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Chemical Modification of Intracellular Cytosine Deaminase from *Chromobacterium violaceum* YK 391

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Abstract Cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1) stoichiometrically catalyzes the hydrolytic deamination of cytosine and 5-fluorocytosine to uracil and 5-fluorouracil, respectively. Amino acid residues located in or near the active sites of the intracellular cytosine deaminase from *Chromobacterium violaceum* YK 391 were identified by chemical modification studies. The enzymic activity was completely inhibited by chemical modifiers, such as 1 mM NBS, chloramine-T, *p*-CMB, *p*-HMB and iodine, and was strongly inhibited by 1 mM PMSF and pyridoxal 5'-phosphate. This chemical deactivation of the enzymic activity was reversed by a high concentration of cytosine. Furthermore, the deactivation of the enzymic activity by *p*-CMB was also reversed by 1 mM cysteine-HCl, DTT and 2-mercaptoethanol. These results suggested that cysteine, tryptophan and methionine residues might be located in or near the active sites of the enzyme, while serine and lysine were indirectly involved in the enzymic activity. The intracellular cytosine deaminase from *C. violaceum* YK 391 was assumed to be a thiol enzyme.

Keywords: cytosine deaminase, chemical modification, *Chromobacterium violaceum* YK 391, intracellular cytosine deaminase

INTRODUCTION

Cytosine, unlike other nucleobases, is not only directly degraded, but also does not act as a substrate for Salvage synthesis. Cytosine is certainly hydrolyzed to uracil by cytosine deaminase, and is therefore involved in the recycling of pyrimidine nucleobases to the pyrimidine nucleotide pool, or entirely degraded by dehydrogenase. Cytosine deaminases from *Serratia marcescens* [1] and *Pseudomonas aureofacience* [2] were first purified to homogeneity in 1975. These enzymes have also been purified to homogeneity from *Escherichia coli* [3], *Salmonella typhimurium* [4] and *Aspergillus fumigatus* [5]. Extracellular cytosine deaminase was purified from *Chromobacterium violaceum* YK 391 [6]. *C. violaceum* YK 391 has been produced not only as an extracellular enzyme, but also as an intracellular cytosine deaminase [7]. Furthermore, cytosine deaminase activity remains to be not observed in any mammalian or plant organ [8]. For these reasons, cytosine deaminase appears to be a unique enzyme, not only in the catabolism of pyrimidine nucleotides, but also in the Salvage synthesis of pyrimidine nucleotides. Cytosine deaminase (cytosine aminohydrolase; EC 3.5.4.1) stoichiometrically catalyzes the hydrolytic deamination of cytosine, 5-fluorocytosine (5-FC) and 5-methylcytosine (5-MC) to uracil, 5-fluorouracil (5-FU) and thymine, respectively. 5-FC possesses no antitumor or bacteriostatic activity, and exhibits little clinical toxicity [9]. However, while 5-FU does possesses antitumor activity and a strong broad-range antimicrobial spectrum, it is also, unfortunately, quite toxic [10]. 5-FC, after its conversion into 5-FU by cytosine deaminase, exhibits antineoplastic activity and acts as a selective fungicide [11,12]. This antifungal activity of 5-FC has been attributed to the participation of cytosine deaminase in the fungi themselves, such that the enzyme deaminates 5-FC to 5-FU.

The full nucleotide sequence of the cytosine deaminase gene (*codBA*) has been determined from *E. coli* [13]. Yergatian *et al.* [14] have shown that cysteine, histidine, and possibly arginine residues are involved in the catalytic functions of cytosine deaminase in baker's yeast. Also, tryptophan, methionine, lysine and cysteine residues are located in or near the active sites of the cytosine deaminase in *Aspergillus fumigatus* [15]. Furthermore, cysteine and methionine residues are located in or near the active sites of the extracellular cytosine deaminase in *C. violaceum* YK 391, while the lysine, histidine and serine residues might be indirectly involved in enzymic activity [16]. However, the amino acid residues involved in the active centers of intracellular cytosine deaminase from *C. violaceum* YK 391 remain to be reported.

In previous papers, the purification of intracellular cytosine deaminase from *C. violaceum* YK 391 has been

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detailed. This enzyme activity was strongly inhibited by Hg^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and Fe^{3+}, and α,α'-dipyridyl, ρ-CMB, NBS and chloramine-T at 1 mM [17].

