Mutations Affecting Transition-State Stabilization by Residues Coordinating Zinc at the Active Site of Cytidine Deaminase

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ABSTRACT: Cytidine deaminase from *Escherichia coli* contains 1 mol of tightly bound zinc per enzyme subunit (Yang, C., Carlow, D., Wolfenden, R., & Short, S. A. (1992) *Biochemistry 31*, 4168–4174). When the metal liganding residues Cys-129 and Cys-132 were replaced by Ala, and His-102 was replaced by Ala, Asn, or Gln, deaminase activities of cell extracts containing these mutant enzymes were decreased by several orders of magnitude relative to that of the wild-type enzyme. After purification, each mutant protein was found to contain less than 0.2 mol of zinc per enzyme subunit, except mutant H102Q, which contained 1 mol of zinc per subunit. The activity of each mutant enzyme increased in the presence of added zinc but never attained wild-type activity. Mutant H102N was unique in that this protein could be purified as a stable apoenzyme, activated by added zinc, and then inhibited by EDTA. This mutant enzyme bound zinc with an apparent K_d value of 6.0×10^{-10} M and regained maximal activity in the presence of 1 mol of zinc per subunit. Affinities of the mutant cytidine deaminases for the transition-state analogue, 5-fluoropyrimidin-2-one ribonucleoside (3,4) hydrate, were found to decrease in rough proportion to k_{cat}/K_m over a range spanning several orders of magnitude. This variation in catalytic efficiency arose mainly from effects on k_{cat}, indicating the involvement of zinc coordination in the catalytic process rather than in substrate binding.

Cytidine deaminase (CDA, EC 3.5.4.5) from *Escherichia coli* catalyzes the hydrolytic deamination of cytidine, producing uridine and ammonia which can be utilized as carbon and nitrogen sources, respectively, to support bacterial growth. This enzyme is a dimer composed of identical subunits (M_r = 31 540), each containing one atom of tightly bound zinc (Yang et al., 1992; Betts et al., 1994). CDA is strongly inhibited by compounds such as 5-fluoropyrimidin-2-one ribonucleoside (FZEB), whose rare 3,4-hydrated species mimics an intermediate formed by the direct water attack on C-4 of the pyrimidine ring (Frick et al., 1989). For cytidine deamination, the active-site zinc may function to bind and activate a water molecule for attack on the pyrimidine ring, followed by elimination of ammonia.

The gene encoding *E. coli* CDA has been cloned, its nucleotide sequence determined, and the deduced amino acid sequence used in homology searches against prokaryotic and eukaryotic deaminases (Yang et al., 1992). These homology comparisons, and an X-ray structure of the enzyme complexed with 5-fluoropyrimidin-2-one ribonucleoside (3,4) hydrate (Betts et al., 1994), suggested that the active-site zinc atom is coordinated by one histidine residue and two cysteine residues (Figure 1). In other enzymes (for review see, Vallee & Auld, 1990), zinc atoms that serve catalytic functions are predominantly liganded to imidazole nitrogens and/or carboxylate groups, as in carbonic anhydrase (Liljas et al., 1972; Kannan et al., 1975), adenosine deaminase (Wilson et al., 1991; Sharff et al., 1992), and carboxypeptidase A (Rees et al., 1983). Only one class of enzymes, the eukaryotic alcohol dehydrogenases, has been shown to contain a catalytic zinc atom that is coordinated by one histidine residue and two cysteine residues (Brandén et al., 1975), but these enzymes have no known hydrolytic activity. In this respect, cytidine deaminase appears unusual among zinc metalloproteins and unique among hydrolytic enzymes. Recently, however, a similar arrangement of potential zinc coordinating residues has been identified for T4-phage deoxycytidylate deaminase (Moore et al., 1993) and proposed for the p27 catalytic subunit of the apolipoprotein B mRNA editing enzyme (Navaratnam et al., 1993).

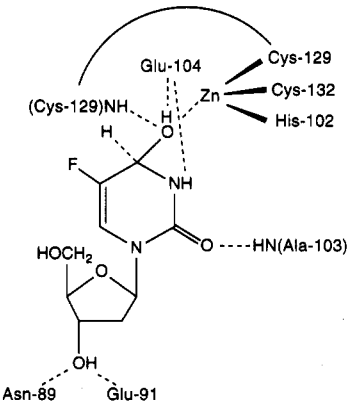

FIGURE 1: Arrangement of the amino acid residues about the active site of cytidine deaminase and their proposed interaction with the transition state analog inhibitor, 5-fluoropyrimidin-2-one ribonucleoside (3,4) hydrate (FZEB-hydrate), based on solution of the CDA:FZEB complex crystal structure (Betts et al., 1994).

