# The Role of Zinc in *Bacillus subtilis* Cytidine Deaminase[†]

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ABSTRACT: Cytidine deaminase (CDA) from *Bacillus subtilis* is a zinc-containing enzyme responsible for the hydrolytic deamination of cytidine to uridine and 2′-deoxycytidine to 2′-deoxyuridine. Titration of the cysteinyl groups of the enzyme with *p*-hydroxymercuriphenyl sulfonate (PMPS) resulted in release of one zinc ion per subunit. Addition of EDTA to chelate the zinc and dithiothreitol (DTT) to remove PMPS, followed by removal of the low molecular weight compounds by gel filtration, resulted in an apoenzyme with no enzymatic activity. The apoenzyme was almost fully reactivated by addition of zinc chloride, indicating that the zinc ion played a central role in catalysis, in keeping with what has been observed with *Escherichia coli* CDA [Betts, L., Xiang, S., Short, S. A., Wolfenden, R., and Carter, C. W. J. (1994) *J. Mol. Biol. 235*, 635−656]. Addition of $Cd^{2+}$ or $Co^{2+}$ caused partial reactivation of the apoenzyme. Zinc reconstitution of the apoenzyme was strictly dependent on the presence of reducing agents, suggesting that the zinc-ligating cysteines, when unligated, participated in disulfide bond formation. An enzymatically active isoform of the tetrameric CDA protein, containing an extension of 13 amino acids at the C-terminus of each subunit, was used in conjunction with the wild-type CDA in subunit−subunit dissociation studies to show that the zinc ion does not assist in the thermodynamic refolding of the protein. After treatment with PMPS and EDTA, the enzyme existed as unfolded unassociated subunits. Immediately following DTT addition to remove PMPS, the subunits refolded into a tetrameric structure, independent of the presence of zinc.

CDA (CDA, EC 3.5.4.5)[1] from *Bacillus subtilis* is a homotetrameric zinc enzyme containing one zinc atom per 15 kDa subunit (*1*, *2*). The enzyme participates in nucleotide metabolism, where its main function is to scavenge cytidine and deoxycytidine for UMP synthesis (*3*). CDA from *Escherichia coli* has been extensively studied, and its crystal structure has been solved (*4*). The *E. coli* enzyme is homodimeric and contains one zinc ion per 32 kDa subunit. Each subunit consists of two structurally very similar core domains, where the N-terminal domain contains the active site with the zinc ion coordinated by the thiolate groups of two cysteines and a nitrogen atom of a histidine residue. The fourth ligand of the zinc ion is a deprotonated water molecule positioned by the zinc ion for attack on the pyrimidine ring with consequent elimination of ammonia. The zinc ion is deeply buried in the pyrimidine nucleoside binding pocket, completely sequestered from the solvent by the C-terminal domain of the other subunit.

The amino acid sequence of the CDA subunit from *B. subtilis* is 26% identical to the zinc-containing N-terminal core domain of *E. coli* CDA, and alignment studies suggested that the two enzymes are structurally homologous (*4*), and that the zinc ion was coordinated to three cysteine residues in the *B. subtilis* enzyme (*2*). This kind of coordination is unusual for a catalytic zinc ion, whereas structural zinc frequently is found coordinated to three or four cysteine residues (for reviews, see *5*, *6*).

In the present study we have studied the effect of removing zinc from *B. subtilis* CDA in order to establish whether the function of the metal ion is purely catalytic, or whether it also has a structural function. We observed that the metal-free apoenzyme had lost all catalytic activity. However, the enzyme could be reactivated by the addition of $Zn^{2+}$, and to some extent also $Cd^{2+}$ or $Co^{2+}$ ions, provided the apoenzyme was kept in a reduced state.

It was previously reported that a small amount (16%) of the *B. subtilis* CDA subunits synthesized in vivo are extended by 13 amino acids as a result of a rare −1 ribosomal frameshift, occurring during translation of the 3′-terminal end of the *cdd* mRNA (*7*). The extended subunits form mixed tetramers with the normal subunits, resulting in the presence intracellularly of about 36% of various heterotetrameric forms of the enzyme. These hybrid CDA forms have the same specific enzyme activity as the homotetrameric enzyme, but are easily distinguished on polyacrylamide gels due to their larger size. The extended subunits were used in the present work as protein markers in subunit−subunit interaction

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[1] Abbreviations: bp, base pair(s); CDA, cytidine deaminase (EC 3.5.4.5); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PAR, 4-(2-pyridylazo)resorcinol; PMPS, *p*-hydroxymercuriphenyl sulfonate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

