J. Biochem. 127, 955–963 (2000)

Expression, Purification, and Characterization of Blasticidin S Deaminase (BSD) from *Aspergillus terreus*: The Role of Catalytic Zinc in Enzyme Structure[1]

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Received November 11, 1999; accepted March 1, 2000

We established an efficient overproduction-purification system for blasticidin S deaminase (BSD) using the cDNA cloned from *Aspergillus terreus*. The estimated molecular mass of the purified enzyme indicated BSD was a tetramer. This tetrameric form was very resistant to denaturation by SDS and showed heat-modifiable behavior on SDS-PAGE; *i.e.*, BSD migrated much slower (as a single band of 36 kDa) in its active conformation than its completely denatured polypeptide (13 kDa) if heat treatment in 2% SDS was not performed before electrophoresis. As predicted from the presence of the catalytic zinc-coordinating sequence motif conserved in the cytosine nucleoside/nucleotide deaminase family, BSD also contained one zinc per deaminase subunit. However, the predicted catalytic function appeared not to be the only role of this zinc in the enzyme. First, titration of the zinc-chelating -SH groups with *p*-hydroxymercuriphenylsulfonate led to dissociation of the BSD tetramer into unstable monomers or dimers. Second, depletion of zinc on reconstitution of chemically denatured BSD (with either guanidine-HCl or acidic pH) resulted in improper folding of the polypeptide. These results suggest that zinc also plays a structural role in maintenance of the protein structure. When we introduced mutations at Glu-56 (the proposed active site) and Cys-91 (a proposed catalytic zinc-binding Cys) in BSD, none of the resulting mutants (E56D, E56Q, C91A, C91S, and C91H) showed any detectable activity, as judged with the spectrophotometric assay. Replacements of Cys-91 resulted in gross perturbation of the enzyme structure although the catalytically essential Glu-56 was not necessarily required for proper folding of the enzyme. These results further support our proposal that the catalytic zinc coordinated by the conserved sequence motif is also structural in BSD.

Key words: catalytic and structural zinc, cytosine nucleoside/nucleotide deaminases, heat-modifiable protein, protein folding and stability, site-directed mutagenesis.

Blasticidin S (BS), an aminoacylnucleoside antibiotic produced by *Streptomyces griseochromogenes*, is the first successful microbial fungicide in Japan (*1*). In the course of studies on the fate of BS in the environment, enzymatic detoxification was discovered in a strain of *Aspergillus terreus* (*2*). An enzyme responsible for this resistance, named BS deaminase (BSD, EC 3.5.4.23), was purified from the fungus and its enzymatic properties were examined. BSD exhibited high specificity towards BS and some of its analogues, and no deamination activity was detected toward cytosine, cytidine, deoxycytidylate, or other purine nucleosides (*3*).

The cDNA encoding BSD has been cloned, its nucleotide sequence determined (*4*), and the deduced amino acid sequence compared with that of *bsr*, another BS deaminase gene identified in *Bacillus cereus* (for a review see Ref. *5*). Interestingly, no overall similarity was observed between the peptide sequences of these BS deaminases except in the significant local region corresponding to residues 85–95 of BSD and 97–107 of BSR (*5*). This short span of contiguous amino acids has also been found in cytidine deaminases (CDAs) (*6–8*), which exhibit no hydrolytic activity toward BS. The results of X-ray crystallographic studies on *Escherichia coli* CDA [EC 3.5.4.5] revealed that this region belongs to a part of a novel zinc binding motif (*9*) which is conserved in the cytosine nucleoside/nucleotide deaminase family (*10, 11*). As shown in Fig. 1, a His (or Cys) and two Cys (boxed) residues were proposed to coordinate a zinc atom essential for catalysis, and a Glu (bold) to be required for protonation of the pyrimidine in the active center (*9–11, 15, 16*).

Previous studies revealed some enzymatic characteristics

[1] This research was supported in part by a grant for a Biodesign Research Group from RIKEN.

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Abbreviations: AP-I, *Achromobacter* protease I; Arg-EP, arginylendopeptidase; ATCase, aspartate transcarbamoylase; BS, blasticidin S; BSD, blasticidin S deaminase from *Aspergillus terreus*; BSR, blasticidin S deaminase from *Bacillus cereus*; CDA, cytidine deaminase; PAR, 4-(2-pyridylazo)resorcinol; PMPS, *p*-hydroxymercuriphenylsulfonate.

of BSD; *i.e.*, inhibitor experiments defined the essential functional groups for the enzyme to be active, and classical kinetic analyses suggested features of the active center (*12*) as well as structural requirements of substrates for binding (*13*). However, some of the data must be looked over again based on the recently obtained crystallographic knowledge of CDA, which belongs to the same deaminase family.[4] Thus, to facilitate analysis of the enzyme structure, we constructed an expression vector for *E. coli* and established an efficient purification system involving the affinity chromatography technique. Furthermore, the recombinant BSD was crystallized with polyethylene glycol 8000 (*14*), and its X-ray crystallographic structure has been recently determined (manuscript in preparation).

In this paper, we report the results of biochemical experiments that were designed based on the structural knowledge of CDAs, and propose a new role of the catalytic zinc in BSD.

EXPERIMENTAL PROCEDURES

Chemicals, Proteins, and Enzymes—BS was obtained from Funakoshi (Tokyo); *p*-hydroxymercuriphenylsulfonate (PMPS) was from Sigma; metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) was from Tokyo Kasei Industries, and Chelex 100 resin was from Bio-Rad. The residue-specific protease kit, containing arginylendopeptidase (Arg-EP), *Achromobacter* protease I (AP-I), TPCK trypsin, *Staphylococcus aureus* V8 protease, and endoprotease Asp-N, was purchased from Takara Shuzo and used according to the manufacturer's instructions.