This paper describes genetic and biochemical studies exploring the role of His-102, Cys-129, and Cys-132 as determinants of the activity of cytidine deaminase. Each of these potential zinc liganding residues was replaced, and mutant proteins were then tested for zinc content, cytidine deaminase activity in the presence and absence of added zinc, and affinity for ground-state and transition-state analogue inhibitors.

MATERIALS AND METHODS

Recombinant DNA Procedures. The subcloning and sequencing of *cdd* gene constructs was performed as described

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by Yang et al. (1992). To facilitate production of the wild-type protein, the *cdd* gene and the upstream promoter/operator sequences necessary for high-level enzyme expression were cloned into pBR322, yielding plasmid pAA5970. Specific amino acid substitutions were made via site-directed mutagenesis of the wild-type *cdd* gene as described by Taylor et al. (1985), using a kit from Amersham Life Sciences. Following mutagenesis, the region of each mutant gene containing the codon change was verified by nucleotide sequence determination and subcloned as a *Nsi*I-*Bgl*II ended DNA fragment into unmutagenized and *Nsi*I-*Bgl*II digested pAA5970. This procedure ensures that only the desired mutations are present in the final *cdd* clone used for enzyme expression.

Enzyme Purification. Plasmids harboring the wild-type *cdd* gene and mutant alleles were transformed into *E. coli* SS6130 (*cytR* Δ*cdd*) for protein production. In this strain, transcription from the plasmid-borne *cdd* gene is completely derepressed and CDA protein is expressed only from the plasmid-borne gene. The bacterial cultures were grown for 16–18 h at 37 °C in LB (Miller, 1972) containing ampicillin (100 μg/mL). The cell pellet collected from this culture (3 g, wet weight) was suspended in 12 mL of buffer containing KCl (50 mM), Tris·HCl (50 mM, pH 7.5), and 10% v/v glycerol. Cell extracts were prepared by one pass through a French press cell at 11 000 psi. Intact cells and cell debris were removed by low speed centrifugation (15 000 rpm for 15 min in an SS-34 rotor), and the crude supernatant was centrifuged at 100000*g* for 1 h. The high-speed supernatant was then applied to a Poros II Q anion-exchange column (1 × 8 cm, Perseptive Biosystems, Cambridge, MA) equilibrated in breaking buffer. Under these conditions, approximately 90% of the enzyme was recovered in the flow-through fraction giving an approximate 4-fold purification. The flow-through fraction was diluted 3-fold with Tris·HCl (50 mM, pH 7.5) containing 10% glycerol (vol/vol, buffer B) and applied to a HR 10/10 MONO-Q anion-exchange column (Pharmacia, Piscataway, NJ) equilibrated in buffer B. Under these conditions, enzyme was eluted at a salt concentration of 0.15 M KCl. Analysis by SDS–PAGE indicated that the enzyme was at least 95% homogeneous.

The wild-type enzyme and each His-102 replacement mutant were purified without addition of $ZnCl_2$ to the breaking and column buffers. Attempts to purify the mutant proteins in which Cys-129 or Cys-132 had been replaced with Ala were unsuccessful: both proteins precipitated after passage through the first column and activity could not be restored by addition of zinc. Mutant CDA C132A lost all activity after the first column even in the presence of 50 μM $ZnCl_2$ and could not be reactivated by addition of more zinc.

Enzyme Assays. The deaminase activities of wild-type and mutant enzymes were routinely measured at 30 °C in a reaction (1.0 mL) containing cytidine (300 μM) in Tris·HCl buffer (50 mM, pH 7.5), with added $ZnCl_2$ (50 μM) when specified. Mutant enzymes requiring zinc activation were diluted into Tris·HCl (50 mM, pH 7.5) containing $ZnCl_2$ (50 μM) and incubated for 10 min at 4 °C prior to assay. Cytidine deamination was measured at 282 nm [$\Delta\epsilon$ (mM) = −3.6] using a Perkin-Elmer Lambda 6 spectrophotometer as described earlier (Cohen & Wolfenden, 1971; Yang et al., 1992). To measure inhibition by product uridine, the reaction was followed at 290 nm [$\Delta\epsilon$ (mM) = −2.2]. Kinetic constants were obtained by nonlinear regression analysis (Leatherbarrow, 1990).

Zinc Analysis. The zinc content of each purified protein was determined using an Instrumentation Laboratories S-12 flame atomic absorption spectrometer. In general, zinc contamination was avoided by the procedures of Falchuck et al. (1988). All solutions were extracted with dithizone (0.2% in CCl_4) and stored over Chelex 100 resin (Bio-Rad, Richmond, CA) to eliminate adventitous metal ions. Plastic ware was rinsed with 20% HNO_3 and metal-free water prior to being used. Dialysis tubing was freed of metal impurities as described by Auld (1988). Before metal analysis, excess or loosely bound metal ions were removed from the protein (0.5–1.0 mg/mL) by dialysis for 48 h at 4 °C against metal-free HEPES·HCl (10 mM, pH 7.0).