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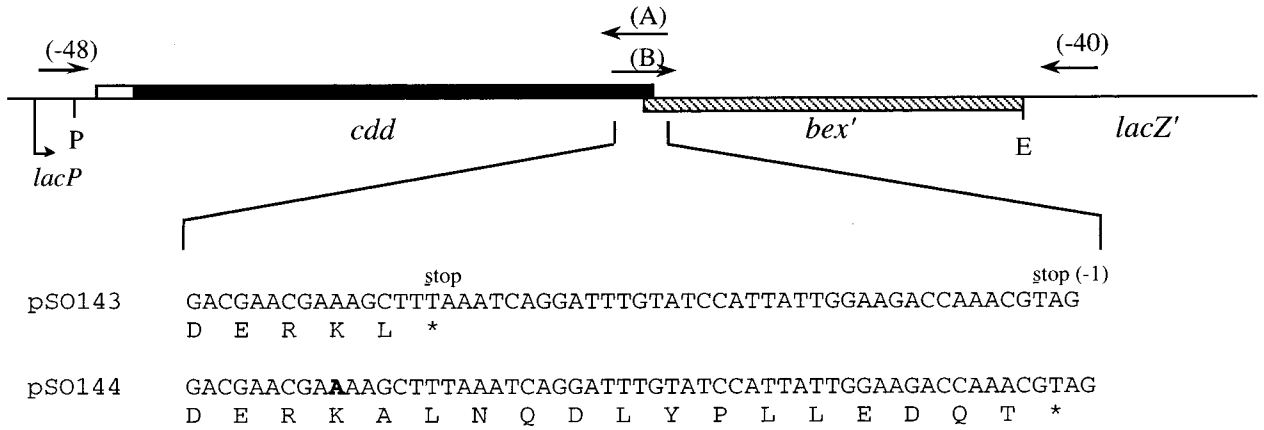


FIGURE 1: Structures of the pUC19-based plasmids pSO143 and pSO144. Thin lines, pUC19 DNA; solid bar, coding region of the *B. subtilis cdd* gene; open bar, leader region of the *cdd* gene; hatched bar, coding region of the 5′ end of the *B. subtilis bex* gene; *lacP*, vector-borne *lac* promoter. Restriction endonuclease sites: E, *Eco*RI; P, *Pst*I. Primers used are indicated by arrows: (−48), 24-mer reverse sequencing primer of M13/pUC; (−40), 23-mer sequencing primer of M13/pUC; (A) and (B), complementary primers used in the construction of pSO144. The nucleotide sequences and the deduced amino acid sequences of the 3′ end of the *cdd* genes in the two constructs are shown.

experiments, and to investigate the role of zinc in the folding and association of the subunits into the tetrameric form.

## MATERIALS AND METHODS

*Materials*. Ampicillin, Brilliant Blue R250, 2′-deoxycytidine, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), glutathione, *p*-hydroxymercuriphenyl sulfonate (PMPS), 4-(2-pyridylazo)resorcinol (PAR), streptomycin sulfate, and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical (St. Louis, MO). Restriction endonucleases were from New England Biolabs (Boston, MA) or Promega (Madison, WI), and T4 DNA polymerase was from Boehringer Mannheim (Germany). Other chemicals were analytical grade from Merck (Darmstadt, Germany). Sephadex G-100 was supplied by Pharmacia Biotech (Uppsala, Sweden) and diethylaminoethyl cellulose (DE52) from Whatman Biosystems Ltd. (Maidstone, England). Protein markers were purchased from Bio-Rad Laboratories (Hercules, CA). Oligodeoxyribonucleotide primers were synthesized by DNA Technology, ApS (Aarhus, Denmark).

*Plasmids*. Plasmid pSO143 (*1*) harbors the *B. subtilis cdd* gene without its native promoter but with its natural ribosomal binding site on a 740 bp *Kpn*I−*Eco*RI fragment in pUC19 (Figure 1). In this construct, the *cdd* gene is expressed from the plasmid-borne *lac* promoter. Plasmid pSO144 was constructed to achieve overproduction of the 13 amino acid-extended CDA subunit observed in vivo as a result of a −1 ribosomal frameshift (*7*). The DNA was constructed in such a way that the amino acid sequence of the carboxy-terminal part of the resulting CDA was DERKALNQDLYPLLEDQT instead of DERKL (Figure 1). One megaprimer (540 bp) was made by PCR amplification with the 24-mer reverse sequencing primer (−48) of M13/pUC and the 23-mer primer A, 5′-CCTGATTTAAAGCTT**T**TCGTTCG-3′ (the inserted base is shown in boldface type). The other megaprimer (360 bp) was made by PCR amplification using the 17-mer sequencing primer (−40) of M13/pUC and the 23-mer primer B complementary to primer A, 5′-CGAACGA**A**AAGCTTTAAATCAGG-3′. In both reactions, pSO143 was used as the template. The two megaprimers were used in a third round of PCR amplification in the presence of the two vector-specific primers. The resulting fragment was digested with *Eco*RI and *Pst*I and inserted into the multiple cloning site of pUC19. The nucleotide sequence of the modified *cdd* gene was verified by DNA sequencing using the BigDye Terminator Cycle Sequencing Kit from *PE* Applied Biosystems and an ABI PRISM 310 Genetic Analyzer.