Plasmid Construction—The cDNA for *bsd* was modified by PCR for insertion into the expression vector pET-12a (Novagen). The complete ORF in pBluescript SK$^-$ was amplified from pBSA712 (*4*) with primer UB-1 (5′-CTCATTG-TCCATATGCCTTTGTCTC-3′) containing the initiation codon (bold-face letters) and primer DB-1 (5′-GAATGTAC-AGGATCCATGGCTGGTGC-3′) complementary to the region immediately downstream of the termination codon. Primers UB-1 and DB-1 contain *Nde*I and *Bam*HI restriction sites (underlined), respectively. After digestion with *Bam*HI and *Nde*I, the DNA fragment was cloned into these sites of pET-12a to give the expression plasmid pET-12a-*bsd*. The absence of PCR errors in the amplified fragment was confirmed by sequencing the 0.44 kb *Xba*I–*Bam*HI fragment of pET-12a-*bsd*. Plasmid pET-12a-*bsd* was then transformed to *E. coli* BL21 (DE3) (F$^-$ *ompT hsdS*^{B} r^{B-}m^{B-} *dcm gal*) and used for enzyme production.

Preparation of Affinity Columns—The substrate BS was used as a ligand for affinity purification of BSD. After equilibration of a HiTrap NHS-activated column (Pharmacia Biotech) with 1 mM HCl, a solution of the ligand (70 mM BS in 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), prepared immediately before use, was injected into the column, followed by incubation for 30 min at 25°C. The coupling reaction proceeded so rapidly that it was not necessary to synthesize the alkaline stable ligand pyrimidinoblasticidin S, as described previously (*12*). The excess active groups in the column were deactivated and washed away according to the manufacturer's instructions.

Purification Scheme for BSD—Recombinant BSD was efficiently isolated from soluble fractions of *E. coli* BL21 (DE3)/pET-12a-*bsd* by the following protocol. The expression strain was cultured in 2 liter fresh LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) containing 100 μg/ml ampicillin at 37°C for 4–6 h until the OD_{600} reached to 0.6. IPTG was added to a final concentration of 1 mM, followed by further incubation for 2–3 h. The cells were harvested by centrifugation and then suspended in 40 ml of buffer A (10 mM Tris-HCl, pH 7.5). Cell lysis was completed by sonication on ice, followed by centrifugation

[4] A previous paper on the purification/characterization of BSD (Ref. *3*) described the enzyme as a monomeric glycoprotein of 30 kDa. The kinetics of BSD suggested that His is the essential catalytic residue involved in deaminohydroxylation (Ref. *12*). However, available knowledge on CDAs suggests that BSD should be a tetramer with a 222 symmetry structure, with its catalytic Glu residue at the interface of subunits. Recent X-ray crystallographic data on BSD firmly support this model (manuscript in preparation).

[I] Cytidine deaminase				
Escherichia coli (P13652)	98 ~ 108	QQTVHAEQSAI	124 ~ 137	VNYTPCGHCRQFMN
Bacillus subtilis (P19079)	49 ~ 59	SMCNCAERTAL	81 ~ 94	GPVSPCGACRQVIS
Human (P32320)	61 ~ 71	PLGICAERTAI	94 ~ 107	DFISPCGACRQVMR
[II] Deoxycytidylate deaminase				
Saccharomyces cerevisiae (P06773)	229 ~ 239	CLCLHAEENAL	255 ~ 268	CDTCPCLTCSVKIV
T4 phage (P16006)	100 ~ 110	KNEIHAELNAI	127 ~ 140	VTLSPCPDCAKAIA
Human (P32321)	80 ~ 90	PYVCHAELNAI	105 ~ 118	VALFPCNECAKLII
[III] Blasticidin S deaminase				
Aspergillus terreus (P78986)	50 ~ 60	TGGPCAELVVL	83 ~ 96	GILSPCGRCRQVLL
Bacillus cereus (P33967)	55 ~ 65	RVTVCAEAIAI	95 ~ 108	RVVSPCGMCRELIS
[IV] Apo B mRNA editing deaminase				
Rat (P38483)	57 ~ 67	NTNKHVEVNFI	88 ~ 101	LSWSPCGECSRAIT
Human (P41238)	57 ~ 67	NTTNHVEVNFI	88 ~ 101	LSWSPCWECSQAIR
[V] Functionally unidentified protein				
"ORF17" *Bacillus subtilis* (P21335)	49 ~ 59	RSIAHAEMLVI	78 ~ 91	VTLEPCPMCAGAVV
"ORF178" *Escherichia coli* (P30134)	64 ~ 74	DPTAHAEIMAL	93 ~ 106	VTLEPCVMCAGAMI
"ORF2" *Bacillus subtilis* (P32393)	66 ~ 76	ARTIHAEMNAI	93 ~ 106	VTHYPCIQCCKSII
"16.6 kDa" *Vivrio fischeri* (P33968)	63 ~ 73	LKTLHAEENAI	87 ~ 100	VTHFPCPNCAAKII

Fig. 1. **Alignment of regions that comprise the zinc-binding site in members of the cytosine nucleoside/nucleotide deaminase family.** Only representatives of each subfamily are shown. The zinc-binding residues are boxed. The active site Glu is shown in bold. The first zinc-binding residue [within the (CH)-A-E sequence] could be either His or Cys, whereas the second and third zinc-binding residues (within the P-C-X$^{(2)}$-C sequence) were exclusively Cys in the cytosine nucleoside/nucleotide deaminases isolated so far.

at 20,000 ×*g* for 30 min, and the supernatant fraction was passed through a 0.2 μm filter (Minisart-plus, Sartorius). This crude enzyme solution was directly loaded onto an affinity column pre-equilibrated with buffer A. After washing the column with ten bed volumes of buffer A, BSD was eluted with buffer A containing 10 mM BS. The active fractions were identified by means of the spectrophotometric enzyme assay (*4*) and then applied to a FPLC HR 5/5 Mono Q column (Pharmacia Biotech) pre-equilibrated with buffer A. The Mono Q column was extensively washed (about 200-fold bed volumes) with buffer A to remove excess BS in the active fractions, and then the recombinant enzyme was eluted with a linear gradient of 0–500 mM NaCl in 20 ml of buffer A. BSD was eluted at around 350 mM NaCl.