Affinity and Stoichiometry of Zinc Binding to CDA H102N. The zinc dissociation constant of mutant H102N was determined by measuring the enzymatic activity regained after equilibration of the enzyme with different concentrations of free zinc, using nitrilotriacetate as a zinc buffer. Zinc concentrations between 10^{-8} and 10^{-12} M were established with this buffer system. Association constants for the 1:1 and 1:2 complex between zinc and nitrilotriacetate were the values reported by Anderegg (1982). After correction for pH and ionic strength differences (pH 7.5, 22 °C in 0.1 M KCl), the values were estimated as $10^{8.43}$ and $10^{1.37}$, respectively. The activation mixtures contained H102N (4 μM) in Tris·HCl (0.1 M, pH 7.5) containing nitrilotriacetate (0.02 M), KCl (0.1 M) and enough $ZnSO_4$ to give the desired free zinc concentration. An incubation time of 30 min was routinely used, since the half-time for recovery of activity was never greater than 3 min. Values of K_d were calculated by nonlinear regression analysis (Leatherbarrow, 1990).

Ligand Binding by Wild-Type and Mutant Enzymes. The binding of [5,6-^{3}H]uridine (Moravek Biochemicals, Inc., Brea, CA) by mutant proteins was measured using a nitrocellulose filter to separate the bound from the free ligand. The binding assay (100 μL) was initiated by addition of enzyme (10 μM final concentration) to the radiolabeled ligand in Tris·HCl buffer (0.01 M, pH 7.5) containing $MgCl_2$ (0.05 M). Following a 10-min incubation period at room temperature, the [^{3}H]uridine bound by the CDA proteins contained in 80 μL of assay mix was collected on prewashed, nitrocellulose membrane filters (Millipore HAWP02500; Millipore Corp., Bedford, MA) at a filtration rate of approximately 2 mL/min. The filters were washed once with the same buffer (300 μL), air-dried, and dissolved in 3 mL of Packard Filter-Count LSC cocktail (Packard Instrument Co., Meriden, CT), and the radioactivity of each sample was determined using a Packard Model 1900TR scintillation counter. Specific binding of uridine by each protein was calculated after correction for nonspecific sticking of radiolabeled ligand to the filters. The binding data were analyzed using a nonlinear curve-fitting program (Leatherbarrow, 1990).

Other Analytical Procedures. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of cell-free extracts and purified proteins was performed using mini slab gels (0.75 × 7 cm) and the buffer described by Thomas and Kornberg (Thomas & Kornberg, 1975). The slab gels consisted of a 6-cm 16.5% separating gel (200:1 acrylamide:bisacrylamide) overlaid with a 1-cm 4.5% stacking gel (36:1 acrylamide:bisacrylamide). Electrophoresis of the proteins through the stacking and separating gels was carried out at a constant voltage of 50 and 200 V, respectively. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. The protein concentrations of crude fractions were measured by the Bradford dye binding procedure using

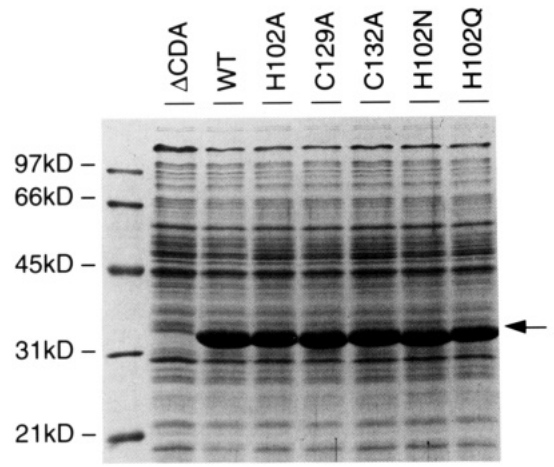

FIGURE 2: Protein profiles obtained following polyacrylamide electrophoresis of cell extracts (25 μg of protein) prepared from SS6130 clones harboring plasmids encoding the wild-type enzyme, the mutant proteins, and the profile for this strain having a plasmid vector lacking the *cdd* coding sequence. The CDA polypeptide (M_r ~32 000) is indicated by the arrow at the right of the figure.

bovine γ globulins as the standard (Bradford, 1976). The concentration of purified enzyme was determined from the absorbance at 280 nm, using an extinction coefficient of 39 000 M^{-1} cm^{-1} (Yang et al., 1992).