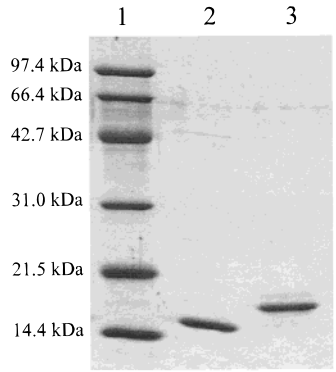


FIGURE 2: SDS−PAGE of purified recombinant *B. subtilis* CDA forms. Lane 1, protein markers; lane 2, CDA purified from strain JF611 harboring pSO143 encoding the wild-type subunit, [S]; lane 3, CDA purified from strain JF611 harboring pSO144 encoding the extended subunit, [$S^{ext}$].

*Purification of Recombinant CDA*. The recombinant wild-type ($[S]_4$) and extended ($[S^{ext}]_4$) homotetrameric enzymes were purified from cells of *E. coli* JF611 (*cdd pyrE argE his proA thr lac thi*) harboring pSO143 and pSO144, respectively, as described previously (*7*). Cells were grown with vigorous shaking at 37 °C in Luria broth (*8*) supplemented with ampicillin (200 $\mu$g/mL) for 16 h, and harvested by centrifugation. The purity of the enzyme preparations was checked by SDS−PAGE (Figure 2).

*Enzyme Assays*. The deamination of 2′-deoxycytidine in 0.1 M Tris-HCl buffer, pH 7.6, was monitored at 290 nm ($\Delta\epsilon_M = -1700\ M^{-1}\ cm^{-1}$) using cuvettes maintained at 25 °C (*9*).

*Titration of Sulfhydryl Groups with DTNB*. CDA subunits (10 $\mu$M) in 0.1 mM potassium phosphate, pH 7.3, were reacted with 175 $\mu$M DTNB. The increase in absorbance at 412 nm was determined, and the amount of thiols reacting with DTNB was calculated as described by Riddles et al. (*10*).

*Titration of Sulfhydryl Groups with PMPS*. Native CDA (14.7 nmol in subunits) in 20 mM Tris-HCl, pH 7.6, 90 mM KCl in a total volume of 600 $\mu$L was titrated with successive additions of 3 $\mu$L aliquots of 4.0 mM PMPS. The reaction of sulfhydryl groups on the enzyme with PMPS was monitored spectrophotometrically at 250 nm (*11*−*13*). The displacement of zinc from the enzyme during titration with PMPS was followed spectrophotometrically at 500 nm by performing the titration in the presence of the high-affinity metallochromic indicator PAR. Native CDA (4.5 nmol in subunits) in 20 mM Tris-HCl, pH 7.6, 90 mM KCl, 0.1 mM PAR in a total volume of 600 $\mu$L was treated with successive additions of 2 $\mu$L aliquots of 4.0 mM PMPS. The change in absorbance at 500 nm was followed. The reference cuvette contained 600 $\mu$L of 20 mM Tris-HCl, pH 7.6, 90 mM KCl, 0.1 mM PAR and was titrated simultaneously with 2 $\mu$L aliquots of 4.0 mM PMPS. In the conditions used, $\Delta\epsilon = 6.6 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 500 nm for $(PAR)_2Zn^{2+}$ formation (*13*).

*Preparation of Apoenzyme*. A procedure similar to that described by Giedroc et al. (*12*) was used for preparing metal-free apoenzyme. To 100 $\mu$L of native CDA (55 $\mu$M in subunits) in 0.1 M Tris-HCl, pH 7.6, was added PMPS to 1.4 mM and EDTA to 1.2 mM. Following incubation at room temperature for 10 min, DTT was added to 5 mM, and incubation was continued for 10 min at room temperature. Quick change of buffer and removal of the small molecules were accomplished by using Penefsky columns (*14*). A disposable 1 mL plastic syringe was plugged with glass wool in the bottom, and the syringe was filled to the 1 mL mark with a suspension of Sephadex G-50 (fine) in 0.1 M Tris-HCl, pH 7.6, 1 mM DTT, 20 mM EDTA or 0.1 M potassium phosphate, pH 7.6, 10 mM glutathione, 20 mM EDTA. The column was placed in a test tube, the liquid allowed to drain, and the column subsequently centrifuged at 2500 rpm for 2 min in a tabletop centrifuge. A 100 $\mu$L sample of the enzyme treated with PMPS, EDTA, and DTT was applied to the column and centrifuged as above, and the effluent (apoenzyme) was collected in a fresh tube. Metal ions were eventually added to 80 $\mu$M, and activity was measured after 30 min incubation at 37 °C.