BSD was also purified from *A. terreus*, from which the encoding gene was originally isolated. No difference was observed in the elution pattern of the activity from that of the recombinant enzyme.

Protein Analyses—Purified BSD was dialyzed against HPLC reagent grade water, and then its peptide sequence was determined with an Applied Biosystems 477A gas-phase protein sequencer. The protein concentration of BSD was calculated by amino acid analysis with a Hitachi 835 amino acid analyzer; the weight of each amino acid residue was determined after hydrolysis of the enzyme with 4 N methanesulfonic acid in an evacuated tube (110°C for 20 h).

Electrophoretic Methods—SDS-PAGE was performed as described by Laemmli (*17*). The purified sample was mixed with SDS sample buffer, either incubated at room temperature or heated in a boiling water bath for 2 min, and then electrophoresed on an SDS polyacrylamide gel. Protein markers for SDS-PAGE (Pharmacia Biotech) were used for molecular weight determination of a polypeptide.

Electrophoresis under non-denaturing conditions (native PAGE) was performed as described by Davis (*18*). The molecular mass of a protein was estimated by the method of Bryan (*19*); *i.e.*, K^R (retardation coefficient) for each protein was calculated by means of Ferguson plots (*20*) with five native gel concentrations (8, 9, 10, 11, and 12% polyacrylamide), and its molecular mass was determined by making use of the linear relationship between $\log K^R$ and logM (M; molecular mass). A non-denatured protein molecular weight marker kit for native PAGE (Sigma) was used as molecular mass standards to prepare the calibration curve.

Circular Dichroism (CD) Spectroscopy and Fluorescence Assays—Far UV CD spectra of BSD samples were measured in a 1 mm path-length cuvette using a JASCO model J-720 CD spectropolarimeter. For fluorescence analyses, samples were measured with a Hitachi F-4010 fluorescent spectrophotometer using an excitation wavelength of 295 nm and recording the emission spectra from 300 to 450 nm. In either case, the final spectrum was obtained by subtracting base-line scans of the buffer blank measured in the same cuvette as that used for the sample.

Zinc Analysis—The metal content of the purified BSD was measured using an inductively coupled plasma optical emission spectrometer (Shimadzu ICPS-50). Before analysis, excess or loosely bound metal ions were removed from the protein by repeated dialysis against Chelex 100-treated buffer B (40 mM HEPES/KOH, pH 7.0) at 4°C for 8 h, with four changes each time.

For examination of mercurial-prompted zinc release from BSD, metallochromic indicator PAR was used as described previously (*21*). The PAR stock solution (5 mM) was added to samples (free of metal ions other than Zn^{2+} which were released from BSD) at a final concentration of 100 μM, and then changes in the extinction coefficient at 500 nm that accompany Zn^{2+} binding to this indicator were monitored. The Zn^{2+} concentration was determined from the calibration curve, which showed good proportionality in the range of 0–10 μM. PAR was sensitive to free Zn^{2+}, but relatively insensitive to mercurial or other reagents; *i.e.*, the effect of PMPS on colour development was negligible at the highest concentrations used in the following experiment (maximum 24.5 μM PMPS).

Measurement of Zn^{2+} Released from BSD and Its Residual Activity during Titration with the Mercurial Reagent PMPS—Purified BSD was dialyzed against metal-free buffer B at 4°C for 24 h. The dialyzed enzyme solution was used for titration of the zinc-chelating Cys residues to determine whether or not the metal content and the residual activity exhibit stoichiometric relationships. Aliquots (0–144 μl) of 100 μM PMPS in metal-free buffer B were added to 48 μl aliquots of the enzyme solution (80 μM subunit) to make the amount of PMPS 0–3.75 times that of the BSD subunit. The reaction mixture was then diluted to a final volume of 588 μl with buffer B, and a 490 μl portion (6.53 μM subunit) was mixed with 10 μl of the PAR stock solution to determine the concentration of Zn^{2+} released from the enzyme with a spectrophotometer (recorded after completion of any time-dependent absorbance changes). The rest of the dilution was used to measure the residual activity of BSD.

Reconstitution of BSD Activity after Denaturation—BSD was inactivated by treatment with guanidine-HCl, acid, or PMPS, and then conditions that influence its renaturation were examined. Each 300 μl stock denaturation solution was added to 100 μl of the purified BSD sample, followed by incubation at ambient temperature for 1 h. The samples were then dialyzed against buffer C (buffer A with 5 mM 2-mercaptoethanol), or buffer C containing EDTA or Zn^{2+} at 4°C for 16 h. Subsequent denaturation/renaturation experiments were performed for samples which could not be reconstituted in the first experiment. In these experiments, another divalent metal, Co^{2+}, Cu^{2+}, Mg^{2+}, or Ni^{2+}, was also included in buffer C to examine its effect on the reconstitution of BSD.

Site-Directed Mutagenesis of BSD—Mutants of the *bsd* gene encoding single amino acid substitutions, E56D, E56Q, C91A, C91S, and C91H, were constructed in pBSA-712 (*4*) by the PCR mutagenesis method of Ito *et al.* (*22*). For generation of the first PCR fragment with Glu-56 substitutions, vector primer T7 (5′-AATACGACTCACTATAGGGC-3′), and internal mutagenic primers (sense strands) MUT-D (5′-CCTTGTGCAGATCTCGTGGTGC-3′) and MUT-Q (5′-CCTTGTGCACAGCTCGTGGTGC-3′) were used for E56D and E56Q, respectively (mismatched bases are underlined). For generation of the first PCR fragment with Cys-91 substitutions, vector primer T3 (5′-ATTAACCCTCACTAAAGGGA-3′), and internal mutagenic primers (antisense strands) MUT-A (5′-CACCTGTCGAGCCCGTCCGCA-3′), MUT-S (5′-CACCTGTCGATGCCGTCCGCA-3′), and MUT-H (5′-CACCTGTCGAGACCGTCCGCA-3′) were used for C91A, C91S, and C91H, respectively. These PCR products were mixed with the *bsd* fragment amplified by

PCR with the following vector primers: T3 and *Hin*dIII site elimination primer DB2 (5′-GTATCGATACGCTTGATATC-3′) for Glu-56 substitutions, and T7 and *Bam*HI site elimination primer UB2 (5′-AGTGGATGCCCCGGGCTGCA-3′) for Cys-91 substitutions. The resulting heteroduplexes were used as templates for the second PCR with vector primers T3 and T7. The second PCR product was double-digested with *Bam*HI and *Hin*dIII (*i.e.*, only the PCR fragments derived from the mutated *bsd* have both *Bam*HI and *Hin*dIII sites at both ends), cloned into pBluescript SK$^-$, and then transferred to the pET-12a expression vector. The absence of PCR errors was confirmed by sequencing the 0.44 kb *Bam*HI–*Xba*I fragments of the *bsd* mutants.

