Crystal Structures of Blasticidin S Deaminase (BSD)

IMPLICATIONS FOR DYNAMIC PROPERTIES OF CATALYTIC ZINC*

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The set of blasticidin S (BS) and blasticidin S deaminase (BSD) is a widely used selectable marker for gene transfer experiments. BSD is a member of the cytidine deaminase (CDA) family; it is a zinc-dependent enzyme with three cysteines and one water molecule as zinc ligands. The crystal structures of BSD were determined in six states (i.e. native, substrate-bound, product-bound, cacodylate-bound, substrate-bound E56Q mutant, and R90K mutant). In the structures, the zinc position and coordination structures vary. The substrate-bound structure shows a large positional and geometrical shift of zinc with a double-headed electron density of the substrate that seems to be assigned to the amino and hydroxyl groups of the substrate and product, respectively. In this intermediate-like structure, the steric hindrance of the hydroxyl group pushes the zinc into the triangular plane consisting of three cysteines with a positional shift of ~0.6 Å, and the fifth ligand water approaches the opposite direction of the substrate with a shift of 0.4 Å. Accordingly, the zinc coordination is changed from tetrahedral to trigonal bipyramidal, and its coordination distance is extended between zinc and its intermediate. The shift of zinc and the recruited water is also observed in the structure of the inactivated E56Q mutant. This novel observation is different in two-cysteine cytidine deaminase Escherichia coli CDA and might be essential for the reaction mechanism in BSD, since it is useful for the easy release of the product by charge compensation and for the structural change of the substrate.

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** The abbreviations used are: BSD, blasticidin S deaminase; BS, blasticidin S; Bs, B. subtilis; CDA, cytidine deaminase; Ec, E. coli; MAD, multwavelength anomalous diffraction; Mm, M. musculus; OH-BS, deaminohydroxy-BS; Sc, S. cerevisiae; r.m.s.d., root mean squared deviation.

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...ification of drug-sensitive hematopoietic cells (18). Thus, information on the reaction mechanism, including the charge compensation of the zinc environment, has been analyzed and discussed (19–21).

The zinc ion takes various numbers and kinds of ligands because of its zero ligand-field stabilization energy owing to its having a filled d orbital and its function as a Lewis acid for accepting pairs of electrons. In particular, its coordination flexibility for polypeptide chains appears to be essential in many types of enzymatic reaction. As observed in crystallographic studies to date, the coordination geometry of the zinc ion varies and the zinc ion appears to act as a powerful electrophilic catalyst through the activation of an accompanying water molecule and a carboxylate group. The zinc ion is classified as a borderline cation according to its polarizability in hard and soft acid/base (HSAB) theory; it mostly prefers moderate nitrogen ligands although it can also accept hard and soft anions. As a result, four amino acid side chains, i.e. histidine, aspartic acid, glutamic acid, and cysteine, have been observed as protein ligands. Most reaction mechanisms of zinc enzymes have been discussed in terms of bond valence buffer theory. However, the dynamic properties of catalytic zinc remain to be elucidated, since the measurement of the properties is difficult because of the silent character of zinc in various spectroscopic methods.

To elucidate the novel substrate recognition and reaction mechanisms; here we report the crystal structures of BSD in six states: native, substrate-bound, product-bound, cacodylate-bound, substrate-E56Q mutant, and R90K mutant. The appearance of two different zinc coordination structures suggests charge compensation resulting from dynamic adjustments in coordination in several crystalline states.

EXPERIMENTAL PROCEDURES

Structural Determination of BSD—Recombinant BSD and its mutant were expressed in E. coli, purified and crystallized according to the procedure described in previous reports (5, 22). Crystallization was carried out using a solution of 20% (w/v) PEG8000, 50 mM magnesium chloride, and 0.1 M sodium cacodylate at pH 7.0 as precipitant. The cacodylic ion occupied the active site of BSD in the crystal obtained from this solution; therefore, it was called a cacodylate-bound crystal, and the cacodylate buffer was changed to 50 mM Tris (pH 7.0) to obtain completely native crystals. These crystals belong to the space group P212121, and one asymmetric unit contains one BSD tetramer.

