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The Flexible C-Terminal Region of *Aspergillus terreus* Blasticidin S Deaminase: Identification of Its Functional Roles with Deletion Enzymes

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Although the four polypeptides of blasticidin S (BS) deaminase (BSD) are packed rather tightly coordinated to the "structural and catalytic" zinc atom of each subunit, the C-terminal region of the enzyme was suggested to be somewhat molten and flexible [M. Kimura, S. Sekido, Y. Isogai, and I. Yamaguchi (2000) *J. Biochem.* 127, 955–963]. To understand roles of this flexible region, we constructed five C-terminal deletion variants of BSD (each successively deleted from the C-terminal end up to five residues) and analyzed their biochemical properties focusing on the structure and activity of the enzyme. BSD and all of the deletion mutants showed the unique rigid conformation (e.g., characterized by their stabilities in SDS solution) and high levels of resistance against protease digestions. Furthermore, both the wild-type and deletion apoenzymes exhibited similar physical properties in thermodynamic refolding into the stable tetramer conformation. However, these small C-terminal deletions exerted deleterious effects on the catalytic efficiency of the enzyme as indicated by their strongly reduced k_{cat}/K_m value. Judging from the altered kinetic parameters and unaltered structural properties of the deletion variants, these C-terminal residues appear to be directly involved in enzyme–substrate interaction. In this short flexible region, Tyr-126, Trp-128, and Gly-130 were the key residues. Most notably, removal of Gly-130 markedly increased K_m for BS without affecting its k_{cat} value. These results indicate that the flexible C-terminal region is important for catalytic function and that a single Gly residue at the C-terminal end of BSD contributes significantly in facilitating access of a substrate to the active site. © 2002 Elsevier Science

Key Words: **cytidine deaminase; C-terminal deletions; protein folding and stability; structural and catalytic zinc; zinc enzyme.**

Blasticidin S (BS) deaminase (BSD, EC 3.5.4.23) from *Aspergillus terreus* is a homotetrameric enzyme that contains a tightly bound zinc atom per subunit (1, 2). The gene encoding the enzyme, *bsd*, is now widely used as a marker gene in transformation of various organisms including fungi, plants, and mammalian cells (3–6). BSD acts on the cytosine moiety of BS and inactivates the antibiotic by a highly specific deamination reaction; i.e., neither cytosine, cytidine, deoxycytidylate, nor other purine nucleosides are deaminated by the enzyme (7). We have shown that BSD shares similar structural and mechanistic features with cytidine deaminases (CDAs) that are the most extensively studied enzymes in the cytosine nucleoside/nucleotide deaminase family (1, 2).

Biochemical and X-ray crystallographic studies revealed that the zinc atom of *Escherichia coli* CDA (EC 3.5.4.5) is essential for catalytic activity and lies deeply buried within the active site coordinated by His-102, Cys-129, and Cys-132 (8). The enzyme is homodimeric and each 32-kDa subunit contains two structurally similar core domains. Only the N-terminal domain of subunit contains the active site with the "catalytic" zinc atom. In contrast, CDAs from *Bacillus subtilis* and human are tetramers of identical subunits (15 and 16 kDa per subunit, respectively) and each subunit is comprised of a single active domain (9, 10). In such small tetrameric CDAs, Cys residues are found at the position corresponding to the His residue in the first

Abbreviations used: BS, blasticidin S; BSD, blasticidin S deaminase; CDA, cytidine deaminase.

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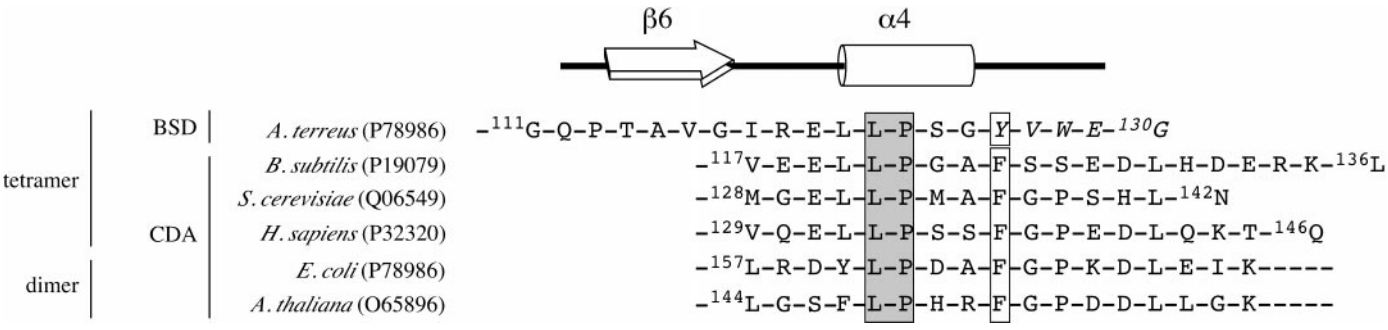

