Structures of dCTP Deaminase from Escherichia coli with Bound Substrate and Product

REACTION MECHANISM AND DETERMINANTS OF MONO- AND BIFUNCTIONALITY FOR A FAMILY OF ENZYMES*

Eva Johansson†‡, Mathias Fanø†‡, Julie H. Bynek‡, Jan Neuhard*‡, Sine Larsen††‡, Bent W. Sigurskjold‡, Ulla Christensen‡‡, and Martin Willemoës§§

From the †Centre for Crystallographic Studies, Department of Chemistry, University of Copenhagen Universitetsparken 5, DK-2100, Copenhagen, Denmark, the ‡European Synchrotron Radiation Facility, BP 220, F-38043 Grenoble Cedex, France, the ‡Department of Biochemistry, August Krogh Institute, University of Copenhagen Universitetsparken 13, DK-2100, Copenhagen, Denmark, the *‡Department of Biological Chemistry, Institute of Molecular Biology, University of Copenhagen Sølvgade 83H, DK-1307, Copenhagen, Denmark, and the ††Department of Chemistry, Laboratory for Physical Chemistry, University of Copenhagen Universitetsparken 5, DK-2100, Copenhagen, Denmark

dCTP deaminase (EC 3.5.4.13) catalyzes the deamination of dCTP forming dUTP that via dUTPase is the main pathway providing substrate for thymidylate synthase in Escherichia coli and Salmonella typhimurium. dCTP deaminase is unique among nucleoside and nucleotide deaminases as it functions without aid from a catalytic metal ion that facilitates preparation of a water molecule for nucleophilic attack on the substrate. Two active site amino acid residues, Arg115 and Glu138, were identified for nucleophilic attack on the substrate. Two active site amino acid residues, Arg115 and Glu138, were identified for nucleophilic attack on the substrate. Two active site amino acid residues, Arg115 and Glu138, were identified for nucleophilic attack on the substrate.

The atomic coordinates and structure factors (codes 1XS4, 1XS1, and H11545) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† These authors contributed equally to this work.

‡ To whom correspondence should be addressed: Centre for Crystallographic Studies, Dept. of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100, Copenhagen, Denmark. Tel.: 45-35-32-02-39; Fax: 45-35-32-02-99; E-mail: martin@ccs.ki.ku.dk.

§§ These authors contributed equally to this work.

* This work was funded by the Danish National Research Foundation and the Danish National Science Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The main source of dUMP, the precursor for dTTP in the Gram-negative bacteria Escherichia coli and Salmonella typhi-

muri um, is obtained via a pathway where dCTP is deaminated by dCTP deaminase (EC 3.5.4.13) to yield ammonia and dUTP that subsequently is hydrolyzed by dUTPase to generate dUMP and pyrophosphate (1). In contrast, Gram-positive bacteria and eukaryotic organisms synthesize dTTP from dUMP obtained by deamination of dCMP by the zinc-containing enzyme dCMP deaminase (2). Recently, a bifunctional enzyme from the archaean Methanocaldococcus jannaschii has been identified (3, 4) that possesses both the dCTP deaminase and dUTPase activities in one polypeptide suggesting that at least in some Archaea, dCTP serves as a source for dUMP. The structure of this archaean enzyme is now known and the subunit shares an overall fold with dUTPsases as well as the organization of subunits in a trimer (5). In the present work we demonstrate that dCTP deaminase from E. coli is yet another member of this family of enzymes, even though significant differences are found in the C-terminal stretch that closes the active site upon catalysis.

One very interesting feature of the dCTP deaminase is that the deamination reaction proceeds without aid from a metal cofactor. Other nucleoside or nucleotide deaminases such as cytosine deaminase (6, 7), cytidine deaminase (8), adenosine deaminase, (9) and adenine deaminase (10) all require a catalytic metal ion, Zn2+, Mn2+, Fe2+, which is either tightly bound in the active site or has to be added together with the substrate to the enzyme. Here, we report the identification of catalytically important amino acid residues of E. coli dCTP deaminase. Furthermore, we present the first structure of a monofunctional dCTP deaminase in a distinct complex with the reaction product dUTP and Mg2+ (dUTP-Mg2+) as well as the same mutant enzyme in complex with bound substrate dCTP and Mg2+ (dCTP-Mg2+) as well as the same mutant enzyme in complex with bound substrate dCTP and Mg2+ (dCTP-Mg2+) as well as the same mutant enzyme in complex with bound substrate dCTP and Mg2+ (dCTP-Mg2+) as well as the same mutant enzyme in complex with bound substrate.