The multiwavelength anomalous diffraction (MAD) method was used to determine phases, and the data set was collected under cryogenic conditions using the Rigaku R-AXIS IV at the BL45XU-PX station of SPring-8, Harima, Japan, as the first attempt to perform a trichromatic procedure (23). This procedure enables us to take diffraction images with a rapid alternation of three-wavelength x-ray beams. Hence, the dispersive differences of anomalous effects can be estimated very accurately (24). The data for structural refinements were also collected separately. All diffraction images were processed using an HKL suite (25) (see Table 1). The positions of the four zinc atoms were determined using Bijvoet and dispersive anomalous Patterson maps. The initial MAD phase set was calculated using the program MLPHARE (26) and further improved using DM (26) by noncrystallographic symmetry averaging for the tetrameric assembly of BSD. The electron density maps allowed the unambiguous modeling of 125 residues in each subunit except for one N-terminal and four C-terminal residues. Densities corresponding to cacodylic ions as well as zinc ions were observed in the anomalous difference Fourier map using the collected data (the f^2 value of arsenic atom is ~3.5e^-2 at an x-ray wavelength of 1.02 Å); thus, the ion models were also included. The Ramachandran plot of the refined model was sound, because all the residues except glycines and prolines were in their most favored and allowed regions (27). All the structural models in this study were constructed using program O (28) and refined with CNS (29) and Refmac (26).

Structural Determination of Substrate and Product Complexes—All the substrate/product-bound crystals were obtained by cocrystallization in the presence of 1 mM BS/OH-BS under the same crystallization conditions in the cacodylate-bound state. OH-BS was produced from BS by an enzymatic process. A marginally active E56Q mutant and a charge-compensation residue R90K mutant were constructed by site-directed mutagenesis using a PCR technique. All the crystals except one had a similar lattice parameter with the same space group to the crystal in the native state, and their initial structures were immediately determined by rigid body refinement of the native structure. On the other hand, only the crystal of the substrate-native enzyme complex belonged to a different space group, P4_2_2_2, despite the similar crystallization conditions. The crystal contained two subunits in an asymmetric unit and its structure was determined by the molecular replacement method on the basis of the structure of the substrate-unbound state using the program AMoRe (26). During the structural refinement of the substrate-native complex, 2 F_B – F_C maps showed a double-headed electron density near the catalytic zinc ion that was likely to be the reaction intermediate of BS. The corresponding molecular models of such BS states (see Figs. 1 and 2, and text) were constructed with MOLDA (30) and optimized with MOPAC7 (31). The structural restriction of the model was included except the torsion and bond angle between the pyrimidine and carbohydrate rings in the structural refinement with CNS and Refmac.

| TABLE 1
Crystallographic statistics for MAD phasing |
|-----------------------------------------------|
| Parameter                                    | Value  |
| Space group                                  | P2_1_2_1 |
| Cell dimensions (Å)                          | a = 54.7, b = 69.8, c = 145.8 |
| Wavelength (Å)                               | 1.0200 |
| Resolution limit (Å)                         | 2.2 |
| Observations                                 | 65,709 |
| Unique reflections                           | 23,532 |
| Completeness (%)                             | 80.6 (68.7) |
| Rmerge                                        | 4.8 (17.0) |
| Phasing                                      | 20.0–2.2 |
| Number of zinc sites                         | 4 |
| FOM^a                                        | 0.49/0.90 |

^a Rmerge = Σ_i |I_i(hkl)| – <|F_i(hkl)|]|Σ_i |I_i(hkl)|. Mean figure of merit.
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RESULTS