FIG. 1. Sequence alignment of the C-terminal region of *A. terreus* BSD with the corresponding regions of tetrameric and dimeric CDAs. SWISS-PROT accession numbers are shown in parentheses. Identical residues among these deaminases are shaded in gray. The position of the highly conserved residues (Tyr for BSD and Phe for CDA; see Discussion) is boxed. The secondary structure elements of BSD are indicated on top of the sequence. The residues comprising the "swinging-arm" of BSD are italicized. The K_m values of CDAs for cytidine are as follows: 39 μM for human CDA (19); 120 μM for *E. coli* CDA (21); and 150 μM for *A. thaliana* CDA (22).

part of the zinc-binding motif of *E. coli* CDA (i.e., His-102). This zinc coordination pattern (by three Cys residues) is quite unusual for "catalytic" zinc of a hydrolytic enzyme, although it is common for "structural" zinc in many proteins (11).

The active tetrameric structure of BSD was highly resistant to a strong denaturing reagent SDS (i.e., the activity of BSD was not lost in 0.2% SDS solution) and showed a unique property in SDS–PAGE; i.e., the native enzyme migrated much slower (apparent M_r of 36 kDa) than its actual subunit size of 13 kDa if samples were not heated in SDS loading buffer before the analysis (1). In accordance with the results, the CD spectra of the enzyme were not changed under similar conditions in SDS solution. In spite of such overall structural integrity of BSD, the C-terminal region (which contains Trp-128, the only Trp residue in the BSD polypeptide; see Fig. 1) appeared to be somewhat molten and flexible as suggested from large changes of the Trp fluorescence spectra by SDS (1).

To determine possible roles of the flexible C-terminal region for the enzyme, it will be useful to prepare and characterize a series BSD mutants deleted from the C-terminus. In this report, we show biochemical evidences that this short flexible region is directly involved in recognition of substrates.

MATERIALS AND METHODS

Materials. BS was obtained from Funakoshi Co. (Tokyo), residue-specific protease kit and LA-Taq (high fidelity *Taq* polymerase) from Takara Biomedicals Co. (Ohtsu, Japan), PAGEL precast mini gels (15% SDS–polyacrylamide gels for low molecular weight proteins) from ATTO Co. (Tokyo), and *p*-hydroxymercuriphenylsulfonate (PMPS) from Sigma (St. Louis, MO). Other chemicals were analytical grade from Wako Ltd. (Osaka). The molecular weight protein markers for SDS–PAGE were purchased from Pharmacia Biotech.

Construction of C-terminal deletion mutants. Plasmid pBSA712 (3) contains the complete ORF of *bsd* in pBluescript SK$^-$. Using this plasmid, we introduced a synonymous mutation (i.e., without amino acid substitution) that generated a unique *Sna*BI site between Tyr-126 (5′-TAC-3′) and Val-127 (5′-GTA-3′) of *bsd*. This was achieved by site-directed mutagenesis using PCR as follows (see Ref. 1 for the primer sequences which are not shown here). Briefly, one fragment was made by PCR with primers SNA (5′-CCATACGTAACCAGAGGGCAG-3′; hereafter mismatched bases are underlined) and T3, and the other fragment was by primers UB-2 and T7. These two PCR products were annealed and extended, and the resulting heteroduplex was used in a second round of PCR amplification with primers T3 and T7. After digestion with *Bam*HI and *Hin*dIII, only the *bsd* fragment with the desired mutation was cloned into the corresponding sites of pBluescript SK$^-$.