Cytosine deaminase is not only a unique enzyme that controls the metabolism of pyrimidine nucleotide, but also a clinically important enzyme with potential to be used as an adjuvant chemotherapeutic agent. The intracellular cytosine deaminase from *C. violaceum* YK 391 has already been purified [17]. Now, it is proposed to assess the differences between the amino acid residues in the active sites of this enzyme and other types of cytosine deaminase by characterizing the amino acid residues involved in the active sites.

This paper details the results of chemical modification studies of intracellular cytosine deaminase purified from *C. violaceum* YK 391.

MATERIALS AND METHODS

Materials

The *N*-acetylimidazole, *N*-bromosuccinimide (NBS), sodium ρ-toluenesulfonchloramide (chloramine-T), 1,2-cyclohexanedione, ρ-chloromercuribenzoate (ρ-CMB), ρ-hydroxymercuribenzoate (ρ-HMB), diethyl pyrocarbonate (DEPC), diethyl pyrocarbonate, glyoxal, 2-hydroxy-5′-nitrobenzyl bromide (HNBB), phenylmethylsulfonyl fluoride (PMSF), pyridoxal-5-phosphate, phenylgyloxal, 2-mercaptoethanol (2-ME) and *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K, WRK) were obtained from Sigma Co., USA. The peptone, meat extract and yeast extract were purchased from Difco (Lab. Inc., USA).

Microorganism and Growth Conditions

C. violaceum YK 391 [7] was grown at 30°C for 3 days in a 5-L mini jar fermentor (SY-500, Korean Fermentor Co. LTD., Korea), containing 4 L of medium (pH 7.0) consisting of 0.75% soluble starch, 1.5% peptone, 0.1% meat extract, 0.1% yeast extract, 0.05% K_2HPO_4, 0.01% NaCl and 0.01% $MgCl_2 \cdot 7H_2O$, with 0.1% (v/v) silicone KM-70 (Shin-etsu Chemical Industry Co., Tokyo, Japan) as an antiformer under aeration. The cells were harvested at the early stationary phase of growth by centrifugation at 13,000 × g for 20 mins, washed twice with saline and stored at -20°C until use.

Enzyme Assay

The cytosine deaminase activity was assayed spectrophotometrically, based on the absorbance of cytosine and uracil in 0.1 N HCl at 290 nm, as previously described [1,7]. The assay system contained 0.2 mL 5 mM cytosine, 0.6 mL of 0.2 M Tris-HCl buffer (pH 7.5) and the enzyme solution, in a final volume of 1.0 mL. The reaction was initiated by the addition of cytosine solution after preincubation at 37°C for 5 mins. After incubation at 37°C for 30 mins the reaction was terminated by the addition of 4 mL of 0.1 N HCl. If a precipitate formed, it was removed by centrifugation and the supernatant then used for analysis. One unit of cytosine deaminase activity was defined as the amount of enzyme that could decompose 1 μmol of cytosine per hour.

Protein Assay

The protein content was determined by the method of Bradford [18] using protein assay dye reagent concentrate (Catalog 500-0006, Bio-Rad Laboratories, CA, USA) with bovine serum albumin as a standard protein, or by measuring the absorbance at 280 nm.

Preparation of Cell-Free Extract

The washed cells were suspended in 0.2 M Tris-HCl buffer (pH 7.5) and the suspension subjected to the action of a 120 kHz ultrasonic oscillator (Sonics and Materials Inc., USA) at temperatures not exceeding 5°C. Cells and debris were removed by centrifugation at 15,000 × *g* for 30 mins and then discarded. The resultant supernatant was referred to as the cell-free extract and was used as the crude enzyme preparation.

Purification of Cytosine Deaminase

The intracellular cytosine deaminase from *C. violaceum* YK 391 was purified by ammonium sulfate fractionation, DEAE-Sephacel, Ultrogel A6 and Sephadex G-100 column chromatographies, as described by Kim *et al.* [17].