*Polyacrylamide Gel Electrophoresis (PAGE)*. For denaturing SDS gels, protein samples were incubated for 2 min at 100 °C in 2 × SDS loading buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.2% bromophenol blue) and applied to a 15% polyacrylamide−SDS gel (*15*) in a Bio-Rad Mini-PROTEAN II cell. Gels were run at 40 mA for 70 min, stained with Coomassie Blue (10% acetic acid, 25% 2-propanol, 0.05% Brilliant Blue R250), and destained in 10% acetic acid. For native gels, protein samples were mixed with 2 × native loading buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 0.2% bromophenolblue) and loaded on 12.5% native polyacrylamide gels made as described above but without SDS. The gels were run, stained, and destained as described above.

*Fast Protein Liquid Chromatography (FPLC)*. An 800 $\mu$L sample of heterotetrameric CDA (70 $\mu$M in subunits) was applied to an ion exchange column (Q5, Bio-Rad) previously equilibrated with 20 mM Tris-HCl buffer, pH 7.6, and connected to an FPLC apparatus (Pharmacia). The column was washed with 3 volumes of 20 mM Tris-HCl buffer, pH 7.6, and eluted with a gradient of KCl (0−0.4 M) in the same buffer.

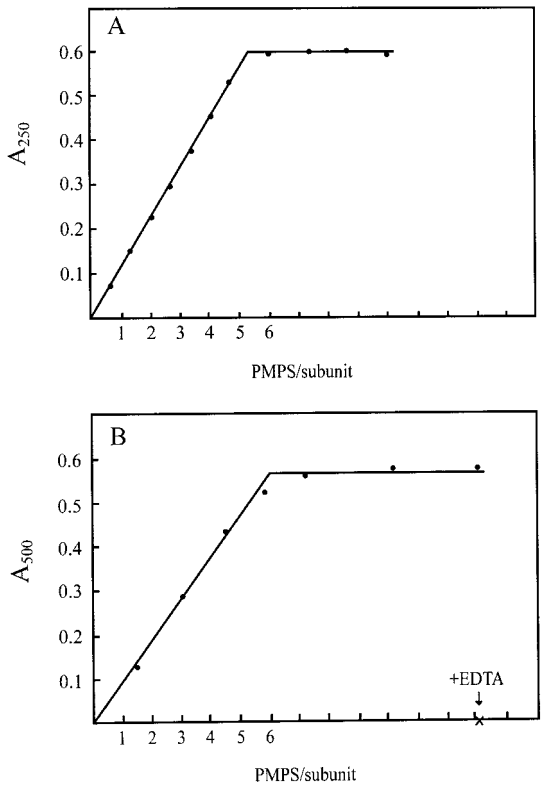


FIGURE 3: Release of $Zn^{2+}$ from CDA during titration with the mercurial reagent PMPS. (A) The absorbance at 250 nm was monitored following successive additions of 3 $\mu$L aliquots of 4.0 mM PMPS to 0.6 mL of 24.5 $\mu$M CDA subunits in 20 mM Tris-HCl, pH 7.6, 90 mM KCl. (B) The absorbance at 500 nm was monitored following successive additions of 2 $\mu$L aliquots of 4.0 mM PMPS to 0.6 mL of 7.5 $\mu$M CDA subunits in 20 mM Tris-HCl, pH 7.6, 90 mM KCl, 0.1 mM PAR. The absorbance values were measured relative to a reference cuvette with all agents added except enzyme and were adjusted for dilution effects. X, absorbance following addition of 2 mM EDTA to the cuvette after end of titration.

## RESULTS

*Sulfhydryl Reactivity of CDA and Removal of Zinc.* The activity of CDA in the presence of 1 mM EDTA at 37 °C showed a half-life of 36 h (data not shown). The half-life of the activity in the presence of 1 mM 1,10-phenanthroline was shorter (around 4 h), and the decrease in activity was accompanied by visual denaturation of the protein. Thus, a different approach was employed for removing the firmly bound zinc from the protein without denaturation. Based on amino acid sequence similarity with the *E. coli* enzyme, the zinc ion in *B. subtilis* CDA was proposed to be coordinated by three specific cysteine residues out of the six present per subunit (*4*). Under native conditions, none of the six cysteine residues were sensitive to treatment with the fast reacting sulfhydryl agent DTNB. In the presence of 8 M urea or 6 M guanidinium chloride, 6 mol of SH groups reacted with DTNB per mole of enzyme subunits (data not shown).