Overall Structure—The tetrameric BSD structure is 45 × 45 × 40 Å (Fig. 1B). The refined BSD model consists of four identical peptides of 125 amino acids (A-, B-, C-, and D-chains). All subunits have identical folding patterns and are related by a 222 point group symmetry. In fact, the root mean square differences among the four subunits are within 0.4 Å for the main chain atoms. Each subunit is composed of a six-stranded β-sheet, sandwiched by α-helices (Fig. 1C) and is classified into the modified Greek key motif similarly to CDAs. The structure of the N-terminal-half of one monomer (1–80) begins with an α-helix (α1, Gln5–Ser29). This α-helix is embraced by an anti-parallel β-sheet (β1, Val29–Ser36; β2, Ile40–Val44, β3, Cys73–Gly78). An additional α-helix (α2, Ala45–Ala63) protrudes from the other side of the β-sheet wall. A long lariat-like region (loop β2–α2: Asn45–Pro53) connects the β-sheet (β1, β2, β3) and α2. A very short strand (β4, Ile54–Leu55) and a continuous loop connect the latter half of the monomer. This portion contains two anti-parallel strands (β5, Lys103–Lys107; β6, Thr114–Gly117) and two α-helices (α3, Cys88–Leu96; α4, Arg119–Leu121). In this domain, all secondary structures have the same orientation except α4. The crystal structures of the native and substrate-bound states (Fig. 1, B and C) are very similar, as indicated by the r.m.s. difference for all protein atoms at ca. 0.6 Å despite of the different space groups.

Active Site Pockets and Substrate Recognition—The active site is located at the bottom of a deep (10 Å in depth) and narrow (5 Å in width) pocket formed by the helices α2 (Tyr47A, Cys54A, Glu56A), α3 (Cys88A, Cys91A) and residues from two adjacent subunits (Tyr126B and Phe49C) (Fig. 1C). The three aromatic residues form the side wall of the pocket, and Tyr126 from the adjacent subunit likely plays the role of a lid for the pocket entrance. Part of the pocket is composed of a loop connecting β2 and α2 in the adjacent subunit. The pocket surface is positively charged because of Arg42 and the zinc ion (Fig. 2). Furthermore, a flexible C-terminal region (Val127–Gly130) extends into the pocket.

The recognition scheme with the pyrimidine ring of BS is similar to that between EcCDA and cytidine (16). However, four amino acids (Ser78, Phe49, Arg82, and Tyr126) interact with the carbohydrate (enopyranuronic acid) portion and the additional tail portion including the guanidium group (Fig. 2). To expand the active site pocket for accepting the larger tail por-
tion, BSD folds the loop regions in a more compact manner than other CDAs: (i) strand $\beta 1$ is shortened, (ii) loop $\alpha 1-\beta 1$ protrudes to the molecular surface, and (iii) loop $\beta 2-\alpha 2$ occupies the inside of the molecule.

Tetramer Formation—Subunit interfaces of the tetramer are located near active site pockets. The interface is formed by the loop (residues 47–52) of $\beta 2$ to $\alpha 2$ and four $\alpha 2$ helices from all subunits at the symmetric center. Tyr$^{47}$, His$^{48}$, Phe$^{49}$, and Asp$^{97}$ form a hydrogen bond network to stabilize subunit association. The mutation of these residues probably influences tetramer formation, and the H48Q mutant of BSD shows no catalytic activity (data not shown). Additionally, two interactions tightly support the tetramer: (i) $\alpha 2$ and the loop (residues 23–28) of $\alpha 1-\beta 1$, and (ii) C-terminal residues 124–126 and two helices $\alpha 3$ from neighboring subunits.

In the formation of the tetramer architecture, each Arg$^{90}$ of four subunits may play a key role. Each arginine faces each other around the catalytic center. The positions of the water molecules, and the tetrameric center recruits a chloride ion that might compensate for the positive amino group of Lys$^{90}$ (Fig. 3B).

