4,9] in which **3** was not detected.

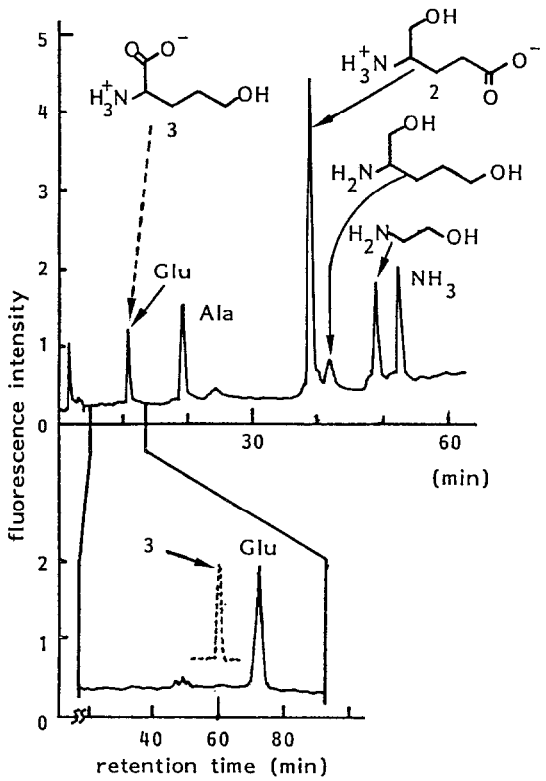


Fig.2 Up: Chromatogram of the products of the $LiBH_4$ reduction followed by hydrolysis of AE-1 tetramethyl ester. Condition; see Ref. 3.
Down: Chromatogram showing separation of compound 3 and Glu (using more dilute buffer and slower flow rate). Dotted line: authentic compound 3. Solid line: the hydrolysate.

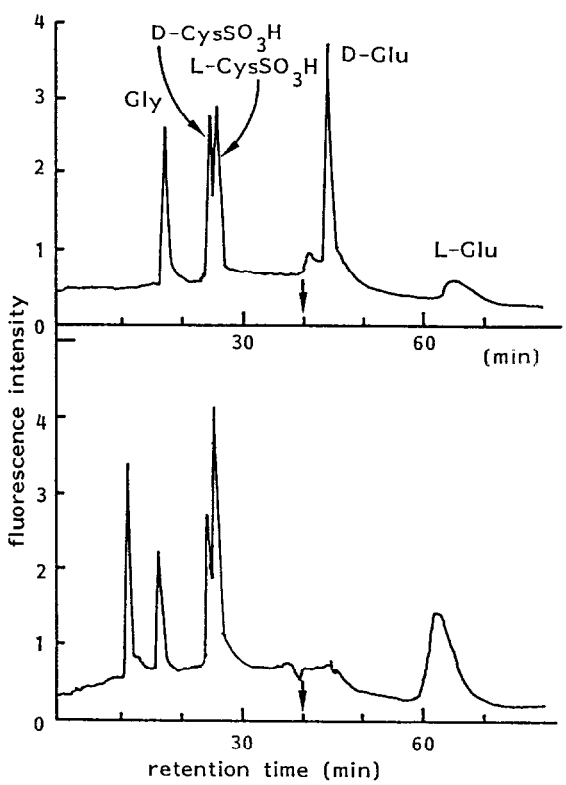


Fig. 3. Determination of absolute configuration of amino acids by Hare's method.[10]
Up: Authentic mixture (each 1 nmole)
Down: Hydrolysate of the performic acid oxidation product of 1.
Flow rate was increased twice at 40 min (arrow).

The absolute configuration of each amino acid was determined using the performic acid oxidation product of **1**.[2] The oxidation product (8 μg) was hydrolyzed with 6N HCl at 110 °C for 24 hr or 72 hr and the products were analyzed by Hare's method[10] to separate enantiomeric D- and L-Glu, and D- and L-$CysSO_3H$. The method involves formation of the diastereomeric chelates of the given D- and L-amino acids with N,N-di-n-propyl-L-alanine through Cu(II) ions. After separation by an ODS column the copper complexes were detected by the OPA-post-labeling method (Fig. 3). Following amino acids were detected: D-Glu(0.0 mol), L-Glu(3.0 mol), D-$CysSO_3H$(0.9 mol), L-$CysSO_3H$(1.7 mol) and Gly(0.7 mol). The particular ratio of D- and L-$CysSO_3H$ [1.0 : 1.9] indicated one of the three Cys residues is D-form; location of which is not determined yet, but will be uncovered by a total synthesis of **1**.

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9. Authentic samples of compound 2 and 3 were prepared as follows: glutamic acid γ-methyl ester was treated with benzoyl chloride (2.5 equiv) and triethylamine (5.0 equiv) in dichloromethane at room temp for 12 hr to form the crude benzamide. Reduction of the half ester with $LiBH_4$ (2 equiv) in THF at 50 °C for 10 hr afforded the mono-ol, which was hydrolyzed with 6N HCl under N_2 at 110 °C for 12 hr to give in 45% overall yield 3 [pmr (D_2O):δ 1.92-2.36(4H, m), 3.74(2H, t, J= 6.0), 4.10(1H, t, J= 5.5 Hz)][Ref: S.P.L. Sørensen, Bull. Soc. Chim. Fr., 33, 1052 (1905)]. Compound 2 was similarly prepared from L-glutamine [pmr (D_2O): δ 1.91(2H, d,t, J= 7.0, 7.0), 2.36(2H, t, J= 7.0), 3.38(1H, d,d,t, J= 4.2, 6.5, 7.0) 3.66(1H, d,d, J= 6.5, 12.0), 3.88(1H, d,d, J= 4.2, 12.0)][Ref. 7].
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