The terminal sequences of the *bsd* fragment were modified by PCR with primers UB-1 and DB-1, or UB-1 and Delta-126 (5′-GCGGATCCCTCCCACACTTAACCAG AGGGC-3′), and cloned between the *Nde*I and *Bam*HI sites on the polylinker of pET-12a vector (Novagen). The latter plasmid was used for a high level expression of Δ(126–130). The short *Sna*BI–*Bam*HI segment of the former plasmid was further replaced with the synthetic adaptors (Table 1) and used for high level expressions of deletion enzymes, Δ130, Δ(129–130), Δ(128–130), and Δ(127–130). The nucleotide sequences of all these modified *bsd* genes were verified by DNA sequencing.

Polyacrylamide gel electrophoresis. For SDS–PAGE, samples were incubated for 2 min, either at room temperature or at 100°C, in SDS loading buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, and 0.1% pyronin Y) and applied to a 15% polyacrylamide-SDS gel. Gels were run at 20 mA in running buffer (0.2% SDS) until the dye reaches the bottom. For Native-PAGE, samples were loaded on

TABLE 1

Adaptors Used for Construction of Deletion BSDs

BSD variants	Adaptor sequences with translations
Δ130	GTG TGG GAG TAA GGG CAC ACC CTC ATT CCC CTA G Val Trp Glu ***
Δ(129–130)	GTG TGG TAA GGG CAC ACC ATT CCC CTA G Val Trp ***
Δ(128–130)	GTG TAA GAG GGG CAC ATT CTC CCC CTA G Val ***
Δ(127–130)	TAA TGG GAG GGG ATT ACC CTC CCC CTA G ***

8–12% polyacrylamide gels and run as described above but without SDS (1).

Preparation of apoenzymes. A procedure similar to that described by Giedroc *et al.* (12) was used for preparing metal free apoBSDs. The purified deletion enzymes were dialyzed against buffer A (40 mM Hepes/KOH, pH 7.0) before titration of the –SH groups with PMPS. To 200 μl of the enzyme solution (0.1 mM subunit), four μl each of 100 mM PMPS in buffer A and 0.5 M EDTA was added to release the tightly bound zinc. Following incubation at 10 min, removal of these reagents was accomplished by repeated concentration and dilution of the reaction mixture using the Centricon YM-10 centrifugal filter device (Millipore Co., Bedford, MA). For deprotection of the Cys residues that reacted with the mercurial reagent, 2-mercaptoethanol was added at approximately 100-fold excess of the subunit molecules. ApoBSDs obtained in this way were finally replaced to buffer B (10 mM Tris–HCl, pH 7.5) using Centricon YM-10 as described above.

Kinetic parameters of enzymes. The concentration of each mutant enzyme was determined based on the molar extinction coefficient (ϵ_{280}), calculated by the total numbers of Tyr and Trp residues in its polypeptide sequence (13). Specific activity and kinetic parameters for the deletion mutants, as well as for wild-type BSD, were determined in buffer B by monitoring the decrease of absorbance at 282 nm (1).

Thermal inactivation of enzymes. Each mutant protein (1 mg/ml) was incubated in 1 ml of buffer B, plus varying concentrations of BS at 65°C. An aliquot (20 μl) was removed at each time point to monitor the residual activity. Half-lives for the enzyme variants were determined from the plots of $\ln(A_0/A_t)$ versus time, where A_0 and A_t represent the enzyme activity at time = 0 and time = t, respectively (14).

RESULTS

Purification and Characterization of C-Terminal Deletion Enzymes

All the deletion enzymes, Δ(126–130), Δ(127–130), Δ(128–130), Δ(129–130), and Δ130, were expressed at high levels in *E. coli* and recovered as soluble proteins. They could routinely be purified as active enzymes using the same protocol as described for the wild-type enzyme (1).

Purified BSDs were analyzed by SDS–PAGE (15% polyacrylamide) either with or without the step of sample heating in SDS loading buffer. The deletion variants, as well as the wild-type enzyme, migrated much slower through the gel (i.e., at apparent M_r of 36 kDa) if the heating step was not included in the analysis (Fig. 2). Furthermore, they were resistant to digestion by a mixture of proteases (trypsin, V8 protease, and endoprotease Asp-N) if the rigid tetrameric structure was not dissociated by PMPS (data not shown). These properties are identical to those of the wild-type enzyme (1).