RESULTS

Synthesis of Wild-Type and Mutant Proteins. Each of the mutant enzymes described in this study was homogeneous. The entire wild-type *cdd* gene had been deleted from the genome of the expression strain so that mixed oligomers composed of mutant and wild-type subunits could not be formed. Thus, the activity measured for each mutant enzyme was due solely to that mutant enzyme. Before exploring the mechanistic consequences produced by replacement of single amino acid residues in the active site of cytidine deaminase, it was first established (1) that the quantity of each mutant protein synthesized was similar to that for the wild-type enzyme, (2) that the mutant enzymes were not truncated, and (3) that the global structures of the mutant proteins resemble that of the wild-type enzyme. The electrophoresis protein profile shown in Figure 2 indicates that substitution for residues His-102, Cys-129, or Cys-132 did not affect expression or produce mutant proteins that were subject to aberrant proteolytic processing. Levels of expression and apparent subunit molecular weights of the mutant enzymes were, in every case, indistinguishable from the wild-type protein (M_r 31 500). No insoluble mutant protein was observed during enzyme purification. Since misfolded proteins in *E. coli* tend to be either unstable and rapidly degraded (Goldberg, 1972; Parsell & Sauer, 1989) or form insoluble inclusion bodies (Pakula et al., 1986), these results suggest that the protein fold of the mutant proteins is comparable with that of the wild-type enzyme. Consistent with this view, the CD spectrum of mutant H102N, recorded in the presence of zinc or EDTA was, within experimental error, superimposable on the spectrum of the wild-type protein (data not shown).

Activity of Mutant Enzymes in Cell Extracts. Table 1 shows that each mutant enzyme exhibited reduced activity compared with that of the wild-type protein, when assayed either in the absence or presence of added zinc. Mutant enzymes were routinely preincubated with 50 μM $ZnCl_2$, a metal concentration that did not affect the activity of wild-type enzyme, and then were assayed in the presence of this same concentration of zinc. The activity measured for the mutant enzymes in cell extracts was stimulated 5–1000-fold by addition of 50 μM zinc chloride to the cell extract. Each mutant enzyme regained maximal activity by addition of 50 μM zinc chloride. Metal concentrations up to 250 μM gave no further stimulation of activity, while higher levels were slightly inhibitory. The measurable levels of activity in cell extracts without zinc addition presumably resulted from traces of zinc present in the cell extract.

Table 1: Influence of Zinc on the Activity of Wild-Type and Mutant Cytidine Deaminases Measured in Cell-Free Extracts

	CDA activity (nmol/min per mg of protein)		fold stimulation
enzyme	− zinc	+ zinc	by zinc
H102A	0.64	12	19
C129A	0.74	5.8	8
C132A	0.15	0.96	6
H102N	1.1	1300	1180
H102Q	5200	26000	5
wild-type	73000	73000	1

Table 2: Zinc Content of Purified Wild-Type and Mutant Cytidine Deaminases Determined by Atomic Absorption Analysis

enzyme	mol of Zn/CDA subunit
wild-type	1.01 ± 0.05
H102Q	1.03 ± 0.05
H102N	0.02 ± 0.01
H102A	0.19 ± 0.02
C129A	0.14 ± 0.03
C132A	0.07 ± 0.02

The largest decreases in deaminase activity were observed for those mutants in which the zinc liganding residues (His-102, Cys-129, Cys-132; Figure 1) had been individually replaced by alanine (Table 1). Even in the presence of added zinc, activities measured for H102A, C129A, and C132A were 1000–70 000-fold lower than that of the wild-type enzyme. Replacement of His-102 by either asparagine or glutamine resulted in mutant enzymes with activities intermediate between that measured for the wild-type enzyme and those measured for the mutants in which alanine replaced His-102, Cys-129, or Cys-132 (Table 1). Mutants H102Q and H102N, with three potential zinc liganding groups, exhibited approximately 40% and approximately 2%, respectively, of wild-type activity when assayed with added zinc. Mutant H102N was unique in that it could be purified as the metal-free apoenzyme (see below) and then activated 1000-fold by zinc. H102N was also activated by Co^{2+}, but not by Cd^{2+}, Cu^{2+}, or Fe^{2+} at concentrations up to 1 mM. In the presence of $CoCl_2$ (50 μM), H102N was 80% as active as the zinc-activated mutant enzyme (data not shown). Attempts to prepare metal-free, zinc-reactivatable wild-type CDA were not successful. Prolonged dialysis of the wild-type enzyme with 10 mM 1,10-phenanthroline removed the zinc, but enzyme inactivation could not be reversed by addition of zinc.