The main source of dUMP, the precursor for dTTP in the Gram-negative bacteria Escherichia coli and Salmonella typhi-

muri um, is obtained via a pathway where dCTP is deaminated by dCTP deaminase (EC 3.5.4.13) to yield ammonia and dUTP that subsequently is hydrolyzed by dUTPase to generate dUMP and pyrophosphate (1). In contrast, Gram-positive bacteria and eukaryotic organisms synthesize dTTP from dUMP obtained by deamination of dCMP by the zinc-containing enzyme dCMP deaminase (2). Recently, a bifunctional enzyme from the archaean Methanocaldococcus jannaschii has been identified (3, 4) that possesses both the dCTP deaminase and dUTPase activities in one polypeptide suggesting that at least in some Archaea, dCTP serves as a source for dUMP. The structure of this archaean enzyme is now known and the subunit shares an overall fold with dUTPsases as well as the organization of subunits in a trimer (5). In the present work we demonstrate that dCTP deaminase from E. coli is yet another member of this family of enzymes, even though significant differences are found in the C-terminal stretch that closes the active site upon catalysis.

One very interesting feature of the dCTP deaminase is that the deamination reaction proceeds without aid from a metal cofactor. Other nucleoside or nucleotide deaminases such as cytosine deaminase (6, 7), cytidine deaminase (8), adenosine deaminase, (9) and adenine deaminase (10) all require a catalytic metal ion, Zn2+, Mn2+, Fe2+, which is either tightly bound in the active site or has to be added together with the substrate to the enzyme. Here, we report the identification of catalytically important amino acid residues of E. coli dCTP deaminase. Furthermore, we present the first structure of a monofunctional dCTP deaminase in a distinct complex with the reaction product dUTP and Mg2+ (dUTP-Mg2+) and also the structures of a mutant enzyme (E138A) in complex with bound substrate dCTP and Mg2+ (dCTP-Mg2+) as well as the same mutant enzyme in complex with bound substrate dCTP and Mg2+ (dCTP-Mg2+) as well as the same mutant enzyme in complex with bound substrate dCTP and Mg2+ (dCTP-Mg2+) as well as the same mutant enzyme in complex with bound substrate.

The main source of dUMP, the precursor for dTTP in the Gram-negative bacteria Escherichia coli and Salmonella typhi-

muri um, is obtained via a pathway where dCTP is deaminated by dCTP deaminase (EC 3.5.4.13) to yield ammonia and dUTP that subsequently is hydrolyzed by dUTPase to generate dUMP and pyrophosphate (1). In contrast, Gram-positive bacteria and eukaryotic organisms synthesize dTTP from dUMP obtained by deamination of dCMP by the zinc-containing enzyme dCMP deaminase (2). Recently, a bifunctional enzyme from the archaean Methanocaldococcus jannaschii has been identified (3, 4) that possesses both the dCTP deaminase and dUTPase activities in one polypeptide suggesting that at least in some Archaea, dCTP serves as a source for dUMP. The structure of this archaean enzyme is now known and the subunit shares an overall fold with dUTPsases as well as the organization of subunits in a trimer (5). In the present work we demonstrate that dCTP deaminase from E. coli is yet another member of this family of enzymes, even though significant differences are found in the C-terminal stretch that closes the active site upon catalysis.

One very interesting feature of the dCTP deaminase is that the deamination reaction proceeds without aid from a metal cofactor. Other nucleoside or nucleotide deaminases such as cytosine deaminase (6, 7), cytidine deaminase (8), adenosine deaminase, (9) and adenine deaminase (10) all require a catalytic metal ion, Zn2+, Mn2+, Fe2+, which is either tightly bound in the active site or has to be added together with the substrate to the enzyme. Here, we report the identification of catalytically important amino acid residues of E. coli dCTP deaminase. Furthermore, we present the first structure of a monofunctional dCTP deaminase in a distinct complex with the reaction product dUTP and Mg2+ (dUTP-Mg2+) and also the structures of a mutant enzyme (E138A) in complex with bound substrate dCTP and Mg2+ (dCTP-Mg2+) as well as the same mutant enzyme in complex with bound substrate dCTP and Mg2+ (dCTP-Mg2+) as well as the same mutant enzyme in complex with bound substrate.