Reconstitution of Deletion apoBSDs with Zinc

Previous studies on *B. subtilis* CDA took advantage of the difference of electrophoretic mobility between the wild-type and C-terminally extended enzyme to investigate conditions required for re-association of dissociated subunits (15). Here we used a similar approach to determine whether these deleted C-terminal residues are critical for thermodynamic refolding of BSD after removal of the "catalytic and structural" zinc.

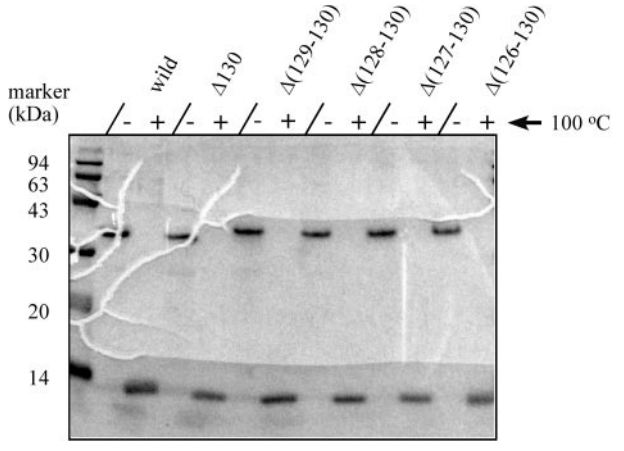

FIG. 2. SDS–PAGE (15% polyacrylamide gel; 3 μg protein each lane) of wild-type and mutant BSDs. The BSD polypeptides were incubated in SDS loading buffer either with or without the heating step. All of these polypeptides were detected apparently at the size of 36 kDa if heat treatments were not done before electrophoresis.

Apoenzymes of C-terminal deletion mutants were prepared by removing the tightly bound zinc by PMPS/EDTA followed by reduction of the Cys residues with 2-mercaptoethanol. These holo- and apoenzymes were subjected to Native-PAGE to see the effects of zinc elimination on their mobility in the polyacrylamide gel. As shown in Fig. 3A, both the wild-type and mutant apoenzymes appeared to be less stable than their corresponding holoenzymes as suggested by smears on the electropherogram (i.e., representing their partially unfolded structures; the marked regions with asterisks in lanes 2, 4, 6, 8 10, and 12). All these apoBSDs were reactivated by addition of $ZnCl_2$, suggesting that zinc depleted BSD could be refolded into the active tetrameric structure independent of the flexible C-terminal residues.

To further characterize conditions under which the deletion variants are reconstituted, apoBSD (wild-type) was mixed with mutant apoBSDs (except for Δ130 whose mobility in the gel was indistinguishable from that of the wild-type enzyme) at the 1:1 ratio, either in the presence or absence of zinc (Fig. 3B). If zinc was added prior to mixing of apoBSDs, only homotetrameric forms of holoBSDs were formed (lanes 1–4). In contrast, heterotetrameric BSDs (i.e., comprising of two wild-type subunits plus two mutant subunits; the middle bands in lanes 5–8) were also detected if zinc was added after mixing these apoenzymes. In either case, reconstituted holoenzymes were fully active. However, the 1:1 mixture of apoBSDs without zinc failed to refold into the active structure