Zinc Content of Purified Enzymes. Determination of the zinc content of purified wild-type and mutant enzymes, following their dialysis to remove loosely bound metal ions, yielded the results shown in Table 2. The wild-type enzyme contained 1 mol of zinc per mole of subunit (Yang et al., 1992), whereas mutants H102N, C129A, and C132A contained less than 0.2 mol of zinc per mole of subunit. H102Q, purified in the absence of zinc, was found to contain 1 mol of zinc per subunit, whereas the activity and zinc stimulation observed originally for this enzyme in cell extracts (Table 1)

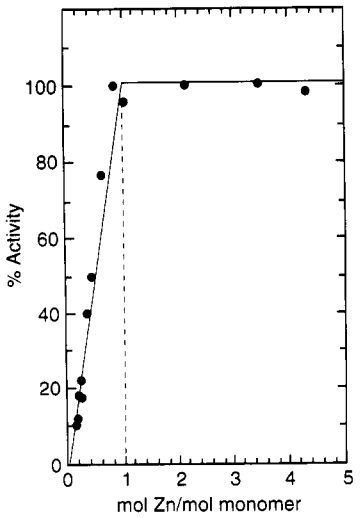

FIGURE 3: Activity titration of cytidine deaminase H102N mutant with zinc. The mutant enzyme (4×10^{-6} M) was incubated for 30 min at 25 °C in 50 mM Tris·HCl (pH 7.5) with zinc at the molar ratios indicated prior to measurement of cytidine deamination.

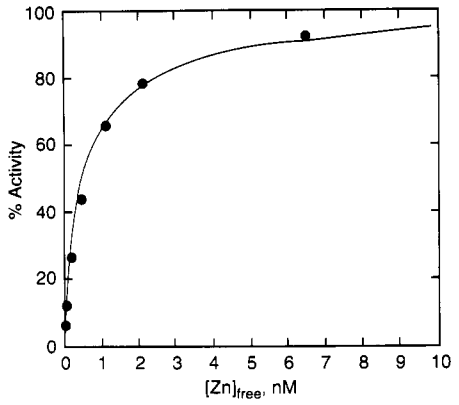

FIGURE 4: Activation of the H102N mutant as a function of the free zinc concentration. The mutant enzyme (1×10^{-6} M) was activated by incubation in 50 mM Tris·HCl (pH 7.5) containing 20 mM nitrilotriacetate and enough $ZnSO_4$ to give the desired free zinc concentration. An apparent K_d value of 6×10^{-10} M was calculated by nonlinear regression analysis.

suggested that it should contain less than 1 mol of zinc per subunit. This enzyme appears to bind zinc much more strongly than the other mutant proteins, but unlike them, the zinc H102Q holoenzyme was not inactivated by EDTA, and assay of the zinc-free form of this mutant protein present in cell extracts required that the cuvettes be rinsed with a solution of EDTA to prevent activation by zinc scavenged from the walls of the cuvette (data not shown). Thus, the high zinc content of purified H102Q may have been due to the acquisition of zinc from buffers and glassware during purification.

Affinity and Stoichiometry of Zinc Binding by H102N. The availability of H102N as a stable apoenzyme that could be reactivated by zinc permitted direct examination of the relationship between cytidine deamination and zinc coordination. When apoH102N (4×10^{-4} M) was titrated with zinc chloride, maximal deaminase activity was achieved at a stoichiometry of approximately 1 mol of zinc per mole of subunit (Figure 3).

The affinity of H102N for zinc was determined by measuring the catalytic activity observed after equilibration of the enzyme with zinc nitrilotriacetate buffer. Figure 4 shows the relationship between the activity of H102N and the concentration of free zinc, yielding an apparent K_d value of 6.0×10^{-10} M. Changes in the concentration of nitrilotriacetate had no effect on activity if the concentration of free zinc was held constant (data not shown). Thus, nitrilotriacetate appears to serve only as a metal buffer and is not itself an inhibitor of the enzyme.

Table 3: Kinetic Parameters for Wild-Type and Mutant Cytidine Deaminases

enzyme	K_m (μM)	$K_{m(mut)}/K_{m(WT)}$	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	$(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{WT}$
wild-type	120		313	2.6×10^6	
H102Q	250	2.1	150	6.0×10^5	2.3×10^{-1}
H102N	200	1.7	10.5	5.3×10^4	2.0×10^{-2}
H102A	390	3.3	0.15	3.8×10^2	1.5×10^{-4}
C129A	1500	12.5	0.3	2.0×10^2	7.7×10^{-5}

Zinc reactivation of the apoenzyme, H102N, was reversible. Thus, metal-activated H102N was completely inactivated by incubation with 1 mM EDTA with a half-time of inactivation of 20 min at 30 °C. In contrast, EDTA (1 mM) did not inhibit either wild-type or mutant H102Q.

Effect of Zinc Ligand Substitution on Catalysis and Inhibitor Binding. Some kinetic consequences of replacing one of the three zinc liganding residues were determined using purified wild-type and mutant proteins, in the presence of $ZnCl_2$ (50 μM), a metal concentration which has been found to be sufficient to give maximal activation of each mutant enzyme. Comparison of the kinetic parameters given in Table 3 shows that substitutions for His-102 did not greatly affect the K_m value of cytidine, whereas in the case of mutant C129A, the K_m value was elevated 13-fold compared with that of the wild-type enzyme. Catalytic efficiency (k_{cat}/K_m) varied with the number of potential zinc liganding residues provided by the mutant enzyme's active site. Thus, the k_{cat}/K_m values for H102Q and H102N, with three potential zinc ligands, were reduced 4- and 50-fold relative to that of wild-type CDA, whereas k_{cat}/K_m values for H102A and C129A, with only two wild-type zinc liganding residues, were reduced 3–4 orders of magnitude. In the case of C132A, which could not be purified in an active form, the reduction in catalytic efficiency seemed to be even greater in cell extracts.