Chemical Modification of the Enzyme by Chemical Modifiers

Modification of the amino acid residues of the cytosine deaminase was carried out as described by Lundblad and Noyes [19]. In order to modify the amino acid residues of the enzyme, the purified intracellular enzyme was incubated with 1 mM chemical modifiers in 0.2 M Tris-HCl buffer (pH 7.5), containing 1 mM cytosine, at 37°C for 30 mins. After termination of the enzyme reaction, the residual enzyme activity was determined. Oxidation of tryptophan residues with NBS was performed, essentially according to the method proposed by Spande and Witkop [20], Yoon and Kim [21] and Kronman *et al.* [22]. Most importantly, the reaction mixture containing NBS and phenylglyoxal was incubated in darkness in order to circumvent photolytic destruction.

Protection against inactivation of the enzyme by ρ-CMB was carried out by preincubation of the enzyme with various concentrations of cytosine in 0.2 M Tris-HCl buffer (pH 7.5) at 37°C for 10 mins, followed by the addition of ρ-CMB. Enzyme reaction mixtures were incubated at 37°C, and the protection of the enzyme activity then determined at given time intervals.

Reactivation of the Enzyme by Reducing Reagents

The cytosine deaminase activity was assayed under

standard reaction conditions by the addition of reducing reagents, such as dithiothreitol (DTT), 2-mercaptoethanol and cysteine-HCl, at indicated concentrations. Relative activity is expressed in comparison with the control value.

RESULTS AND DISCUSSION

Effect of Chemical Modifiers

The tryptophan, methionine, lysine and cysteine residues are located in or near the active sites of the intracellular cytosine deaminase in *A. fumigatus* [6], while a serine residue is indirectly involved in the enzymic activity. Also, the cysteine, histidine and arginine residues appear to be involved in the activity of intracellular cytosine deaminase from baker's yeast [14]. In order to ascertain which amino acid residues are located in or near the active sites of the intracellular cytosine deaminase from *C. violaceum* YK 391, the purified enzyme was incubated with several chemical modifiers.

As shown in Table 1, the intracellular cytosine deaminase was completely inhibited by 1 mM *p*-CMB and *p*-HMB.

The intracellular enzymes of *A. fumigatus* [5], *P. aureofaciens* [23], *E. coli* [3] and baker's yeast [14,25] were strongly inhibited by 1 mM *p*-CMB. Additionally, the extracellular cytosine deaminases from *Arthrobacter* sp. [26] and *Bacillus polymyxa* [27] were completely inhibited by 1 mM *p*-CMB. However, the intracellular enzymes of *S. marcescens* [28] were not inhibited by *p*-CMB. The inhibition of the enzyme by mercury compounds (*e.g.* *p*-CMB) suggests that a sulfhydryl group is also involved in the active site [29,30]. These results indicate that cysteine residue might be another compound involved in the active site of this intracellular cytosine deaminase.

NBS has several modification functions; among these are the reactivities with tryptophan, tyrosine and histidine [20,31]. *A. fumigatus* cytosine deaminase was completely inhibited by 1 mM NBS [5]. *C. violaceum* YK 391 intracellular cytosine deaminase was completely inhibited by 1 mM NBS, but only weakly inhibited by 1 mM HNBB. Conversely, the extracellular cytosine deaminase of *C. violaceum* YK 391 was not inhibited by 1 mM NBS.

The specific or selective modification of methionine residues in proteins and peptides is somewhat difficult to achieve under relatively mild conditions. The majority of the modification reactions used to study methionine involves oxidation either by chloramine-T or H_2O_2 [32].

The intracellular cytosine deaminase from *C. violaceum* YK 391 was completely inhibited by 1 mM chloramine-T, as was the cytosine deaminase of *A. fumigatus* [5] and the extracellular cytosine deaminase from *C. violaceum* YK 391 [16].

The intracellular enzymic activity was strongly inhibited by serine-specific modification reagents, such as PMSF and iodine [33-35], at a concentration of 1 mM. However, the extracellular cytosine deaminase from *C. violaceum* YK 391 was only weakly inhibited by PMSF and iodine [16]. Also, *A. fumigatus* cytosine deaminase [5] was strongly (80%) inhibited by 1 mM PMSF. Lysine-specific reagents, such as pyridoxal-5'-phosphate [33], weakly inhibited the intracellular enzymic activity, as did pyridoxal-5'-phosphate [16].