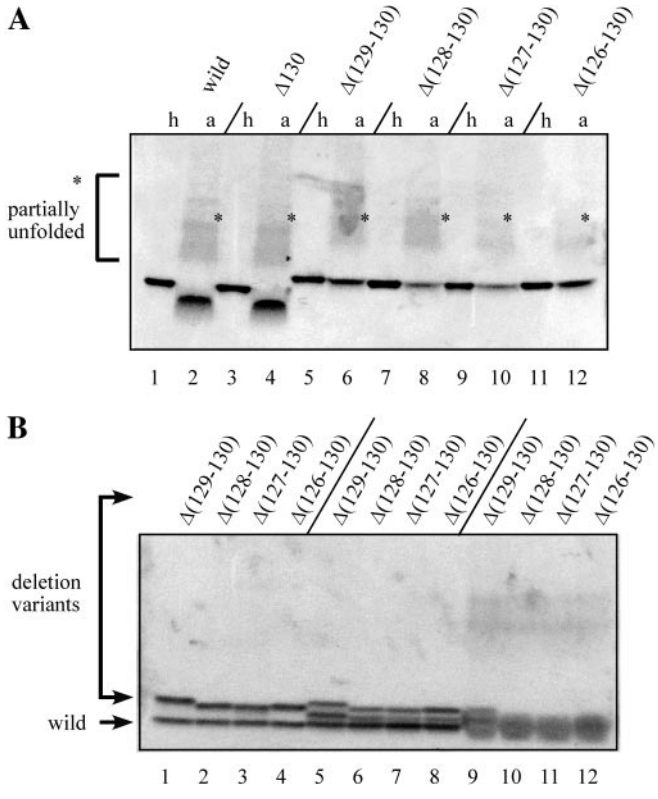

FIG. 3. Conformational changes of wild-type and deletion BSDs as reflected by the electrophoretic mobility on Native PAGE. (A) Inactivation of BSDs by removal of the zinc. Holoenzymes and apoenzymes (indicated as "h" and "a," respectively) of the BSD variants were separated on a 12% polyacrylamide gel (3 μg each variant). (B) Reconstitution of apoenzymes in the presence of zinc. Lanes 1–4, apoenzyme of each deletion variant was mixed with that of the wild-type enzyme after addition of $ZnCl_2$ (final 10 nmol); lanes 5–8, same as lanes 1–4, except that zinc was added after mixing of the apoenzymes; lanes 9–12, same as lanes 5–8, except that zinc was not added. The mixtures of the enzyme variants were separated on an 8% polyacrylamide gel (2 μg each variant).

and appeared as smears on the electropherogram (lanes 9–12). These results suggest that the apoBSD variants are dissociated into unstable dimers in the absence of zinc, but that they still retain the property of wild-type BSD to be reassembled into the active tetrameric form in the presence of zinc. Similar results were obtained in mixing of Δ130 and all the other deletion BSDs (data not shown).

Effects of Deletions on Kinetic Parameters of the Enzyme

The kinetic parameters for wild-type and mutant BSDs are summarized in Table 2. The K_m value for wild-type BSD (estimated to be approximately 1 μM) was much lower than those of CDAs for cytidine (see legend to Fig. 1), indicating that wild-type BSD has an outstandingly high affinity toward the substrate among the cytosine nucleoside deaminase family.

Among the five contiguous deletions, the most striking and unexpected effect was observed when the C-terminus Gly-130 residue was eliminated from the enzyme; i.e., the deletion resulted in a greatly elevated K_m value for Δ130. In addition to this mutant, conspicuous alterations of kinetic parameters were noticed for other BSD variants that have deletions extending over Trp-128; i.e., the catalytic efficiencies (k_{cat}/K_m) of Δ(128–130) and Δ(127–130) were reduced approximately 5-fold compared with those of Δ130 and Δ(129–130). This effect resulted from a combination of decreased k_{cat} (in the range of 2.2- to 2.8-fold of decrease) and increased K_m (2.1- or 2.5-fold of increase) values. Finally Tyr-126 was also found to be an important residue for the catalytic efficiency. The k_{cat}/K_m value of Δ(126–130) further decreased approximately 5-fold relative to Δ(128–130) and Δ(127–130). This reduction was solely attributed to the decreased k_{cat} value, and the K_m value remained constant among the deletion variants Δ(128–130), Δ(127–130), and Δ(126–130).

Thermal Inactivation of Deletion Enzymes in the Presence of Substrate

The rates of thermal inactivation of BSD and its variants were measured at 65°C in the presence or absence of BS (at a concentration of 0, 0.05, 0.1, 0.2, or 0.5 mM). As shown in Fig. 4, the half-lives of the enzyme variants extended in proportion to the concentrations of the substrate with which the enzyme variants were incubated. However, neither cytidine nor cytosine, which does not serve as a substrate for BSD, was able to extend the half-lives of the enzymes (data not shown). This substrate-specific protective effect against thermal inactivation was most evident for wild-type BSD, which has much higher affinity toward the substrate than any other deletion variants (Table

TABLE 2

Kinetic Constants for Wild-Type and Deletion BSDs

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Wild	1[a]	770	7.7×10^8
Δ130	8.1	860	1.1×10^8
Δ(129–130)	9.6	890	9.3×10^7
Δ(128–130)	20	390	2.0×10^7
Δ(127–130)	20	320	1.1×10^7
Δ(126–130)	20	71	3.6×10^6

Note. All measurements were performed at 30°C as described under Materials and Methods. The kinetic parameters were calculated from the s/v–s plots (Hanes–Woolf plots) by the least-square method.