The wild-type enzyme has been shown to bind FZEB as its 3,4-covalent hydrate by UV spectroscopy (Frick et al., 1989). Mutant proteins H102N and H102Q bound FZEB with sufficient affinity to allow observation of their complexes by UV spectroscopy (K_d values of 8×10^{-6} and 5×10^{-7} M, respectively). FZEB (2.4×10^{-4} M) and mutant protein (1×10^{-5} M, with 5×10^{-5} M $ZnCl_2$ added) were placed in separate compartments of a tandem cuvette, and the UV absorption spectrum was determined. The contents were then mixed, and change in the spectrum was recorded. The difference spectra of these mutant enzymes were similar in form and magnitude to that of the wild-type enzyme, suggesting that these mutant enzymes bind FZEB as its 3,4-covalent hydrate.

As the catalytic efficiencies of the mutants decreased, their affinities for a transition-state analogue inhibitor, FZEB-hydrate,[1] also decreased relative to that of wild-type CDA (Table 4). For H102Q and H102N, the 4- and 50-fold decreases in k_{cat}/K_m were matched by 3.6- and 57-fold

[1] From the rapid onset of inhibition, it seems clear that the inhibitor FZEB encounters the enzyme as the unhydrated species (Frick et al., 1989). Hydration then occurs rapidly within the active site, in a process analogous to generation of a hydrated intermediate in cytidine deamination. By the principle of microscopic reversibility, FZEB is presumably released by the enzyme as the unhydrated species.

Table 4: Inhibition of Cytidine Deamination by Uridine, a Ground-State Competitive Inhibitor, and the Transition-State Inhibitor 5-Fluorozebularine

	K_i determined for		
enzyme	uridine (M)	5-F zebularine[a] (M)	k_{cat}/K_m (s^{-1} M^{-1})
wild-type	8.0×10^{-4}	1.4×10^{-7}	2.6×10^{6}
H102Q	6.4×10^{-4}	5.0×10^{-7}	6.0×10^{5}
H102N	6.0×10^{-5}	8.0×10^{-6}	5.3×10^{4}
C129A	1.5×10^{-3}	3.6×10^{-5}	2.0×10^{2}
H102A	6.0×10^{-4}	2.0×10^{-4}	3.8×10^{2}

[a] In the wild-type, H102Q, and H102N enzymes, FZEB was shown to be bound as the 3,4-hydrated species, so that the true K_i value of the hydrate is lower than the apparent K_i value by a factor of approximately 0.0041 (see text).

increases, respectively, in their K_i values for FZEB[2] (Table 4). These K_i values, after correction for a hydration equilibrium constant of 0.0041 in water (D. Carlow & R. Wolfenden, unpublished data), would be lower by this factor for at least these three enzymes (wild-type, H102Q, and H102N). For mutant enzymes with Ala substitutions, binding affinity changed with k_{cat}/K_m, but the correlation was less exact.

No such relationship was observed for inhibition of the mutant enzymes by product uridine. Compared with wild-type enzyme, H102N showed a 49-fold decrease in catalytic efficiency but a 13-fold *increase* in binding affinity for uridine. To explore the possibility that the presence of zinc might affect this result, the binding affinity of apoH102N for radiolabeled uridine was determined in the absence and presence of zinc (50 μM). Uridine K_d values were 51 μM in the presence of zinc, 4 μM in buffer alone, and 7 μM in buffer containing EDTA (50 μM). The K_d value for uridine in the presence of zinc, obtained by direct binding studies, 51 μM, was similar to the K_i value of uridine obtained from kinetic experiments, 60 μM (Table 4). When the binding of uridine by H102N was measured in the absence of zinc, however, its K_d value decreased to 4–7 μM.