RESULTS AND DISCUSSION

Expression and Purification of Recombinant BSD—Using the T7 transcription system, *bsd* was overexpressed in *E. coli* BL21(DE3) and the recombinant enzyme was purified to homogeneity by affinity chromatography, with an overall yield of 40–50%. When the purified enzyme was separated on SDS-PAGE (0.2% SDS–15% polyacrylamide gel) after heat treatment (100°C) in 2% SDS, the protein gave a single protein band of 13 kDa, this value corresponding to the subunit size of the BSD polypeptide. When heat treatment was not carried out prior to sample loading, the enzyme showed an apparent molecular mass of 36 kDa (referred to as the "apparent 36 kDa form" hereafter). Surprisingly, over 90% of the activity was detected when BSD was dissolved in 2% SDS, diluted 10-fold with the buffer, and then incubated at room temperature for 2 h, *i.e.*, similar treatment to that is performed during SDS-PAGE without heating of samples. This result suggests that BSD is quite resistant to denaturation by SDS.

What is the "Apparent 36 kDa Form" of BSD?—It is obvious that the retarded mobility of the "apparent 36 kDa form" of BSD on SDS-PAGE was not caused by non-specific aggregation of polypeptides through disulfide bridges, since a sufficient amount of 2-mercaptoethanol was included in the SDS sample buffer. Rather, this feature is reminiscent of heat-modifiable proteins, such as the *E. coli* OmpA, OmpC, OmpF, and OmpT outer membrane proteins (*23*, *24*). These proteins are not denatured by SDS under non-heating conditions and are electrophoresed in their native conformations on SDS-PAGE (*25*). To examine the conformational states of the protein, we compared the CD and fluorescence spectra of BSD in the presence and absence of SDS.

Figure 2A shows the CD spectra of these samples with or without heating in 2% SDS. The far UV CD spectrum of native BSD (curve 1) exhibits a broad minimum at 207 nm and the zero crossing at 202 nm. A similar CD spectrum was obtained for the BSD sample in SDS solution without heat treatment (curve 2; final 0.2% of SDS). In contrast, the negative intensity of the CD band was dramatically increased by heat treatment of BSD in SDS solution (curve 3; final 0.2% SDS). Heating (100°C for 2 min) was more effective for causing the structural change of BSD than keeping it in a 10 times higher concentration of SDS without heat treatment (curve 4; final 2% SDS).

Figure 2B shows the tryptophan fluorescence spectra of these samples. Treatment with SDS alone resulted in an increase in the tryptophan fluorescence intensity by 32%, without a shift in the emission peak at 350 nm (curves 1 and 2). This spectrum change is expected to reflect an environmental change of Trp-128, which is the only Trp residue in BSD. Heating in SDS solution caused an increase of as much as 94% in the maximum fluorescence intensity, and further caused a shift of the emission peak to 345 nm (curve 3). This implies a significant structural change of the polypeptide, with the Trp-128 exposed to a more hydrophobic environment. Incubation of BSD with a higher concentration of SDS (final 2%) without heating resulted in an 81% increase in the maximum fluorescence intensity, but the shift of the emission peak was not observed (curve 4). The results again indicate that heat treatment in SDS is indispensable for complete denaturation of BSD.

Takagi *et al.* (*26*) showed that binding of SDS to a protein causes the negative CD intensities (at 220 nm) to reach their maximum values at SDS concentrations below 1 mM (equal to 0.029% w/v). Actually, the secondary structures of many proteins (*e.g.*, molecular weight protein markers) are sufficiently denatured without heating in an SDS solution, and they are electrophoresed according to their molecular