Table 1. Effect of chemical modifiers on the intracellular cytosine deaminase activity from *Chromobacterium violaceum* YK 391

Chemical modifiers	Target amino acid	Residual activity (%)
N-bromosuccinimide	Tryptophan	0
Chloramine-T	Methionine	0
p-CMB	Cysteine	0
p-HMB	Cysteine	0
Iodine	Serine	0
PMSF	Serine	20
Pyridoxal 5'-phosphate	Lysine	38
HNBB	Tryptophan	70
WRK	Aspartic acid, Glutamic acid	87
N-Acetylimidazole	Tyrosine	98
Glyoxal	Tyrosine	100
Phenylglyoxal	Arginine	88
1,2-Cyclohexadione	Arginine	100
Diethyl pyrocarbonate	Histidine	100
None		100

The cytosine deaminase activity was assayed under standard reaction conditions in the presence of several modifiers (1 mM) and expressed in terms of the relative activity compared to that with no modifier.
Chloramine-T; sodium *p*-toluenesulfonchloramide, *p*-CMB; *p*-chloromercuribenzoate, *p*-HMB; *p*-hydroxymercuribenzoate, PMSF; phenylmethylsulfonyl fluoride, HNBB; 2-hydroxy-5-nitro-benzylbromide, WRK; Woodward's reagent K.

These results suggest that the tryptophan, methionine, cysteine and serine residues are located in or near the active sites of the intracellular cytosine deaminase from *C. violaceum* YK 391, while lysine appears to be indirectly involved. Glyoxal, *N*-acethylimidazole, 1,2-cyclohexanedione, phenylglyoxal and diethyl pyrocarbonate had no effect on the intracellular cytosine deaminase from *C. violaceum* YK 391, as shown in Table 1. This suggests that the tyrosine, arginine and histidine residues are not located in or near the active sites of this intracellular cytosine deaminase.

From these results, it was speculated that the tryptophan, methionine, cysteine and serine residues may be located in or near the active sites of the intracellular cytosine deaminase from *C. violaceum* YK 391, while lysine is only indirectly involved in the enzymic activity.

Inhibition Constant

The apparent inhibition constant (*Ki*) was calculated

from a Dixon plot [36]. The apparent *Ki* values for chloramine-T, *p*-CMB, NBS and iodine were determined as 2.1×10^{-5}, 3.9×10^{-6}, 2.1×10^{-5} and 3.0×10^{-2} M, respectively (data not shown). For the cytosine deaminase from *A. fumigatus*, the apparent *Ki* values for chloramine-T and *p*-CMB were found to be 7.4×10^{-8} and 63.5×10^{-8} M, respectively [6]. Furthermore, in the extracellular cytosine deaminase from *C. violaceum* YK 391 the apparent *Ki* values for chloramine-T, *p*-CMB and α,α′-dipyridyl were measured to be 2.56×10^{-5}, 6.77×10^{-5} and 12.8×10^{-5} M, respectively [16]. The *Ki* values associated with the intracellular cytosine deaminase from *C. violaceum* YK 391 were found to be different from those associated with the extracellular cytosine deaminase from *C. violaceum* YK 391 and the *A. fumigatus* enzyme in chemical modification studies.

Protection of the Inactivated Enzyme Activity with Chemical Modifiers by Substrates

The inactivation of a specific enzyme activity by chemical modification reagents for certain amino acid residues does not always directly imply that such residues are present at the active sites [30,37]. However, protection of an enzyme activity against inactivation by substrates would suggest that the amino acid residues protected from chemical modifications, such as *p*-CMB, and chloramine-T, NBS and iodine, are located in or near the active sites.

Modification reactions with *p*-CMB were performed both at low and high level substrate concentrations, so that the protection of the enzyme activity by the substrates could be tested. In order to determine the effects of the substrates on the intracellular cytosine deaminase activity that had been deactivated by *p*-CMB, the enzyme reaction mixture was preincubated with 0.004 mM *p*-CMB, followed by the addition of 0.5 to 2.0 mM cytosine, and the enzyme activity then assayed at the given time intervals. The concentrations of chloramine-T, NBS and iodine used were 0.2, 0.02 and 30 mM, respectively, and the concentration of chemical modifiers that resulted in a 50% inhibition of the enzymic activity.