DISCUSSION

We have previously identified the *E. coli* cytidine deaminase as a metalloprotein containing 1 mol of zinc per enzyme subunit and suggested three enzyme residues (His-102, Cys-129, and Cys-132) that could function in zinc coordination (Yang et al., 1992). Recently, the CDA:FZEB-hydrate complex crystal structure was solved, revealing that these potential zinc coordinating residues were located in the active site and were positioned appropriately for interaction with the enzyme-bound metal (Betts et al., 1994). The present study provides further evidence that His-102, Cys-129, and Cys-132 function in zinc coordination. All of the mutant enzymes, except for H102Q, exhibited zinc stoichiometries that were markedly lower than that found for the wild-type protein (Table 2). The activity of each mutant enzyme as isolated, including H102Q, was less than that measured for the wild-type enzyme and was increased following addition of zinc, although complete wild-type activity was never attained (Table 1). Each of the mutant proteins was homogeneous; the *cdd* alleles were expressed in a *E. coli* strain lacking a chromosomal copy of the wild-type gene, and misincorporation of the wild-type amino acid into the mutant proteins was minimized as much as feasible by the choice of the codons used during site-directed mutagenesis. The physical data suggest that, with the possible exception of C132A which could not be purified for examination, effects on enzyme activity resulting from replacement of individual zinc liganding residues did not result from gross changes in the conformation of the mutant proteins. Therefore, changes in activity for the mutant deaminases presumably arose from the altered patterns of metal coordination in the active site of the mutant enzymes.

Examination of the active site in the CDA:FZEB-hydrate complex crystal structure (Betts et al., 1994) suggests that cytidine deamination involves interaction of the C-4 hydroxyl group of the altered substrate in the transition state with (1) Glu-104, the general base, (2) the zinc atom which is uniquely coordinated to one His and two Cys residues, and (3) the NH group of Cys-129. The position of C-4 is relatively fixed due to hydrogen bonds formed between the ribose 3′- and 5′-OH groups and the interaction of the hydrophobic edge of the pyrimidine ring with aromatic enzyme residues. Replacement of Glu-104 by Ala produced a mutant enzyme that did not differ detectably from the wild-type enzyme in its conformation and zinc content, but whose k_{cat}/K_m was reduced by approximately 8 orders of magnitude (manuscript in preparation).

The mutant enzymes constructed for the present study can be divided into two broad categories: (1) enzymes in which amide side chains, potential zinc ligands, replace the side chain of His-102 and (2) enzymes with Ala residues substituted for each amino acid residue coordinated to zinc. The properties of the enzymes of the first class, H102Q and H102N, suggest that the tertiary structure of their catalytic centers approximates that found in the wild-type protein. H102Q showed the highest level of activity of all the mutants examined in this study. This enzyme's catalytic efficiency and affinity for substrate cytidine, product uridine, and the transition-state analogue FZEB were each reduced less than 5-fold compared with those of wild-type enzyme (Tables 3 and 4). The affinity of H102Q for zinc, like that of wild-type protein, was high as indicated by its resistance to inactivation by EDTA. When His-102 was replaced by asparagine, the deaminase activity of this mutant enzyme, in the absence of added zinc, was reduced (Table 1) but was activated 1000-fold by addition of zinc (Figure 3). When cytidine deamination by H102N was measured in the presence of 50 μM $ZnCl_2$, this mutant enzyme had a K_m value similar to that determined for the wild-type enzyme (Table 3). In addition, the catalytic efficiency of H102N and its affinity for FZEB were each reduced ~50- and ~15-fold relative to the wild-type and H102Q enzymes, respectively (Tables 3 and 4). Unlike the other two enzymes containing three zinc liganding residues, H102N did not bind zinc with high affinity. This protein could be prepared as a stable apoenzyme, activated by zinc, and inhibited by excess EDTA following zinc supplementation. Somewhat surprisingly, the affinity displayed by H102N holoenzyme for product uridine was *increased* approximately 10-fold compared with affinities of the wild-type or H102Q enzymes for product uridine (Table 4) but was approximately 15-fold less than that of the apoenzyme. In this mutant, the presence of zinc in the active site greatly enhances the enzyme's ability to discriminate between inhibitors representing different stages in substrate transformation.

In the second class of mutants, alanine replaces one of the three amino acid residues formerly involved in zinc coordination. The missing zinc liganding residue of the protein may well have been replaced by another amino acid, by a ligand

[2] These K_i values, after correction for a hydration equilibrium constant of 0.0041 in water (D. Carlow & R. Wolfenden, unpublished data), would be lower by this factor for at least these three enzymes (wild-type, H102Q, and H102N).

from solution, or by water itself.[3] The activities and stabilities of these mutants were markedly different from those observed for the enzymes with three zinc liganding residues. Mutant H102A was purified as an apoenzyme that could be activated by addition of zinc (Table 1). The affinities of this enzyme for substrate [$(K_m)_{mut}/(K_m)_{WT}$ = 3.3; (Table 3)] and for product [$(K_i)_{mut}/(K_i)_{WT}$ = 0.75; (Table 4)] were similar to those of the wild-type enzyme. However, the catalytic efficiency of H102A was reduced 7000-fold compared with the efficiency of the wild-type enzyme (Table 3), and its affinity for the transition-state analogue inhibitor FZEB was the lowest among this set of mutant enzymes (Table 4). These findings indicate that the His-102 → Ala substitution affects the stability of the transition state profoundly, leaving the ground state relatively unaffected. The detailed structural basis of these contrasting effects, currently under investigation, will be of extreme interest.