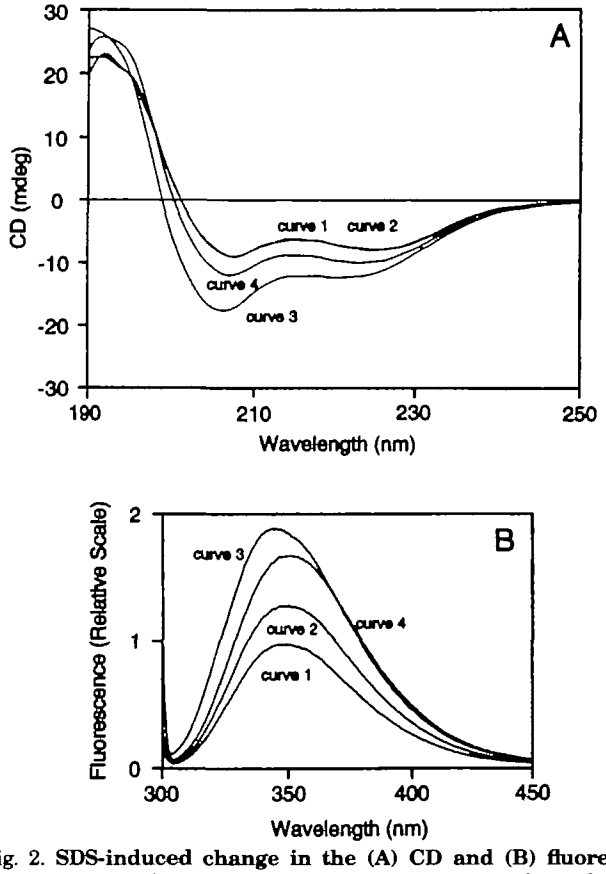

Fig. 2. **SDS-induced change in the (A) CD and (B) fluorescence spectra of BSD.** Curve 1, native BSD; curve 2, BSD in final 0.2% SDS without heat treatment; curve 3, BSD in final 0.2% SDS with heat treatment; curve 4, BSD in 2% SDS without heat treatment. BSD was dissolved in 2% SDS, followed by incubation at either room temperature (curves 2 and 4) or 100°C (curve 3) for 2 min. The concentration of SDS was then reduced to 0.2% (curves 2 and 3) or kept at 2% (curve 4). After 2 h incubation at room temperature, the spectra were measured. The concentrations of BSD used for measurements (curves 1–4) were 2.5 μM (A) and 5 μM (B), respectively.

weight. In the case of BSD, however, its secondary structure was substantially unchanged in the absence of heat treatment (Fig. 2A; curves 1 and 2). As demonstrated for the *E. coli* outer membrane proteins (*25*), the heat-modifiable behavior of BSD proved to depend on the strong stability of the enzyme against SDS. Thus the "apparent 36 kDa form" of BSD on SDS-PAGE can be used as a convenient and reliable marker for monitoring the active conformation of BSD (Fig. 2A, see also Fig. 4A), as was the case for the *in vitro* assembly assays of *E. coli* porin proteins (*27, 28*).

Biochemical Properties of Recombinant BSD—Fifteen residues of the N-terminal sequence of BSD were determined to be PLSQEESTLIERATA. The N-terminus was probably digested by methionine aminopeptidase of *E. coli* after translation (*29*). The molecular weight and molar extinction coefficient (ε_{280}) of recombinant BSD were calculated to be 13,337 Da and 9,100 M^{-1} cm^{-1} per subunit, respectively. This ε_{280} value is in good agreement with the theoretical value of 9,150 M^{-1} cm^{-1}, given by three Tyr and one Trp residue per subunit (*30*). When BSD was analyzed for its zinc content by inductively coupled plasma optical emission spectroscopy, tightly bound zinc was found at a concentration of 112 μM Zn^{2+}/125 μM subunit, indicating one zinc per subunit. No other metals, such as Ni^{2+}, Co^{2+}, and Mg^{2+}, were present in significant quantities.

The molecular mass of recombinant BSD was estimated to be 49 kDa by native PAGE as described under "EXPERIMENTAL PROCEDURES," and 54 kDa by gel permeation chromatography on a Superdex 75 HR10/30 column (Pharmacia Biotech). A similar value of 52 kDa was obtained on ultracentrifugal sedimentation equilibrium analysis (data not shown). These results indicate the homotetrameric nature of recombinant BSD. The enzyme purified from *A. terreus* showed exactly the same molecular mass on gel permeation chromatography, and also gave exactly the same sized bands (13 kDa with heat treatment, and apparently 36 kDa without heat treatment) on 15% SDS-PAGE. Sugar residues were not detected on PAS-staining of the gel. The N-terminal residues (1st–10th residues) of the fungal BSD were completely identical to those predicted from the cDNA sequence. Thus we conclude that recombinant BSD is identical to the fungal BSD except for the lack of the N-terminal Met residue.[5]

Release of Zinc during Titration of BSD with PMPS—Figure 3 shows plots of the concentration of Zn^{2+} complexed with $(PAR)_2$ during titration with PMPS. A small amount of Zn^{2+} was detected in the sample dialyzed against the metal-free buffer even in the absence of PMPS, but most of the catalytic zinc (91%) still remained tightly bound to the enzyme molecule. When BSD was titrated with PMPS, Zn^{2+} was released in proportion to the amount of PMPS added and increased up to 6.5 μM, which is equal to the concentration of the BSD subunit in the reaction mixture. Inactivation of the enzyme also depended linearly on the amount of PMPS, and all the activity was lost when the molar ratio of PMPS/BSD subunit reached 2.7. This implies that PMPS at three (2.7/0.91) times the amount of zinc-binding subunit was necessary for complete inactivation. The three Cys residues which are thought to be involved in zinc coordination (Fig. 1) could have been titrated with PMPS, and this chemical modification would lead to the release of Zn^{2+} from BSD in the presence of PAR.

Hunt *et al.* observed similar linearity of the plot of released Zn^{2+} *versus* PMPS added for *E. coli* aspartate transcarbamoylase (ATCase), which contains six structural

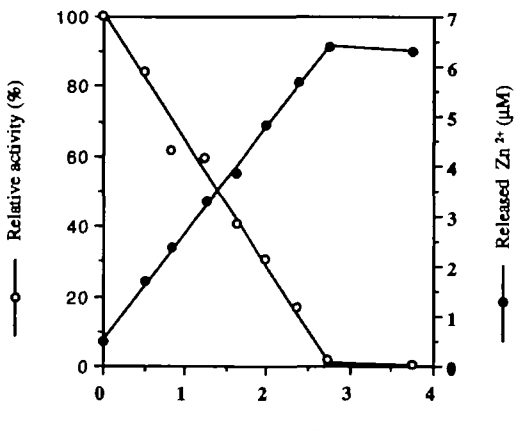

Fig. 3. **Release of zinc from BSD during titration with the mercurial reagent PMPS.** After the addition of PMPS, the concentration of released Zn^{2+} complexed with PAR was calculated by monitoring the absorbance at 500 nm. A_{500} was determined by adding a 1/49 volume of the PAR stock solution (5 mM) to the reaction mixture for BSD (6.53 μM subunit). The enzymatic activity was calculated after each addition of PMPS.