One way of displacing sulfhydryl-coordinated zinc from a protein is to add equivalents of an organomercurial such as PMPS (*13*, *16*). When 25 $\mu$M CDA was titrated with PMPS, the absorbance at 250 nm increased due to the formation of mercury−thiolate charge-transfer complexes (Figure 3A). The titration indicated the presence of six thiol groups per CDA subunit. By repeating the PMPS titration

Table 1: Activity of Metal-Free ApoCDA and Reactivation by Metal Ions[a]

| sample | buffer | activity (units/mg) | activity (%) |
|---|---|---|---|
| holoenzyme | TrisHCl, pH 7.6, 1 mM DTT | 120 | 100 |
| apoenzyme | TrisHCl, pH 7.6, 1 mM DTT | 2 | 2 |
| apoenzyme + 80 $\mu$M $ZnCl_2$ | TrisHCl, pH 7.6, 1 mM DTT | 100 | 84 |
| apoenzyme + 80 $\mu$M $CdCl_2$ | TrisHCl, pH 7.6, 1 mM DTT | 22 | 20 |
| holoenzyme | phosphate, pH 7.6, 10 mM glutathione | 120 | 100 |
| apoenzyme | phosphate, pH 7.6, 10 mM glutathione | 0 | 0 |
| apoenzyme + 80 $\mu$M $ZnCl_2$ | phosphate, pH 7.6, 10 mM glutathione | 87 | 73 |
| apoenzyme + 80 $\mu$M $CdCl_2$ | phosphate, pH 7.6, 10 mM glutathione | 20 | 17 |
| apoenzyme + 80 $\mu$M $CoCl_2$ | phosphate, pH 7.6, 10 mM glutathione | 23 | 19 |
| apoenzyme + 80 $\mu$M $MgCl_2$ | phosphate, pH 7.6, 10 mM glutathione | 0 | 0 |

[a] Holoenzyme was treated like apoenzyme except that PMPS was omitted.

in the presence of the zinc binding dye PAR, the release of zinc from the enzyme was followed by monitoring the absorbance at 500 nm resulting from the formation of the $PAR_2Zn$ complex (Figure 3B). It should be noted from Figure 3B that the absorbance at 500 nm increased linearly with the amount of PMPS added, and that the end-point of titration occurred at the same value of PMPS/subunit as did the plot of $A_{250\ nm}$ versus PMPS. By employing the value $\Delta\epsilon = 6.6 \times 10^4\ M^{-1}\ cm^{-1}$ at 500 nm for the $(PAR)_2Zn^{2+}$ complex (*13*), the amount of $Zn^{2+}$ released per 15 kDa subunit in the experiment shown in Figure 3B corresponded to 1.15.

*Preparation and Activity of ApoCDA and Reconstitution with Zinc and Other Metal Ions.* Zinc-free apoCDA was prepared by reacting the native enzyme for 10 min with 25 equiv of PMPS in the presence of 1.2 mM EDTA, followed by the addition of excess DTT to displace the enzyme-bound PMPS. Subsequently, PMPS and $Zn^{2+}$-EDTA were removed by centrifugation through a gel filtration column. The apoenzyme recovered was totally inactive (Table 1). As shown in Table 1, 84% of the activity was recovered by addition of 80 $\mu$M $ZnCl_2$ to the apoenzyme and 20% by the addition of $CdCl_2$. The presence of a reducing agent was essential for this reactivation. Other divalent metal ions, such as $Fe^{2+}$, $Ni^{2+}$, $Mn^{2+}$, $Mg^{2+}$, and $Cu^{2+}$, failed to promote reactivation. It was not possible to test for reactivation of the apoenzyme with cobalt ions in the presence of DTT, since $Co^{2+}$ reacted with DTT, forming an insoluble brown precipitate. However, when glutathione in phosphate buffer was used as the reducing environment, reactivation was observed (Table 1). Even though $Co^{2+}$ could substitute for $Zn^{2+}$ with 20% activity, the cobalt-substituted enzyme lost all activity following incubation at 37 °C overnight, whereas the zinc- and cadmium-containing enzymes remained active for several days under the same conditions (data not shown).