[a] The value must be looked at with some caution, because the substrate concentrations were all higher than K_m due to the unavoidable detection limit of the method to determine the specific activity. What is clear, however, is that the K_m of wild-type BSD was much lower than those of its deletion variants.

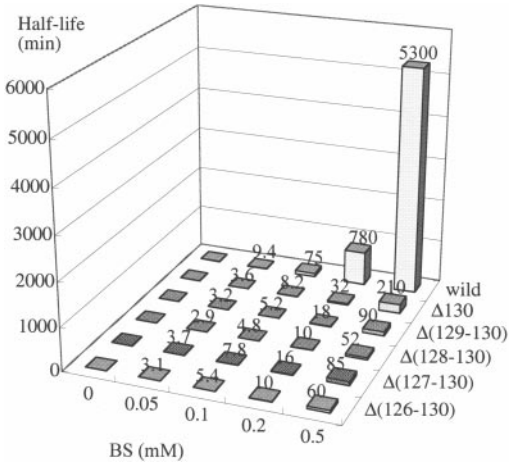

FIG. 4. Comparison of thermostability of BSD variants in the presence of BS. Values on top of the bars represent half-lives (min) for the enzymes at 65°C.

2). The half-lives of BSD variants generally correlated with their affinities for the substrate, but not with the k_{cat}/K_m or k_{cat} value.

DISCUSSION

A number of studies were carried out to identify possible roles of the C-terminal residues of BSD. These residues were previously suggested to be somewhat molten and flexible by the Trp fluorescence study (1). This is also supported by the X-ray diffraction study of the native BSD crystal to 1.5 Å resolution; i.e., the electron density map of the unliganded crystal structure did not reveal the position of the last five residues even in the cryo-condition (Yamamoto *et al.,* manuscript in preparation). Based on the knowledge of structural flexibility in this region, we have constructed the five C-terminal deletion enzymes (Fig. 1) and characterized their biochemical properties.

All the mutant enzymes inherited the overall structural integrity of wild-type BSD as suggested by (i) the heat-modifiable behavior of proteins in SDS–PAGE, and (ii) insensitivity of proteins against the proteases. These properties proved to reflect the tightly packed tetrameric structure of BSD in our previous study (1). This suggests that the last five C-terminal residues are *not* involved in maintenance of the rigid structure characteristic of BSD. In addition, these residues proved *not* to contribute to structural organization of BSD: they did not assist in thermodynamic refolding of the protein (Fig. 3) and were simply characterized as components of the flexible tail attached to the rigid core sphere. Judging from these overall structural similarities and properties of the deletion enzymes, the observed differences in the activity could be interpreted as an evidence of direct interaction of the substrate with the flexible C-terminus.

As demonstrated by kinetic analysis of BSDs, all of these deletion mutants impaired the function as a catalyst to deaminate BS. Comparison of k_{cat}/K_m among the deletion variants revealed three key residues, Gly-130, Trp-128, and Tyr-126, which play important roles in interaction with the substrate. The involvement of the C-terminal region for substrate recognition was also supported by the 1.5-Å X-ray crystal structure of the BSD-BS binary complex: side chains of three additional C-terminal residues stabilized by substrate binding (i.e., -Y126-V127-W128-) were uniquely defined in space by the electron density map (Yamamoto *et al.,* manuscript in preparation). In this respect, this short flexible region might be referred to as a "swinging-arm" that facilitates entry and alignment of the substrate in the active site. Although there are many examples of flexible regions that are critical for the catalytic efficiency, they concern the linker region connecting the two domains in a polypeptide. BSD can be distinguished from such enzymes in that the flexible C-terminus itself has direct interaction with the substrate. The only documented instance of an enzyme with such features is dUTP pyrophosphatase of a few organisms (16–18).