TABLE I. **Relative activities of BSD in the reconstitution experiment.** Italicized letters represent inactivation conditions and plain letters represent additives in the reconstitution buffer; see "EXPERIMENTAL PROCEDURES." *Tris* represents dilution of BSD 4-fold with buffer (176 and 44 μM subunit for the first and second reconstitution experiments, respectively). *GdmCl*, *pH 3.5*, and *PMPS* represent inactivation of BSD by adding a 3-fold volume of 8 M guanidine-HCl, an acidic solution (0.1 M citrate buffer, pH 3.5), and 1.33 mM PMPS, respectively. For reconstitution of the enzyme activity, BSD was dialyzed against the buffer supplemented with various trace elements. –EDTA represents no additives, and +EDTA, $+Zn^{2+}$, and $+Me^{2+}$ represent 1 mM EDTA, 25 μM Zn^{2+}, and 25 μM of another metal (Co^{2+}, Cu^{2+}, Mg^{2+}, or Ni^{2+}), respectively. Activity is expressed as a percentage of that of the stock BSD sample. N.D. = not detected. Detection limits: 0.018% (a) and 0.071% (b).

	Inactivation/Reconstitution	Activity (%)
1	*Tris*/−EDTA	82
2	*Tris*/+EDTA	72
3	*GdmCl*/−EDTA	71
4	*GdmCl*/+EDTA	N.D.[a]
5	*pH 3.5*/−EDTA	14
6	*pH 3.5*/+EDTA	N.D.[a]
7	*PMPS*/−EDTA	81
8	*PMPS*/+EDTA	76
9	*Tris*/−EDTA/*Tris*/−EDTA	63
10	*GdmCl*/+EDTA/*GdmCl*/−EDTA	N.D.[b]
11	*GdmCl*/+EDTA/*GdmCl*/$+Zn^{2+}$	28
12	*GdmCl*/+EDTA/*GdmCl*/$+Me^{2+}$	N.D.[b]

[5] In Ref. *3*, the molecular mass of BSD was determined to be 46–54 kDa by Sephadex G-100 gel permeation chromatography. However, its molecular weight was suggested to be 30 kDa on SDS-PAGE. Yamaguchi *et al.* explained this difference could be due to an interaction of putative sugar residues in BSD with the Sephadex G-100 matrix (*3*). The molecular mass of BSD thus estimated by conventional SDS-PAGE on a disk gel (*33*) most likely corresponds to "the apparent 36 kDa form" of BSD (Laemmli method on a slab gel) in this study.

Zn^{2+} (each coordinated by four Cys residues in one subunit) essential for maintaining its quaternary structure (*31*). They explained this stoichiometric relationship as the preference of PMPS to react with the protein -SH groups in great excess; *i.e.*, the attack of these groups by PMPS is not random, and once the first of the four -SH groups in one ATCase subunit forms a mercaptide, the rest of the zinc chelating -SH groups in the same subunit rapidly react to give the linear release of one Zn^{2+} for every four equivalents of PMPS added to the reaction mixture. Although the number of Cys residues involved in zinc coordination is different in BSD, the linear relationship in Fig. 3 suggests similar mechanisms of zinc release leading to instability of the protein to as reported for ATCase.

Effects of Divalent Metals on Renaturation of Denatured BSD—To show that Zn^{2+} is essential for organization of the active enzyme, we performed a reconstitution experiment (Table I). Native BSD was not inhibited by metal chelating agents such as EDTA (see lines 1 and 2) or *o*-phenanthroline (data not shown), suggesting that the zinc atom is tightly bound to the enzyme molecule. The presence of EDTA (1 mM) in the reconstitution buffer, however, completely blocked renaturation of BSD which had been denatured with guanidine-HCl or acid (lines 3–6 in Table I). But, interestingly, renaturation of PMPS-inactivated BSD was not affected by the presence of EDTA (lines 7 and 8). These results suggest that PMPS-inactivated BSD retains some structures which can loosely trap zinc within the enzyme molecule even in the presence of EDTA. There might be some significant structural differences between guanidine-HCl or acid-denatured BSD and PMPS-inactivated BSD (see also the discussion section of Fig. 4B).

We performed a second reconstitution experiment for BSD (*GdmCl*/+EDTA) which could not be reactivated in the first experiment (line 4 in Table I). The inactive enzyme was dialyzed against the reconstitution buffer containing various metal ions. Zinc-depleted BSD was partially reactivated in the presence of Zn^{2+} but not with other metal ions (lines 9–12). The results indicate that denatured BSD completely lacking zinc could be reactivated by supplementing Zn^{2+} in the reconstitution process, and that the zinc is essential for the enzyme activity. As expected from the catalytic role of the zinc in CDA (*9*), recent X-ray crystallographic data on the BS-BSD complex have revealed that this zinc is catalytic (manuscript in preparation). However, there has been no report so far on the structural role of the zinc in CDAs.

Electrophoretic Analyses of BSD Samples in the Reconstitution Experiment—To examine the conformational changes of the BSD samples after the denaturation/renaturation treatment, they were mixed with SDS sample buffer (without heating) and then analyzed by SDS-PAGE (Fig. 4A). The intensity of the 36 kDa band correlated well with the residual enzyme activity after the denaturation/renaturation treatment. The inactive conformations of BSD, due to the lack of appropriate zinc coordination, did not exhibit the resistance of the enzyme against SDS. Thus, the catalytic zinc may also play an important role in the formation of the BSD structure.