Putative Reaction Scheme—In the structure in the substrate-bound state, the substrates were unambiguously identified in the electron density map. An unexpected feature in the electron density map of BS was observed at the 4-amino (4-NH$_2$) group of the cytidine core near the zinc ion (Fig. 4). Although the density corresponding to the amino group of the substrate or the hydroxyl group of the product was negligible, a double-headed electron density was detected around the catalyzed 4-amino group, where an amino group was exchanged for a hydroxyl group during catalytic reaction. Either of the heads could be assigned to a nitrogen or oxygen atom of unit occupancy, and the temperature factor of each atom was comparable to that of adjoining atoms in the refined model. Thus, in accordance with the suggested reaction scheme (Fig. 1A), the double-headed density could be interpreted as the amino and hydroxyl groups in the reaction intermediate of BS (BS$^*$). However, a tear drop-shaped density was observed in the omit map in subunit A (Fig. 4C), and this density could not be satisfactorily assigned to the hydroxyl group predicted in the model. This suggests that the structure exists in a state between the enzyme-water-BS complex and the tetrahedral intermediate 1 in Fig. 1A, whereby the hydroxyl group might be in an ionic state rather than covalently bonded with the C4 atom of the pyrimidine ring.

Zinc Coordination and Substrate Binding in Six States—The structure of the active center in the native state shows that the zinc ion coordinated in a tetrahedral geometry with Cys$^{54}$, Cys$^{88}$, and Cys$^{91}$ (Fig. 5A and Table 2). This geometry is shared by other CDA structures. As proposed for the reaction mechanism of CDA (16), a water molecule usually accompanying the zinc ion probably replaced the fourth ligand shown as Wat1 in Fig. 5A. This complete native structure with no substrates or inhibitors contains three water molecules, including Wat1, around the catalytic center. The positions of the water molecules correspond to those of the 4-amino group; one of the water molecules, Wat5, occupies the leaving ammonia binding...
site. This geometry is also observed in the OH-BS complex (Fig. 5D) and cacodylic acid binding states.

In the substrate-bound state, the zinc coordination geometry is classified to be distorted trigonal bipyramidal rather than tetragonal (Fig. 5C). From the tetrahedral geometry, the zinc ion apparently shifts ∼0.6 Å toward the hydration water molecule hydrogen-bonded with Arg90 and enters the triangular plane of three cysteine S atoms (Table 2), despite the corresponding structures of the polypeptide chains being nearly identical. At the same time, the water molecule (Wat2) exhibits a small but significant positional shift of ca. 0.4 Å. The positional shift of Wat2 is associated with the alternative conformation of Arg90, which faces the center of enzymatic tetrameric 222 symmetry. Thus, the zinc coordination shift may be influenced by the conformation of Arg90 and the signal of the shift may be transferred to other subunits through Wat3 as shown in Fig. 3A.

The E56Q mutant and BS complex each show a similar conformation to the putative intermediate complex (Fig. 5B). However, this structure showed a weak electron density of the fourth water ligand, and the 4-amino group is far from zinc and interacts with Gln56Oe. We examined four possibilities of ambiguity of the Oe/Ne inversion and substrate/product. The two possibilities of Gln146 χ3 inversion could be distinguished by the temperature factors of Oe and Ne. From the result, we conclude that there is an interaction between the 4-amino group and GlnOe and that there are two possible structures: (i) the substrate BS is maintained in its 4-amino group; and (ii) the substrate is processed to an OH-BS product and the 4-amino group is maintained in its 4-amino group; and cysteine residues is not affected by constraints in the protein structure (37).


discussion

change in coordination geometry—The zinc coordination shift from tetragonal to trigonal bipyramidal is caused by the steric distortion of the pyrimidine ring and the carbohydrate-pyrimidine bond during substrate-product transition. The structure of the EcCDA and uridine complex suggests that the relative angle between the pyrimidine and ribose rings is distorted during the catalytic reaction (20). This distortion compromises the energies of the zinc coordination with the intermediate O4 atom and the hydrogen bonds with the enzyme and ribose ring. If zinc could be moved to the plane of the three cysteine ligands, the distortion energy could be released by the positional shift of zinc. Zinc has a ligand field stabilization energy of zero, therefore, there is no energetic disadvantage for the shift if an additional fifth ligand is adopted.