EXPERIMENTAL PROCEDURES

Construction of an Overexpression Vector for the E. coli dcd Gene and Mutant Alleles—Manipulation of DNA and the preparation and transformation of CaCl2 competent E. coli cells was performed using stand-

This paper is available on line at http://www.jbc.org

Vol. 280, No. 4, Issue of January 28, pp. 3051–3059, 2005
Printed in U.S.A.
and procedures (11). The dcd (12) gene was obtained by PCR using the Pfu polymerase (Stratagene) according to the suppliers manual with chromosomal DNA from a E. coli K12 strain as a template and the deoxyoligonucleotides: DCD-5: GAAATTCATATGCCGCTGACCCGACACCAGC and DCD-3: GCCGGATCTTCATTTTACGCAGTGGC. Lettering in italics indicate the restriction sites incorporated by the primers in the PCR product; NdeI in DCD-5 and BamHI in DCD-3. The PCR product, comprising the reading frame of the dcd gene, and plasmid pET11a (Novagen) were digested with the restriction enzymes NdeI and BamHI, ligated, and the ligation mix was subsequently transformed into BL21(DE3) cells. This yielded a strain, SØ5352, harboring the plasmid pETDCD that allows for overexpression of the dcd gene upon induction with IPTG. Mutant alleles of the dcd gene encoding the R115A, E138A, and E138Q enzymes were constructed using the QuickChange method (Stratagene) with pETT11DCD as the template and the complementary deoxyoligonucleotides 5R115A, TCTCCTCAGGC-GCTCTGGGCGCTGATC; 3R115A, CATCAGCGCGAGACGCCCGGAGT-GAGGA; 5E138A, TGCCATTGTTGCGCCGGTCTCAACCTCC; 3E138A, GAGGTTTGTGACACCCGACAATGCA; SE138Q, TGCCATTGTTGCGCAATTCAACTCC and 3E138Q, GAGGTTTGTGACACCCGACAATGCA-CATGCA. The entire coding region for wild-type and mutant alleles of the dcd gene were verified by sequencing using an ABI PRISM 310 DNA Sequencer as recommended by the supplier (PerkinElmer Life Sciences).

Purification of E. coli Wild Type and Mutant dCTP Deaminases—An overnight culture of BL21(DE3) derivatives harboring a plasmid encoding either wild type (SØ5352) or mutant dCTP deaminases constructed as described above were grown in LB medium at 37 °C supplemented with ampicillin (100 μg ml \(^{-1}\)). These overnight cultures were then used to inoculate 300 ml of the same media as above, and growth was continued at 37 °C to an OD\(\text{600}=1\) when IPTG was added to a final concentration of 0.5 mM. After three hours of induction the cells were harvested by centrifugation in a Sorvall centrifuge at 10,000 rpm at 4 °C. The cell paste was suspended in 5 ml of the same buffer, and the cells were disrupted using a French Pressure Cell. Cell debris was sedimented by centrifugation in a Sorvall centrifuge using an SS34 rotor at 14,000 rpm for 20 min. A freshly prepared 10% (w/w) streptomycin solution was added to the extract to a final concentration of 1% (w/w). The precipitate was cleared by centrifugation as above, and the supernatant was dialyzed against several changes of washing buffer. The dialyzed protein was loaded on to a DEAE 52 column (35 ml) equilibrated with 50 mM potassium phosphate, pH 6.8, which was mounted on a Gradifrac (Amersham Biosciences). The column was eluted with a gradient from 0 to 0.4M KCl in 50 mM potassium phosphate, pH 6.8 of a total of 240 ml. The protein eluted at 0.05M KCl and fractions containing dCTP deaminase were diluted 3-fold with 50 mM potassium phosphate, pH 6.8, and a second run of the DEAE 52 column as above was performed. The fractions eluted from the column were pooled and made 60% saturated (40 g/100 ml) with ammonium sulfate. The precipitate was recovered by centrifugation as above and dissolved in 5 ml of 50 mM Hepes, 2 mM dithiothreitol, pH 6.8 and dialyzed against several changes of the same buffer. This protocol yielded 10–15 mg of dCTP deaminase that migrated as a single protein band in SDS-PAGE (13) of ~20 kD corresponding to the dCTP deaminase monomer with a purity of more than 95%. For crystallization the enzyme was stored at 4 °C whereas for enzymological studies it was stored at 37 °C in 50 mM Hepes, 2 mM dithiothreitol, pH 6.8. The analysis of initial velocity data were performed using Equation 1 for cooperative substrate, [S] represents the concentration of S for half-maximal velocity and n is the Hill coefficient.