Figure 4B shows the electrophoretic profiles of these samples under non-denaturing conditions (native PAGE; 9%). The conformational differences in the native and other inactive BSD samples were again evident from the marked

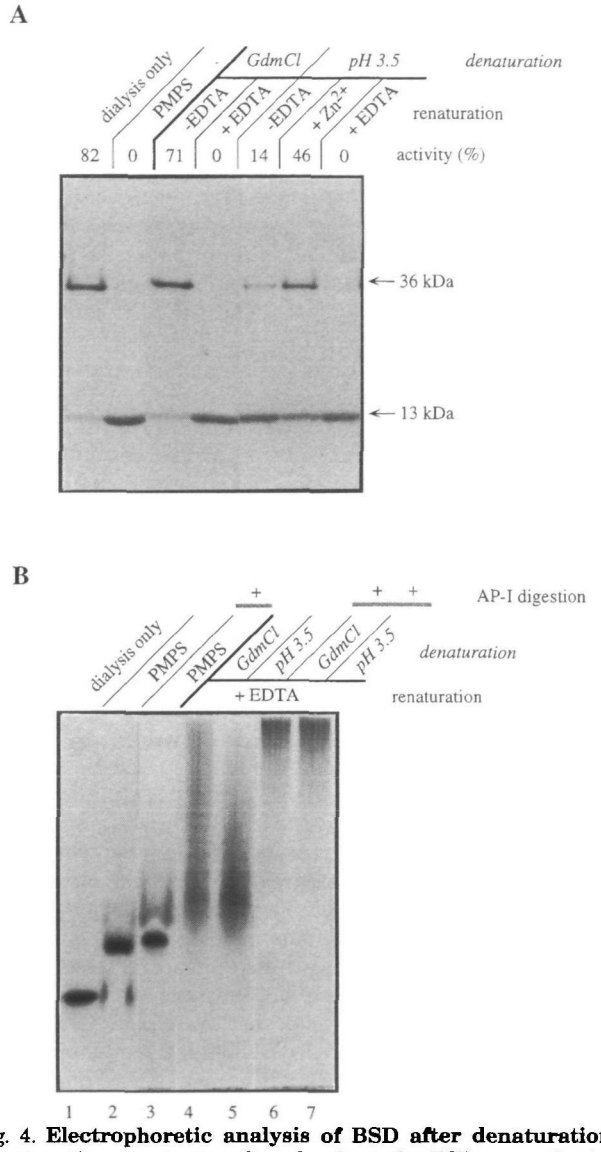

Fig. 4. **Electrophoretic analysis of BSD after denaturation/renaturation treatment.** Samples from the BSD reconstitution experiment were analyzed by electrophoretic methods to examine the conformational differences. The denaturation conditions (italicized) and renaturation conditions (additives in buffer C) are indicated at the top of the gels (for details, see the legend to Table I). Each lane contained 7 μg of BSD polypeptides. (A) SDS-PAGE (15%) of BSD samples without heat treatment. The residual activity is shown at the top of each lane. (B) Native PAGE (9%) of BSD samples. For lanes 3, 6, and 7, the samples were digested with AP-I and then immediately subjected to electrophoresis.

differences in the electrophoretic mobility, further supporting the above idea that zinc is essential for structure organization. The BSD polypeptides which had been incorrectly refolded and/or aggregated in the absence of Zn^{2+} (Table I; lines 4 and 6) migrated as "smeared ladders" (lanes 4 and 5 in Fig. 4B). In contrast, PMPS-inactivated BSD was separated as a sharp band, with an electrophoretic mobility slower than that of the native enzyme (lane 2). The K^R calculated from Ferguson plots (*20*) suggested that PMPS-inactivated BSD has a Stokes radius corresponding to a globular protein of approx 23 kDa (data not shown). Thus zinc release appears to promote the dissociation of the BSD

tetramer into monomers or dimers. When PMPS-inactivated BSD was digested with AP-I and then immediately analyzed by native PAGE, it was detected as a sharp band with slightly reduced mobility (lane 3). Such an electrophoretic pattern was again quite different from those of other chemically denatured BSD samples (see Table I; lines 4 and 6) digested with AP-I (lanes 6 and 7). These results further indicate that the conformation of PMPS-inactivated BSD is significantly different from guanidine-HCl or acid-denatured BSD (see also the discussion section of Table I).

Zinc Coordination is Essential for the Stability of BSD—BSD was quite stable in the native conformation and showed resistance to all residue-specific proteases, such as Arg-EP, AP-I, TPCK trypsin, *Staphylococcus aureus* V8 protease, and endoprotease Asp-N. When the samples were analyzed by SDS-PAGE without prior heat treatment, the "apparent 36 kDa form" of BSD was observed. In contrast, PMPS-inactivated BSD was degraded into fragments of small polypeptides and these fragments were normally electrophoresed according to their predicted molecular weights (data not shown). This increase in protease sensitivity might reflect a somewhat molten (or partially unfolded) structure of PMPS-inactivated BSD in the absence of tightly bound zinc.

In consistent with the results of the above protease sensitivity experiment, the conformation of PMPS-inactivated BSD was more unstable than that of native BSD; *i.e.*, if stored at 4°C for 10 days, it could no longer be detected as a sharp band on native PAGE and its activity could not be restored by dialysis against the reconstitution buffer containing 1 mM EDTA. In addition, this PMPS-inactivated BSD showed an elution pattern suggestive of aggregation and/or unfolding of the polypeptides on gel permeation chromatography, *i.e.*, the protein was not detected as a sharp peak, and was eluted between the positions of the exclusion limit of Superdex 75 HR10/30 and the molecular mass of 20 kDa. These results suggest that PMPS-inactivated BSD might be an unstable folding/unfolding intermediate in the absence of tightly bound zinc, and further support the structural role of the zinc in maintenance of the active conformation of tetrameric BSD.

Analysis of Mutant Enzymes—The conserved sequence motif in Fig. 1 has been proposed to be essential for catalysis by the cytosine nucleoside/nucleotide deaminase family (*9–11*, *15*, *16*). We thus introduced mutations by site-directed mutagenesis at Glu-56 (the proposed active site) and Cys-91 (a proposed catalytic zinc-binding Cys) of BSD to confirm the importance of these residues in catalysis and in zinc binding.

E56D and E56Q were efficiently purified by affinity chromatography using BS as a ligand, suggesting that the structures of these mutants are well preserved. In support of this, E56D and E56Q contained one zinc per deaminase subunit, as revealed with the metal indicator PAR (*21*). Furthermore, the mutants also showed exactly the same heat-modifiable behavior on SDS-PAGE and were resistant to proteases (mixture of TPCK-trypsin, V8 protease, and endoprotease Asp-N) if they were not treated with PMPS (Fig. 5A). These results strongly suggest that there are no quaternary structure differences between the wild and Glu-56 mutant enzymes. As we expected, the activities of the mutant enzymes were not detectable with the spectrophotometric assay (detection limit; 0.004 of wild type). Considering that E56D and E56Q retain the same structure as that of the wild type BSD, the loss of activity indicates that Glu-56 is involved in the catalytic process, but not in the structure maintenance. These results are in consistent with the proposed role of this residue in catalysis (*i.e.*, protonation of pyrimidine in the active center) in CDA (*9–11*, *15*, *16*).