The shift from trigonal bipyramidal to tetragonal might be triggered by releasing the distortion of the pyrimidine ring and the carbohydrate-pyrimidine bond. Subsequently the removal of the ammonium group and formation of the products occur (Fig. 1A), although the probable intermediate appears stably trapped as described before. In concert with the geometrical alteration, the coordination bond lengths of zinc and ligands change. Although the bond lengths are almost equivalent in the tetrahedral geometry, this equality was broken in the trigonal bipyramidal geometry. The Zn-S bond is shorter than the Zn-O bond with the OH group of BS* and the additional water ligand. This observation is consistent with the greater stability of equatorial coordinations than axial coordinations based on the ligand field theory for trigonal bipyramidal geometry. The increase in the distance between zinc and the product would facilitate the exchange of the product and water.

In facilitating movement and coordination shift in zinc, three cysteine ligands might be essential, since sulfur ligands do not stabilize coordination by orientation selectivity as soft acid lacks polarization capability. From the details of the BSD structure, the movement of cysteines is not significant even in large shifts. In contrast, the conformation of the histidine ligand in EcCDA is strictly stabilized by the adjacent Asn234 side chain and Ala232 carbonyl group as the elec-His-Zn motif, where the position and orientation of the zinc ion are maintained by stabilized histidine ring (32). In the motif, a zinc coordination shift would require a large rearrangement including main-chain atoms, as observed in the crystal structures of alcohol dehydrogenase from Thermoaerobacter brockii (TbADH) and human class III alcohol dehydrogenase (HsADH) (33, 34). These shifts might be achieved because of the existence of many possible ligands including the substrate and the product, and their positional shift that includes the main chain. The time-resolved x-ray absorption fine structure analysis of TbADH revealed two types of pentacoordinated zinc formed during catalysis (35). The predicted coordination geometry is distorted bipyramidal trigonal, similar to that in our observation.

A dynamic rearrangement of the coordination structure has also been observed in other metal ions. The iron ion can take two spin states and alters its coordination geometry to bind with the nitrogen and oxygen ligands of heme. The ligand field stabilization energy is a source of the allosteric effect in hemoglobin (36). In contrast, the interaction between the zinc ion and cysteine residues is not affected by constraints in the protein structure (37).

Contribution of Arg90 to Catalysis—In the BSD structure, the coordination shift of zinc would be affected to Arg90 via the fifth water ligand and the side chain of Arg90 should change its distance from the fifth water ligand. However, Arg90 is a key residue for tetramer formation, therefore such conformational change must change both conditions.
The R90K mutant exhibited a larger $V_{\text{max}}$ than the native enzyme in the result of enzymatic assay (result now shown). Arg$^{90}$ is only conserved in tetrameric CDAs from Actinomyces species. In tetrameric $B.\text{subtilis}$ CDA (BsCDA) and $M.\text{musculus}$ CDA (MmCDA), Arg$^{90}$ is replaced with Ala$^{88}$ and Ala, but Arg$^{56}$ and Arg$^{68}$ of BsCDA and MmCDA, respectively, also contribute to tetramerization (11). Because of this amino acid replacement and the size of water pool area in the tetramer interface, BSD and Actinomyces CDAs should be differentiated from other common CDAs.