Mutants C129A and C132A are similar to H102A in that they have lost one zinc coordinating residue but differ from H102A in their stability and activity. Neither C129A nor C132A could be purified in the absence of zinc. Attempts to prepare metal-free mutant proteins resulted in their precipitation during the first step of purification. In cell extracts, activities of these mutant enzymes were stimulated 6–8-fold by zinc (Table 1). Only C129A retained activity during purification, although C132A, purified in the presence of zinc, was soluble and dimeric (data not shown). C129A was the least active of the enzymes purified, with a catalytic efficiency (k_{cat}/K_m) 4 orders of magnitude lower than that of the wild-type enzyme (Table 3). C129A showed the highest K_m value, the lowest affinity for product uridine, and a greater than 200-fold decrease in affinity for the transition-state analogue inhibitor FZEB (Tables 3 and 4). These severe reductions in enzyme activity and on protein stability, resulting from replacement of zinc-coordinating Cys residues by Ala, may reflect changes in zinc coordination at the active site. In this regard it is noteworthy that in the CDA:FZEB-hydrate crystal structure, Cys-129 and Cys-132 are located at the N-terminus and within the first turn of the second helix, respectively, that is part of a unique $\alpha\beta\alpha\beta$ fold in the CDA active site (Betts et al., 1994). Possible changes in the structure of the zinc binding site and state of coordination remain to be examined in detail by spectroscopic methods.

The theory of absolute reaction rates implies that an enzyme should show an affinity for an ideal transition-state analogue that matches the rate enhancement that the enzyme produces when k_{cat}/K_m is compared with the rate of reaction under the same conditions in the absence of enzyme (Wolfenden, 1969). Accordingly, any change in the structure of (1) the substrate or (2) the enzyme that affects the rate of reaction should produce a predictable change in the enzyme's binding affinity for an ideal transition-state analogue inhibitor. In earlier work, the first of these relationships was tested and confirmed by comparing the binding affinities of papain for inhibitory aldehydes (Westerik & Wolfenden, 1972), of thermolysin for inhibitory phosphonamidates (Bartlett & Marlowe, 1983), and of carboxypeptidase A for inhibitory phosphoric acids (Hanson et al., 1989). The availability of enzymes with single amino acid substitutions allows this relationship to be tested in a different way, by comparing the consequences of replacing a specific residue on k_{cat}/K_m with its effect on K_i for a transition-state analogue. Phillips et al. (1992) showed that $1/K_i$ for phosphonate inhibitors varied in strict proportion to k_{cat}/K_m for substrates as the amino acid at position 127 underwent successive alterations in carboxypeptidase A.

In the present study, the affinities of mutant cytidine deaminases for FZEB-hydrate ($1/K_i$) decreased in rough proportion to k_{cat}/K_m (Table 4). This variation in catalytic efficiency arose mainly from effects on k_{cat}, rather than on K_m (Table 3), indicating effects on the catalytic process, rather than merely on substrate binding. Two of the mutant enzymes (H102N and H102Q) showed marked reductions in enzyme activity. These mutant enzymes were found to bind FZEB as the 3,4-hydrate, as does the wild-type enzyme. For these three proteins (H102N, H102Q, and wild-type), affinities for FZEB-hydrate changed in proportion to k_{cat}/K_m, consistent with identification of FZEB-hydrate as a transition-state analogue inhibitor[2] (Table 4).

For two other mutant enzymes (H102A and C129A), k_{cat}/K_m values were reduced to a much greater extent. Their inhibitory complexes with FZEB, although kinetically observable (Table 4), were too weak to observe by UV spectroscopy, preventing determination of whether FZEB was bound as the 3,4-hydrated species. In contrast with the results obtained with the wild-type, H102Q, and H102N enzymes, the observed affinities of mutants H102A and C129A for FZEB were somewhat greater than expected from their observed k_{cat}/K_m values (Table 4). It seems possible that in the H102A and C129A mutant proteins, with drastically reduced catalytic activity, FZEB may be bound partly as a hydrated transition-state analogue inhibitor and also partly as the unhydrated inhibitor. If FZEB could be bound in two forms, and both were competitive with substrate binding, then the amount of free enzyme, at any given concentration of free inhibitor, would be less than if only one of these forms were inhibitory.

The results obtained with this collection of mutant cytidine deaminases support a role for zinc in cytidine deamination and suggest that profound changes in catalytic activity arise when metal coordination is disturbed. This relationship was most evident in the major decreases in enzyme stability, activity, and affinity for inhibitors that accompanied loss of either cysteine ligand. Exact structures of these mutant proteins, currently under examination, should reveal the relative importance of steric and electronic effects on transition-state stabilization.

[3] It is also conceivable that Glu-104 might be recruited as a zinc ligand, as suggested by a reviewer. It is not evident, however, how such an active site could function as a catalyst.

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