None of the five residues in the C-terminal region of BSD was identical in comparison with those of the conserved sequence of CDAs. This might be reasonable considering the involvement of the flexible C-terminus in specific recognition of the substrate. However, there existed a highly conserved amino acid substitution between the enzymes which are collectively divided into two subfamilies; i.e., Tyr-126 of BSD is replaced by Phe in CDAs from various organisms (see Fig. 1). The importance of this position was previously demonstrated by mutational analysis of human CDA, in which replacement of Phe-137 (equivalent to Tyr-126 of BSD) with Trp resulted in strongly decreased k_{cat} value compared to that of the wild-type enzyme (19). Similar negative effect of mutation on k_{cat} of the enzyme was also observed in kinetic analysis of BSD with the deletion variants (Table 2). Therefore, the residue at this position (either Tyr for BSD or Phe for CDAs) is most likely to control bringing residues with catalytic roles to the bound substrate, rather than merely binding the substrate, in members of the cytosine nucleoside deaminase family. The conserved substitution might reflect the difference of the substrate structure between BSDs and CDAs, since another BS deaminase from *Bacillus cereus* (represented as BSR in Refs. 1 and 3–6, as this bacterial enzyme is coded by the *bsr* gene) also has a Tyr residue at this position.

Unexpectedly, as indicated by the markedly increased K_m value of Δ130 compared to that of wild-type BSD, the C-terminus Gly residue proved to play important roles in increasing the affinity of the enzyme toward the substrate. This is quite interesting because Gly-130 does not directly participate in substrate bind-

ing as mentioned above (i.e., -E129-G130-COOH is missing in the cryo-X-ray crystal structure of the binary complex). The significant contribution of Gly-130 to increasing the affinity of the enzyme was also suggested from the result of thermal inactivation experiment: deletion of Gly-130 led to a greatly reduced efficiency of the substrate in protecting BSD against heat denaturation (Fig. 4; wild-type vs deletion variants). Since higher thermostability generally arise from elevated degrees of electrostatic interactions (salt bridges, hydrogen bonds) or hydrophobic interactions within the enzyme molecule, polar and bulky side chains of C-terminal residues (e.g., Trp-128, Tyr-126, but not Gly-130) must be responsible for establishing such interactions with the substrate. The possible role left for Gly-130 may thus be to rotate the "swinging-arm" for the substrate with greater velocity compared to that of Δ130, which could lead to an increased chance of substrate recognition and access to the active site by other side chains of these residues. This hypothesis is supported by determination of a rotational correlation time for each "swinging-arm" by time-resolved fluorescence depolarization method (Furuichi *et al.*, manuscript in preparation).

In conclusion, the results obtained with a series of deletion BSDs suggested roles of the flexible C-terminal region for substrate recognition and binding. Most notably, a single Gly residue at the C-terminal end of the polypeptide proved to contribute significantly in increasing the affinity of the enzyme toward the substrate. It will be interesting to see whether similar functions are identified in the small C-terminal region of CDA, for which development of specific inhibitors is expected to overcome the enzyme's negative effect in pharmaceutical application of cytosine-based anticancer drugs (20).