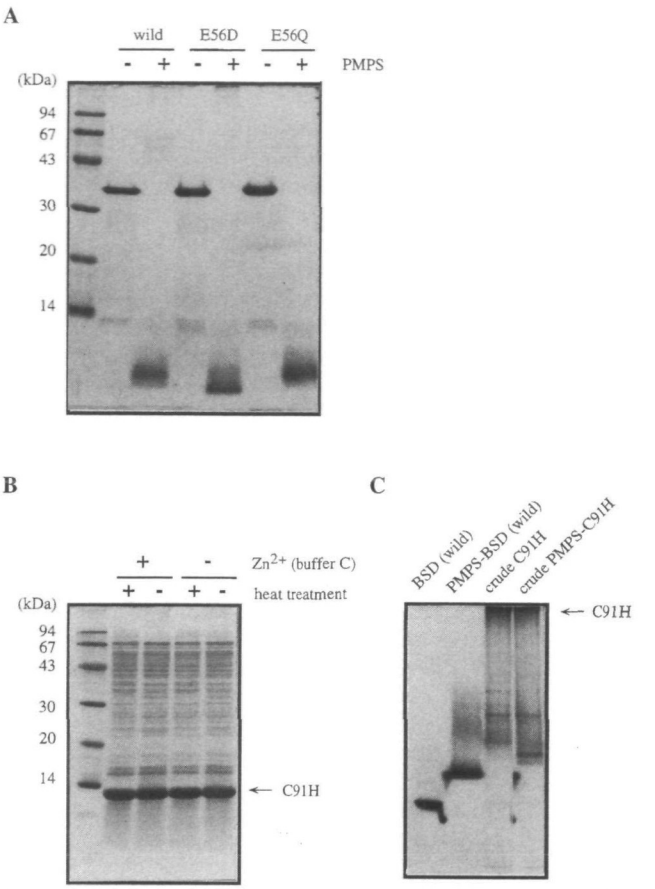

Fig. 5. **Electrophoretic mobility of BSD mutants.** (A) SDS-PAGE (15%) of BSD mutants E56D and E56Q. Samples (intact or PMPS-treated) were digested with a mixture of proteases (TPCK-trypsin, V8 protease, and endoprotease Asp-N) and then analyzed by SDS-PAGE without heat treatment. (B) SDS-PAGE (15%) of mutant BSD (C91H). The inclusion bodies solubilized with 6 M guanidine-HCl were dialyzed against buffer C in either the presence or absence of 25 μM Zn^{2+}. The recovered soluble fractions of C91H were subjected to SDS-PAGE with or without heat treatment in the SDS sample buffer. (C) Native PAGE (9%) of C91H recovered in the soluble fraction. The arrow indicates the C91H polypeptides (dialyzed against buffer C containing Zn^{2+}) aggregated and remaining at the top of the gel.

A previous study showed that mutants of *E. coli* CDA, in which the second (Cys-129) or third (Cys-132) zinc binding residue (boxed in Fig. 1) was replaced by Ala, could not retain zinc in the protein molecule (*32*). Similar results were reported for human CDA, in which each Cys residue in the $P\text{-}C^{99}\text{-}X^{(2)}\text{-}C^{102}$ sequence (boxed in Fig. 1) was replaced by Ala (*15*). Thus, we examined whether one of the highly conserved Cys residues, Cys-91, within the $P\text{-}C^{88}\text{-}X^{(2)}\text{-}C^{91}$ sequence of BSD could be replaced by Ala, His, or Ser. In contrast to the wild type BSD, these mutants were found in inclusion bodies, and no deaminase activities were detected

in any of these recombinant *E. coli* cells. Among these mutants, C91H, in which residue Cys-91 was replaced by another possible zinc ligand, His, was further analyzed. We tried to solubilize and renature C91H according to essentially the same procedure as described for the reconstitution experiment with 6M guanidine-HCl. The solubilized proteins were dialyzed against the reconstitution buffer, in either the presence or absence of 25 μM Zn^{2+}, at 4°C for 16 h. As shown in Fig. 5B, approximately 50% of the C91H polypeptides was recovered in the soluble fraction in either case. However, they exhibited neither the deaminase activity nor the "apparent 36 kDa form" on SDS-PAGE without heating of the samples, which are the unique properties of the wild type BSD. The solubilized C91H polypeptides appeared to have gross perturbations in the protein structure, since the polypeptides aggregated and remained on top of the gel on native PAGE (Fig. 5C). This indicates that Cys-91 of BSD (equivalent to Cys-132 and Cys-102 in *E. coli* and human CDA, respectively) is essential for organization of the active enzyme structure. In contrast, the first part of the zinc-binding residue (*i.e.*, (CH)-A-E sequence) appears not to be required for maintenance of zinc in the active center, since the H102Q (His-102 boxed in Fig. 1) mutant of *E. coli* CDA proved to be active and to contain 1 mol of zinc per subunit (*32*). Our data indicate that it is not possible to introduce mutations at the Cys residues in the latter part of the catalytic zinc-binding motif (*i.e.*, P-C-$X^{(2)}$-C sequence) while maintaining a properly folded structure, and thus this short sequence is also considered to be a structural zinc-binding site in BSD.

The authors wish to thank Mr. M. Chijimatsu of the Division of Biomolecular Characterization of RIKEN for the ultracentrifugal sedimentation analysis, protein sequencing, and amino acid analysis. We are also grateful to Dr. A. Takatsuki of the Animal and Cellular Systems Laboratory of RIKEN for careful reading of the manuscript.

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