As shown in Fig. 1, the enzymic activity that had been deactivated by *p*-CMB did not recover in the presence of 1.0 mM cytosine, but recovered up to 50% of its original activity in the presence of 1.33 mM cytosine and completely recovered in the presence of 2.0 mM cytosine. The intracellular enzymic activity deactivated by *p*-CMB was reactivated by 2.0 mM cytosine, and was completely protected in the presence of excess cytosine. In addition, the enzymic activity deactivated by 0.2 mM chloramine-T, 0.02 mM NBS and 0.05 mM iodine recovered up to 50% of its original activity in the presence of either 1.41, 1.64 or 1.26 mM cytosine, and enzymic activity deactivated by chloramine-T, NBS and iodine was also completely protected in the presence of 2.0 mM cytosine.

The extracellular cytosine deaminase activity in *C. violaceum* YK 391 that had been deactivated by *p*-CMB was reactivated by 3.0 mM cytosine, and the enzyme activity inactivated by *p*-CMB and chloramine-T was completely protected in the presence of excess cytosine [16].

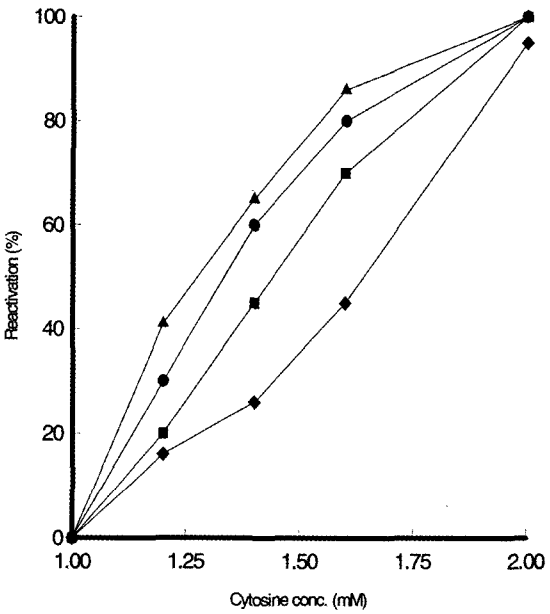

Fig. 1. Reactivation of the intracellular cytosine deaminase deactivated with *p*-CMB, chloramine-T, NBS and iodine by various concentration of cytosine. The reaction was carried out in 0.2 M Tris-HCl buffer (pH 7.5) with 1 to 2 mM cytosine. Symbols: ●, 0.004 mM *p*-CMB; ◆, 0.02 mM NBS; ■, 0.2 mM chloramine-T; ▲, 30 mM iodine.

And, *A. fumigatus* cytosine deaminase activity deactivated by chloramine-T, pyridoxal-5′-phosphate and NBS was completely protected by cytosine at high concentrations [6]. From these results, it became clear that the deactivated cytosine deaminase activity of various types and sources could be protected in the presence of excess cytosine.

The effects on the enzymic activity deactivated at the given substrate concentrations were analyzed by Lineweaver-Burk plots [38]. As shown in Fig. 2, the *Km* values of the enzymic activity deactivated by chemical modifiers were changed, but the *Vmax* values were not. This suggests that the chemical modifiers were competitively binding with the substrate, cytosine, at the active site of the enzyme.

Reactivation of the Enzyme by Reducing and Hydroxyl Reagents

In order to identify reactivation of the deactivated enzymic activity by sulfhydryl reagents, *p*-CMB-modified cytosine deaminase, with up to 50% residual activity, was incubated with 1 mM of the various reducing reagents in 0.2 M Tris-HCl buffer (pH 7.5), and then assayed for reactivated enzymic activity. As shown in Fig. 2, the enzymic activity was inhibited approximately 50% by 1.33 mM *p*-CMB.

As shown in Table 2, the enzymic activity that had been deactivated by *p*-CMB was reversed by incubation with cysteine-HCl, dithiothreitol and 2-mercaptoethanol at con-