**Compensation of Zinc Net Charge**—
The net charge of zinc must be compensated by deprotonated cysteines. An electrospray mass spectrometry study suggests that two of three or four cysteines (HCCC or CCCC) are protonated in zinc finger arrays (38). The temperature factor of only Cys$^{91}$ is higher than the other two cysteines. Its electron density is anisotropically distributed in the complex structure and the two bond distances of Zn–Cys$^{54S}$γ and Zn–Cys$^{91S}$γ are shortened in the substrate-bound structure (Table 2). A similar structure was also observed in EcCDA–uridine complexes. This suggests that the bond valences for both the reaction of the substrate and the release of the product are regulated by a decrease in the distance between Zn and Cys$^{132S}$γ of ~0.1 Å (20). These features would contribute to buffering of the bond valence in the zinc ion.

In the three-cysteine CDAs, a different charge compensation mechanism is only observed in MmCDA. Most three-cysteine CDAs (i.e. BsCDA, ScCDA, HsCDA, and MmCDA) have no fifth ligand corresponding to Wat2 in BSD. However, an arginine residue (Arg$^{68}$ in MmCDA) is located in the vicinity of the zinc environment and the hydrogen bonds formed directly two cysteine ligands (Cys$^{53}$ and Cys$^{89}$) in all the cases. This compensates for part of the negative charge of the cysteine ligands (16). Despite the lack of contribution of the fifth ligand,
there is no structural interference in the movement of the zinc ion for the charge compensation. The movement of zinc might affect all three-cysteine CDAs, although the charge compensation mechanism of the zinc ion during catalytic reaction in three-cysteine CDAs remains unclear.

**Reaction Scheme**—A putative reaction scheme could be proposed based on the five states of BSD structures. The complete native structure, at the beginning of the catalytic reaction, contains three water molecules (Fig. 5A). Wat1 is directly coordinated with the zinc and works as the catalytic water. When entering a substrate BS, an enzyme-water-BS complex is formed, as shown in Fig. 5B. A weak Fo – Fc signal could be assigned to the catalytic water, even though the corresponding model contains no Wat1. Here Wat5 replaces the 4-amino group bound to the leaving ammonia binding site. NH₃ and the deprotonated Wat1 may occupy the site until ammonia leaves,
Crystal Structures of Blasticidin S Deaminase

| TABLE 2 Crystallographic statistics for refinement |
|---------------------------------------------------|
| Crystal Structures of Blasticidin S Deaminase |
| Space group | P2₁₂₁₂₁ |
| Cell dimensions (Å) | a = 54.12 |
| Resolution limit (Å) | 50.0–1.5 |
| Completion (%) | 99.3 |
| Unique reflections | 88,094 |
| Refinement | |
| Refinement | |
| Average B factor (Å²) | 14.9 |
| Non hydrogen atoms | 4,517 |
| Average angle (°) | 33.2 |
| Average distance (Å) | 2.23 |
| Cys91-Zn-Wat2 | 66.4 (66.1–66.6) |
| Cys88-Zn-Wat2 | 84.1 (82.6–85.1) |
| Wat2-Arg90N | 2.30 (2.25–2.33) |
| Cys54-Zn-Wat2 | 66.0 (65.2–67.3) |
| Cys91-Zn-Wat1/BS | 107.7 (105.2–111.6) |
| Cys88-Zn-Wat1/BS | 104.8 (103.7–106.2) |
| Wat2-Ag90N | 2.15 (2.08–2.21) |
| Proximal distal | 3.07 (2.97–3.16) |
| Wat2-Arg90N | 2.15 (2.08–2.21) |
| Cys91-Zn-Wat2 | 84.1 (82.6–85.1) |

and the pyrimidine ring will rotate around the root of the C1’-N1 glycosidic bond. This process has been suggested by Xiang et al. (20). In this state, zinc enters the triangular plane consisting of three cysteines. In the reaction intermediate, deprotinated Wat1 attacks the C4 atom of BS and forms a covalent bond with some rotation of the pyrimidine ring (Fig. 5C). The replacement is completed as shown in Fig. 5D. The pyrimidine ring is located nearest the zinc. The leaving ammonia site is occupied by a water molecule. After ammonium leaves, the product OH-BS will interact with the leaving ammonia site as shown in Fig. 5B to exit the product.