*Subunit−Subunit Exchange in Native CDA.* It has previously been shown that a rare −1 ribosomal frameshift near the 3′ end of the *cdd* gene resulted in the synthesis, in vivo, of small amounts of a CDA subunit ([$S^{ext}$]) extended by 13

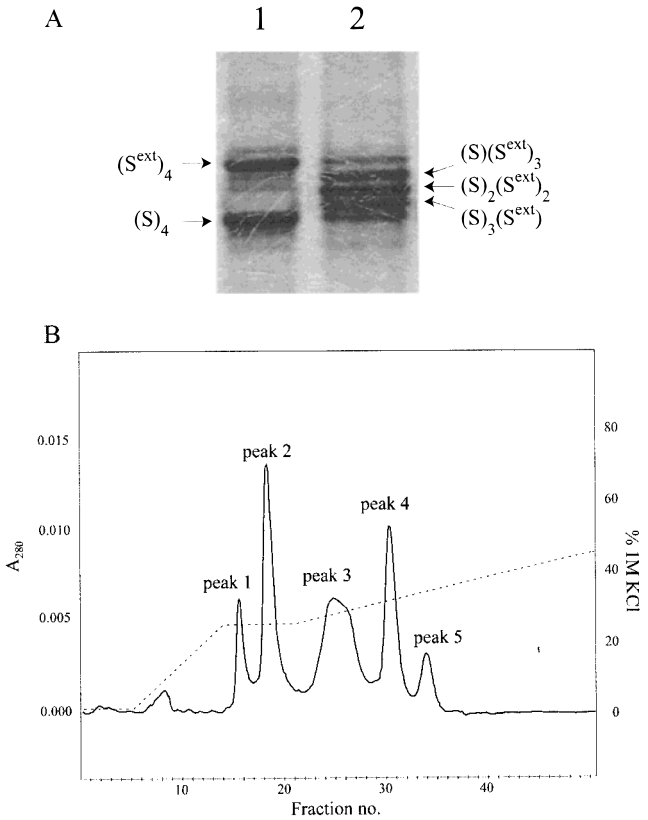


FIGURE 4: Formation of heterotetrameric forms of *B. subtilis* CDA following incubation of a mixture of the two homotetrameric forms, $[S]_4$ and $[S^{ext}]_4$. Each form was present at 20 $\mu$M in 0.1 M Tris-HCl, pH 7.6. (A) Nondenaturing PAGE of aliquots of the mixture. Lane 1, immediately after mixing; lane 2, after 72 h incubation at room temperature. (B) FPLC of 1 mL of the mixture after 72 h incubation at room temperature. The column was eluted with a gradient in KCl. Solid line, absorbance at 280 nm ($A_{280}$); dashed line, salt gradient in percentages of 1 M KCl.

amino acids at the carboxy terminal, and that [$S^{ext}$] formed mixed tetramers with the wild-type subunits, [S] (*7*). The hybrid forms of the enzyme, as well as the homotetramer $[S^{ext}]_4$, showed the same specific enzyme activity as the wild-type homotetramer, $[S]_4$, but were easily distinguished from it on nondenaturing PAGE. Plasmid pSO144, containing a mutant version of the *B. subtilis cdd* gene encoding the [$S^{ext}$] subunit, was constructed by site-directed mutagenesis (see Materials and Methods), and $[S^{ext}]_4$ CDA was purified from cells harboring this plasmid (Figure 2).

A 1:1 mixture of 40 $\mu$M (subunit) solutions of purified $[S]_4$ and $[S^{ext}]_4$ CDA in 0.1 M Tris-HCl, pH 7.6, was incubated at room temperature for 72 h, and the mixture was analyzed both by nondenaturing PAGE (Figure 4A) and by ion-exchange FPLC (Figure 4B). As shown, three new forms of the enzyme were formed. This suggested that subunit−subunit exchange between the two homotetrameric forms had occurred, with the formation of the three possible heterotetrameric forms, $[S]_3[S^{ext}]$, $[S]_2[S^{ext}]_2$, and $[S][S^{ext}]_3$. The subunit composition of each of the five CDA forms eluted from the FPLC column (Figure 4B) was verified by SDS−PAGE and native PAGE as shown in Figure 5. The areas defined by each of the peaks in Figure 4B were determined and showed the following relative sizes: 9% (peak 1), 28% (peak 2), 35% (peak 3), 22% (peak 4), and 6% (peak 5). Thus, the relative amounts of the five tetrameric forms of the enzyme

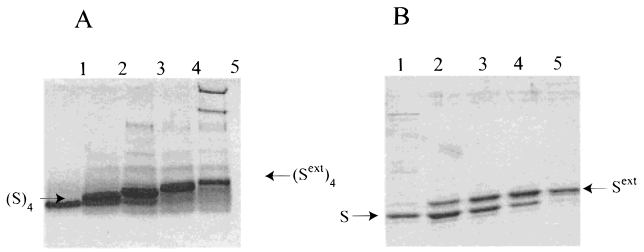


FIGURE 5: Polyacrylamide gel electrophoresis of peak fractions from the FPLC separation shown in Figure 4B. (A) Native PAGE; (B) SDS−PAGE. Lanes 1−5, enzyme collected from peaks 1−5 of Figure 4B, respectively.