REFERENCES

1. Kimura, M., Sekido, S., Isogai, Y., and Yamaguchi, I. (2000) Expression, purification, and characterization of blasticidin S deaminase from *Aspergillus terreus:* The role of catalytic zinc in enzyme structure. *J. Biochem.* **127,** 955–963.
2. Nakasako, M., Kimura, M., and Yamaguchi, I. (1999) Crystallization and preliminary X-ray diffraction studies of blasticidin S deaminase from *Aspergillus terreus. Acta Crystallogr. D Biol. Crystallogr.* **55,** 547–548.
3. Kimura, M., Kamakura, T., Tao, Q. Z., Kaneko, I., and Yamaguchi, I. (1994) Cloning of the blasticidin S deaminase gene (*BSD*) from *Aspergillus terreus* and its use as a selectable marker for *Schizosaccharomyces pombe* and *Pyricularia oryzae. Mol. Gen. Genet.* **242,** 121–129.
4. Tamura, K., Kimura, M., and Yamaguchi, I. (1995) Blasticidin S deaminase gene (BSD): A new selection marker gene for transformation of *Arabidopsis thaliana* and *Nicotiana tabacum. Biosci. Biotechnol. Biochem.* **59,** 2336–2338.
5. Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994) Blasticidin S deaminase gene from *Aspergillus terreus* (BSD): A new drug resistance gene for transfection of mammalian cells. *Biochim. Biophys. Acta* **1219,** 653–659.
6. Kimura, M., and Yamaguchi, I. (1996) Recent development in the use of blasticidin S, a microbial fungicide, as a useful reagent in molecular biology. *Pest. Biochem. Physiol.* **56,** 243–248.
7. Yamaguchi, I., Shibata, H., Seto, H., and Misato, T. (1975) Isolation and purification of blasticidin S deaminase from *Aspergillus terreus. J. Antibiot.* **28,** 7–14.
8. Betts, L., Xiang, S., Short, S. A., Wolfenden, R., and Carter, C. W., Jr. (1994) Cytidine deaminase. The 2.3 Å crystal structure of an enzyme: Transition-state analog complex. *J. Mol. Biol.* **235,** 635–656.
9. Carlow, D., Carter, C. W., Jr., Mejlhede, N., Neuhard, J., and Wolfenden, R. (1999) Cytidine deaminase from *B. subtilis* and *E. coli:* Compensating effects of changing zinc coordination and quaternary structure. *Biochemistry* **38,** 12258–12265.
10. Cambi, A., Vincenzetti, S., Neuhard, J., Costanzi, S., Natalini, P., and Vita, A. (1998) Identification of four amino acid residues essential for catalysis in human cytidine deaminase by site-directed mutagenesis and chemical modifications. *Protein Eng.* **11,** 59–63.
11. Vallee, B. L., and Auld, D. S. (1990) Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* **29,** 5647–5659.
12. Giedroc, D. P., Keating, K. M., Williams, K. R., Konigsberg, W. H., and Coleman, J. E. (1986) Gene 32 protein, the single-stranded DNA binding protein from bacteriophage T4, is a zinc metalloprotein. *Proc. Natl. Acad. Sci. USA* **83,** 8452–8456.
13. Fasman, G. D. (1977) *in* CRC Handbook of Biochemistry and Molecular Biology, Vol. 1, 3rd ed., CRC Press, Boca Raton, FL.
14. Azzariti, A., Vacca, R. A., Giannattasio, S., Merafina, R. S., Marra, E., and Doonan, S. (1998) Kinetic properties and thermal stabilities of mutant forms of mitochondrial aspartate aminotransferase. *Biochim. Biophys. Acta* **1386,** 29–38.
15. Mejlhede, N., and Neuhard, J. (2000) The role of zinc in *Bacillus subtilis* cytidine deaminase. *Biochemistry* **39,** 7984–7989.
16. Mol, C. D., Harris, J. M., McIntosh, E. M., and Tainer, J. A. (1996) Human dUTP pyrophosphatase: Uracil recognition by a β hairpin and active sites formed by three separate subunits. *Structure* **4,** 1077–1092.
17. Vertessy, B. G., Larsson, G., Persson, T., Bergman, A. C., Persson, R., and Nyman, P. Q. (1998) The complete triphosphate moiety of non-hydrolyzable substrate analogues is required for a conformational shift of the flexible C-terminus in *E. coli* dUTP pyrophosphatase. *FEBS Lett.* **421,** 83–88.
18. Nord, J., Kiefer, M., Adolph, H. W., Zeppezauer, M. M., and Nyman, P. O. (2000) Transient kinetics of ligand binding and role of the C-terminus in the dUTPase from equine infectious anemia virus. *FEBS Lett.* **472,** 312–316.
19. Vincenzetti, S., Cambi, A., Maury, G., Bertorelle, F., Gaubert, G., Neuhard, J., Natalini, P., Salvatori, D., Sanctis, G. D., and Vita, A. (2000) Possible role of two phenylalanine residues in the active site of human cytidine deaminase. *Protein Eng.* **13,** 791–799.
20. Bouffard, D. Y., Laliberte, J., and Mompaler, R. L. (1993) Kinetic studies on 2′,2′-difluorodeoxycytidine (Gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. *Biochem. Pharmacol.* **45,** 1857–1861.
21. Smith, A. A., Carlow, D. C., Wolfenden, R., and Short, S. A. (1994) Mutations affecting transition-state stabilization by residues of coordinating zinc at the active site of cytidine deaminase. *Biochemistry* **33,** 6468–6474.
22. Vincenzetti, S., Cambi, A., Neuhard, J., Schnorr, K., Grelloni, M., and Vita, A. (1999) Cloning, expression, and purification of cytidine deaminase from *Arabidopsis thalina. Protein Express. Purif.* **15,** 8–15.