In the case of EcCDA, Xiang et al. (20) has proposed that the distortion of the C1’-N1 glycosidic linkage in the product complex might be the source of substrate leaving force. The careful refinement in BSD shows smaller distortion of the linkage. This might be caused by the fact that the distortion might be released in part by the positional shift of zinc. Thus, we suggest common and diverse features of CDA reaction mechanism. However, more structural studies of various kinds of complexes and mutant BSDs are necessary to clarify the activation mechanism of the three cysteine-coordinated catalytic zinc.

Is It Possible to Maintain the Reaction Intermediate?—Several reasons can be considered in the stabilization of the putative reaction intermediate structure. One is the high affinity of BSD for a product. To adopt the larger substrate of BS, an ancestor of BSD had evolved through the enhancement of the interaction between the enzyme and the substrate. The additional interaction from the tail portion of BS increases the total affinities of BS for both the substrate and the product (substrate $K_m$ and product $K_p$ are 2.1 and 2.3 $\times 10^{-5}$ M at pH 7.5, respectively) (7), which are higher than those for EcCDA ($K_m = 1.2 \times 10^{-4}$ M, $K_p = 3 \times 10^{-4}$ M) (39). Because the differences in $K_m$ and $K_p$ are probably affected only by the replacement of the 4-amino group with the 4-hydroxyl group, BSD of higher affinity might be able to bind to the product.

The amino group of the intermediate is found to be trapped by Glu56Oi and the carbonyl oxygens of Ser86 (Fig. 4B). This interaction for the exiting ammonium ion has been suggested for the EcCDA structure having a similar environment (20), and a trapped ammonium was observed in MmCDA (14). Additionally, the C-terminal region of BSD, ranging from Val127 to Trp129 or Glu129, would contribute to the substrate recognition and the exit of the ammonium ion and product. This region seems to block the exit of the products in the complex structure, whereas this region is not observed in the electron density map of the native structure. The flexibility of the C terminus may control the accessibility of substrates and solvents to the active site pocket. Such a
mechanism is supported by results of a previous deletion mutant study (40).

The crystalline state sometimes provides this type of unfavorable structure due to unusual crystal packing forces and a static environment. The crystal of the putative intermediate was obtained as a tetragonal space group, different from the orthorhombic native crystal. Large structural differences (over 0.5 Å shift) in both states were observed in several regions: residues (i) 4–10, (ii) 35–39, (iii) 83–86, and (iv) 106–117. The differences between these regions might be associated with crystal packing or the binding of the cacodylic ion to Glu<sup>7</sup> and Cys<sup>73</sup> (Fig. 1B). Although these regions are far from active site pockets, the shift in position resulted in Ser<sup>86</sup> moving toward Cys<sup>73</sup> (Fig. 1B). However, has also been negatively argued in some articles: a<sup>13</sup>C structure. The existence of such an unfavorable structure, how-

tion between the intermediate and the protein.

A tetrahedral intermediate has also been reported in the crystal structure of adenosine deaminase, a zinc-dependent enzyme of the purine salvage pathway (41). Inosine, the enzyme product, is observed as a gem-diolate water adduct in such a structure. The existence of such an unfavorable structure, however, has also been negatively argued in some articles: a<sup>13</sup>C NMR study has revealed that this conformation will not be taken in a solution (42), and a crystallographic study of CDA has described that the reason for the misinterpretation might be model bias caused by structural restraint at lower resolution (20). Our result could not exclude the latter possibility, so it should be validated by further structural and functional studies of the enzyme-product complex.

The crystal structures of BSD show that the catalytic zinc ion has a three-cysteine coordination sphere, and provide a new framework for a three-cysteine coordinated zinc active center. The movement of zinc coordination in BSD might be related to the steric hindrance of the cytidine core during catalytic reaction. The double-headed electron density of the substrate is a possible clue to resolving the reaction mechanism.

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