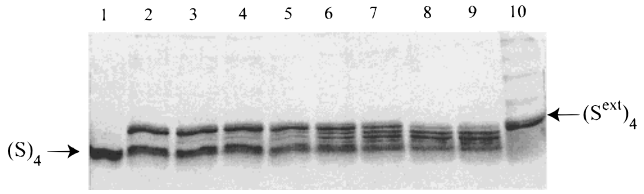


FIGURE 6: Time dependency of subunit exchange between the $[S]_4$ and $[S^{ext}]_4$ homotetramers. Equal volumes of 40 $\mu$M (subunits) solutions of the homotetramers in 0.1 M Tris-HCl, pH 7.6, were mixed and incubated for different lengths of time at 37 °C. Aliquots of the mixtures were submitted to nondenaturing PAGE. Lane 2, 0 min; lane 3, 30 min; lane 4, 45 min; lane 5, 1.5 h; lane 6, 3 h; lane 7, 6 h; lane 8, 18 h; and lane 9, 32 h. The homotetrameric forms were also incubated separately for 32 h and used as controls: lane 1, $[S]_4$; lane 10, $[S^{ext}]_4$, respectively.

were in accordance with the theoretical values 6.25%, 25%, 37.5%, 25%, and 6.25% of a random distribution.

The time course of the subunit−subunit exchange was investigated by incubating mixtures of $[S]_4$ and $[S^{ext}]_4$ homotetramers (20 $\mu$M subunits in 0.1 M Tris-HCl, pH 7.6) at 37 °C for different lengths of time. Each incubation mixture was analyzed by nondenaturing PAGE. As shown in Figure 6, the heterotetrameric forms $[S]_3[S^{ext}]$, $[S]_2[S^{ext}]_2$, and $[S][S^{ext}]_3$ were visible after 3 h, and appeared to have reached equilibrium after 32 h.

A mixture of $[S]_4$ and $[S^{ext}]_4$ homotetramers was diluted to a final concentration of 0.2 $\mu$M, and then concentrated by centrifugation on Ultrafree-MC spin columns and submitted to nondenaturing PAGE. Since no heterotetrameric forms were detected on the electropherogram (data not shown) it was concluded that the enzyme existed as tetramers even in very dilute solutions.

*Reconstitution of Tetrameric Enzyme in the Presence and Absence of* $Zn^{2+}$. Treatment of a 1:1 mixture of $[S]_4$ and $[S^{ext}]_4$ homotetramers (20 $\mu$M subunits of each in 0.1 mM Tris-HCl, pH 7.6) with PMPS and EDTA, followed by addition of DTT, gel filtration through a Penefsky column, and addition of 80 $\mu$M $Zn^{2+}$, resulted in the formation of all combinations of heterotetramers (Figure 7A, lane 1). This indicated that PMPS treatment caused complete dissociation of the oligomeric enzymes into subunits. The time span of the experiment was less than 30 min: therefore, a mixture of $[S]_4$ and $[S^{ext}]_4$ homotetramers treated as above, but without the addition of PMPS, did not show a significant degree of formation of the heterotetrameric forms (control sample, Figure 7A, lane 2). The activity of the apoheterotetrameric forms was reconstituted by zinc equally well as the apohomotetramers.

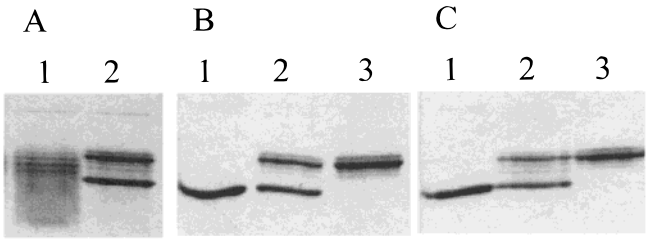


FIGURE 7: Refolding of denatured CDA in the presence and absence of $Zn^{2+}$. (A) Mixtures containing 20 $\mu$M each of $[S]_4$ and $[S^{ext}]_4$ CDA were treated with PMPS and EDTA followed by reconstitution with DTT and $Zn^{2+}$ (lane 1); the same as above but omitting PMPS (lane 2). (B) Solutions containing 40 $\mu$M $[S]_4$ and 40 $\mu$M $[S^{ext}]_4$ were separately treated with PMPS plus EDTA, and followed by addition of DTT. Aliquots of the two samples were submitted to nondenaturing PAGE (lanes 1 and 3, respectively). After DTT treatment, equal volumes of the two samples were mixed in the presence of 80 $\mu$M $Zn^{2+}$, and an aliquot was submitted to nondenaturing PAGE (lane 2). (C) same as in (B), except that the final mixing was done in the absence of $Zn^{2+}$.