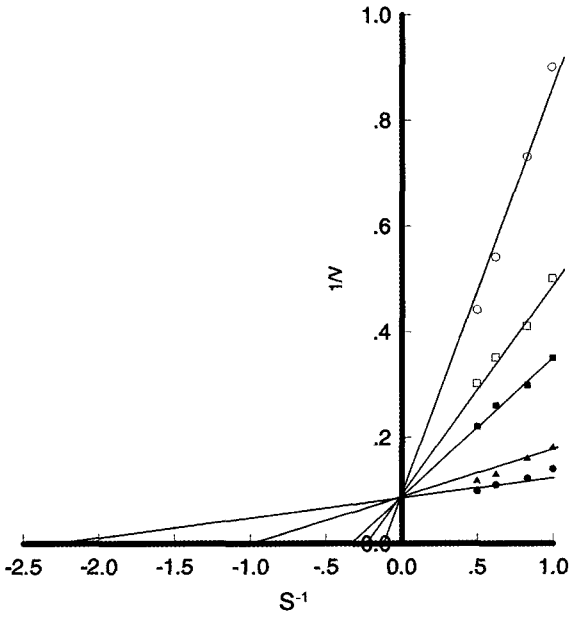

Fig. 2. Protection of the intracellular cytosine deaminase deactivated with chemical modifiers by cytosine. The enzyme activity was assayed in the presence or absence of chemical modifiers. Velocity(v) was expressed by the decrease in the absorbance at 290 nm for 30 mins. Symbols: ●, none; ▲, 30 mM iodine; ■, 0.004 mM ρ-CMB; □, 0.2 mM chloramine-T; ○, 0.02 mM NBS.

Table 2. Reactivation of the intracellular cytosine deaminase that had been deactivated with ρ-CMB by various concentration of thiol compounds

Reducing agent (mM)	Relative activity (%)			
	0	1 mM	3 mM	5 mM
None	100			
ρ-CMB	50			
ρ-CMB + Cysteine-HCl		100	34	3
ρ-CMB + dithiothreitol		99	94	91
ρ-CMB + 2-mercaptoethanol		99	97	100

The enzyme deactivated with ρ-CMB was incubated with 1 to 5 mM thiol compounds.

centrations between 1 and 5 mM, and reactivated up to 90% of the total enzymic activity by 2-mercaptoethanol and dithiothreitol at concentrations between 1 and 5 mM and by 1 mM cysteine-HCl. The enzymic activity that had been deactivated by 1.33 mM ρ-CMB was completely reactivated by 1 mM cysteine-HCl, dithiothreitol and 2-mercaptoethanol. This implies that the reactivation of the chemical-modified enzyme can be attributed to the chemical modification of a cysteine residue in the active site of the intracellular cytosine deaminase from *C. violaceum* YK 391.

The essential amino acid residues in the active site of baker's yeast cytosine deaminase include cysteine, histidine and arginine [14], and that of the *A. fumigatus* enzyme was associated cysteine, tryptophan, methionine and lysine, while serine appears to be indirectly involved in the enzymic activity [6].

Table 3. Differences of amino acid residues of the active sites of the intracellular and extracellular cytosine deaminase from *Chromobacterium violaceum* YK 391

Enzymes	Amino acid residues					
Intracellular[1)]	Cys	Met	Trp	-	-	-
Extracellular[2)]	Cys	Met	-	Lys	-	-
Baker's yeast[3)]	Cys	-	-	-	His	Arg
A. fumigatus[4)]	Cys	Met	Trp	Lys	-	-

[1)] In this study, intracellular cytosine deaminase of *C. violaceum* YK 391 [17]
[2)] extracellular cytosine deaminase of *C. violaceum* YK 391 [16]
[3)] intracellular cytosine deaminase of baker's yeast [14]
[4)] intracellular cytosine deaminase of *A. fumigatus* [15]
Symbols: Cys; cysteine, Met; methionine, Trp; tryptophan, Lys; lysine, His; histidine, Arg; arginine.

In the proceeding papers [16], the authors have reported on the amino acids of the active sites of the extracellular cytosine deaminase from *C. violaceum* YK 391. As shown in Table 3, the tryptophan, methionine and cysteine residues may be located in or near the active site of the intracellular cytosine deaminase in *C. violaceum* YK 391. However, the active site of the extracellular cytosine deaminase from *C. violaceum* YK 391 is, instead, associated with the cysteine, methionine and lysine residues [16]. Furthermore, the cytosine deaminases from *C. violaceum* YK 391, baker's yeast [14,24], *A. fumigatus* [5,6], *E. coli* [3] and *P. aureofaciens* [22] are thiol-enzymes, but *S. marcescens* cytosine deaminase is not [27].

From these results, it can be surmised that the amino acids residues of the active sites of intracellular cytosine deaminases are quite different from those associated with the extracellular enzymes from *C. violaceum* YK 391.

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