Enzyme Kinetics and Recording of CD Spectra—Assays were conducted as described under “Experimental Procedures.” A, concentration of dCTP varied as indicated in the presence of potassium phosphate at fixed concentrations of 0.025 M (closed circles); 50 mM (open circles); 100 mM (closed squares), and 200 mM (open squares). Initial velocity data were fitted to Equation 1. B, plot of the dependence on the phosphate concentration on S\(_{0.5}\) and the corresponding Hill coefficient calculated for the initial velocity data in A.

The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; NCS, non-crystallographic symmetry; CD, circular dichroism.

\[ v = V[S]^n[S_{0.5}]^c + [S]^c \]
using the hanging drop vapor diffusion technique. Small, irregular crystals of the E138A inactive mutant enzyme cocrySTALLlized with dCTP and Mg$^{2+}$ were obtained in both polyethylene glycol and salt conditions in the solubility screen and large crystals with sharp edges appeared in crystal screen I condition 23 (30% (v/v) polyethylene glycol 400, 0.2 M magnesium chloride, 0.1 M Hepes, pH 7.5). The crystals used for structure determinations were grown at room temperature with a hanging drop of 5–8 mg/ml enzyme, 5 mM nucleotide (dCTP or dUTP, 3053) were collected under cryogenic conditions (100 K) at beamline ID11 (Diamond Light Source). Diffraction data for the E138A crystals were recorded to 1.8 Å resolution. Good quality data sets were obtained in both polyethylene glycol and salt conditions were used for structure determination. Since the crystals were nonmerohedrally twinned which made auto indexing of the data nontrivial and as consequence the quality of the sums are over all reflections in the working set and test set, respectively.

**Enzyme:ligand**

| Enzyme/ligand | E138A/ScMet: dCTP-Mg$^{2+}$ | E138A: dUTP-Mg$^{2+}$ |
|---------------|-----------------------------|------------------------|
| **Wild type:** dUTP-Mg$^{2+}$ | 1.087 | 0.969 | 1.542 |
| **Resolution (Å)** | 25–1.80 | 30–2.53 | 25–2.00 |
| **R_{free} (%)** | (1.84–1.80)$^a$ | (2.59–2.53) | (2.05–2.00) |
| **Space group** | P2$_2$(twinned)$^f$ | P2$_2$ | C2$^2$ |
| **Cell dimensions** | a = 63.0, b = 97.5, c = 95.5 | a = 63.3, b = 97.9, c = 95.1 | a = 179.9, b = 63.1, c = 96.4 |
| **Number of reflections** | 335,111 | 137,363 | 282,767 |
| **Number of unique reflections** | 100,503 | 37,005 | 71,843 |
| **Refinement statistics** | | | |
| **Number of reflections (total)** | 90,724 | 36,399 | 66,024 |
| **Number of reflections (working set)** | 85,935 | 34,581 | 62,497 |
| **Number of reflections (test set)** | 4,789 | 1,818 | 3,527 |
| **Number of unique reflections** | 71,843 | 37,005 | 71,843 |
| **Number of atoms** | 9,621 | 9,355 | 9,518 |
| **Average Bfactor water (Å$^2$)** | 28.0 | 21.9 | 25.8 |
| **Bond angle rmsd from ideal (Å)** | 15.5 | 15.5 | 14.9 |
| **Bond length rmsd from ideal (Å)** | 0.007 | 0.020 | 0.021 |
| **Bond angle rmsd from ideal (Å)** | 0.021 | 1.66 | 1.98 |

**Values in parentheses are data for the highest resolution shell.**

**Twinning operator (h,k,l) → (h,k,-h,l), twinning fraction 0.47.**

**R_{free} = \Sigma F_{calc} \cdot I \sigma(F_{calc}) / \Sigma F_{calc}, where F_{calc} and F_{obs} are observed and calculated structure factors, respectively, k is the scale factor, and the sums are over all reflections in the working set and test set, respectively.**