To investigate the role of zinc in this refolding and reassociation process, the homotetramers $[S]_4$ and $[S^{ext}]_4$ were separately treated with PMPS, EDTA, and DTT, and immediately after mixed 1:1 in the presence (Figure 7B) or the absence (Figure 7C) of zinc. If the zinc ion was important for the reassociation of subunits, the mixture would show a heterotetrameric pattern on a native PAGE gel, as was observed in the experiment above (Figure 7A, lane 1). However, only the two homotetrameric forms were observed (Figure 7B,C), implying that the enzyme reassembled into the tetrameric form immediately following DTT addition to the PMPS/EDTA-treated enzyme, and that this reassociation was independent of the presence of zinc.

## DISCUSSION

This study was undertaken to investigate the importance of zinc ions in catalysis and in folding of the homotetrameric *B. subtilis* CDA. Titration of the enzyme with DTNB indicated that none of the six thiolates present per subunit were readily accessible unless the enzyme was denatured with either urea or guanidinium chloride. Titration with the strong dissociating sulfhydryl reagent PMPS in the presence or absence of the high-affinity metal indicator PAR revealed six sulfhydryl groups per subunit, and indicated that $Zn^{2+}$ release was a linear function of the mercurial added with 1 equiv of $Zn^{2+}$ released for every 6 equiv of PMPS added during the titration. This suggests that once the first thiolate group has reacted with PMPS, the subunit will unfold sufficiently to allow for a rapid reaction with the remaining five cysteine residues of the same subunit, resulting in release of $Zn^{2+}$. Thus, by PMPS titration it is not possible to distinguish between the three putative zinc-coordinating cysteine residues (Cys-53, Cys-86, and Cys-89) and the three additional cysteines (Cys-41, Cys-51, and Cys-97). Identification of the three zinc-coordinating thiolate side chains is therefore largely based on amino acid sequence homology with *E. coli* CDA (*2*, *4*). However, supporting evidence is the finding that replacement of either Cys-53, Cys-86, or Cys-89 with Ala resulted in inactive enzymes, whereas mutant enzymes containing C41A, C51A, or C97A substitutions were all enzymatically active (S. Vincenzetti and N. Mejlhede, unpublished results). Very similar results were re-

cently obtained with human CDA. The amino acid sequence of the human enzyme is 40% identical with that of *B. subtilis* CDA (*17*).

We have employed a combination of PMPS and EDTA, followed by release of the enzyme-bound PMPS by addition of DTT, to remove zinc from CDA. The metal-free apoenzyme is enzymatically inactive but can be almost fully reconstituted by addition of $Zn^{2+}$, and to a lesser extent also by $Cd^{2+}$ and $Co^{2+}$, provided the apoenzyme is kept in a reduced state. Thus, besides being involved directly in catalysis, zinc appears to play an antioxidant role by preventing disulfide bond formation in the active site.

$Zn^{2+}$ lacks significant redox activity due to its electron arrangement where two outer electrons are lost, resulting in a pseudo noble gas electron configuration (*18*). $Co^{2+}$, which is an ion often observed to be able to substitute for zinc, is more reactive. The cobalt-substituted enzyme, although being enzymatically active, was found to be more labile. $Cd^{2+}$ has the same electron configuration as zinc and possesses presumably many of the same physical properties, except that it is larger. The $Cd^{2+}$-substituted enzyme, though only 20% as active as the $Zn^{2+}$-enzyme, was considerably more stable than the $Co^{2+}$-enzyme.

Assembly processes and protein−protein interactions depend on the correct folding of the polypeptide chains. If zinc was structurally important for CDA, lack of the metal ion would most likely lead to misfolded subunits unable to assemble into a correct tetrameric structure. Treatment of native CDA with PMPS resulted not only in a loss of metal and in dissociation of subunits, but presumably also in partial unfolding of the subunits as indicated by the apparent cooperativity in sulfhydryl labeling. By using two easily distinguishable forms of CDA subunits, both capable of forming enzymatically active homotetramers, we were able to show that these major structural perturbations caused by titration of the enzyme's thiol groups with PMPS were reversible, and that refolding and reassociation into the tetrameric form, promoted by removal of PMPS, was rapid and independent of the presence of $Zn^{2+}$.

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