**R_{free} value did not reach below 28%. Closer examination** of the cumulative intensity distribution of the diffraction data suggested presence of merohedral twinning. Therefore a twinning operator (h,k,l) → (h,k,-h,l) was introduced and refinement was recaptured using SHELXL (27) resulting in considerably improved refinement statistics. The crystals of E138A in complex with dUTP crystallized in another space group (C2), and the structure was determined using the three-dimensional structure of the E. coli dCTP deaminase in complex with dCTP-Mg$^{2+}$ was determined by a combination of molecular replacement and single wavelength anomalous dispersion methods. First, molecular replacement as implemented in AMoRe (19) was applied using an trimmed polyalanine trimer of the bifunctional dCTP deaminase-dUTPase from M. jannaschii (Protein Data Bank code 1OGH; chain A, residues 1–45 and 86–164) as search model. Diffraction data in the 15–4.5 Å range from a crystal of E138A mutant enzyme not including selenomethionine protein were used (data not shown), and all translational searches were performed using the centered correlation function (20). A solution with high contrast of the correlation coefficient and R-value placed two trimers in the asymmetric unit. Introduction of correct amino acid side chains, refinement using REFMAC5 (21), and rounds of prime and switch in RESOLVE (22) utilizing the 6-fold NCS of the crystal did not improve the electron density maps significantly and large parts (~35%) were missing. An anomalous difference electron density map with phases from the present model and diffraction data from the selenomethionine substituted protein revealed the positions of 24 selenium sites, four for each protein chain. The selenium sites were refined with SHARP (23), DM (24) was subsequently used for density modification and 166 of the 193 amino acids in one of the six protein chains could be automatically traced by ARP-wARP (25). The remaining residues were manually built using O (26) and the five other protein chains were generated by NCS. After one cycle of positional refinement with REFMAC5 (21), dCTP, and a magnesium ion could be introduced in each of the active sites. Cycles of refinement with REFMAC5 using NCS restraints, manual rebuilding in O and water picking with ARP-wARP were performed. The structure of the wild-type enzyme in complex with dUTP-Mg$^{2+}$ was determined using the first model, free from water molecules as well as dCTP and Mg$^{2+}$, as starting point since the crystals both belonged to the same crystal form. After rigid body refinement allowing the six protein chains in the asymmetric unit to move separately, clear electron density was seen for dUTP, Mg$^{2+}$, and the side chain of residue Glu138 and these were model built in O. Further rounds of refinement in REFMAC5, rebuilding in O and water picking using ARP-wARP were performed. Despite electron density maps of high quality, the R_{free} value did not reach below 28%. Closer examination of the cumulative intensity distribution of the diffraction data suggested presence of merohedral twinning. Therefore a twinning operator (h,k,l) → (h,k,-h,l) was introduced and refinement was recaptured using SHELXL (27) resulting in considerably improved refinement statistics. The crystals of E138A in complex with dUTP crystallized in another space group (C2), and the structure was determined using the trimer of the wild-type product complex free from ligands as search model resulting in one clear molecular replacement solution with the
RESULTS AND DISCUSSION

Inhibition of dCTP Deaminase by Inorganic Phosphate—A previous enzyme kinetic analysis of the dCTP deaminase from *S. typhimurium* revealed positive cooperativity for the binding of dCTP with Hill coefficients close to a value of 2 (30). However, during our studies of the enzyme from *E. coli* we discovered that this cooperativity was caused by the presence of phosphate in the assay buffer. Omission of phosphate in the assay incubations gave nearly hyperbolic saturation curves for dCTP binding to *E. coli* dCTP deaminase (Fig. 1). From Fig. 1A it can be seen that the inhibition by inorganic phosphate of the dCTP deaminase activity does not affect the maximal velocity of the enzyme, but only has an effect on the half-saturation with dCTP \( (S_{0.5}) \) and the cooperativity of dCTP binding. Apparently, phosphate is a competitive inhibitor of dCTP binding but at the same time phosphate induces an increase in cooperativity of dCTP binding. Fig. 1B shows a linear correlation between increasing fixed concentrations of phosphate present in the incubation and a resulting increase in \( S_{0.5} \) for dCTP, as well as concomitant increases in the Hill coefficient for dCTP binding. The specific mechanism by which phosphate acts on dCTP deaminase is not yet understood.

Analysis of Mutant dCTP Deaminases—None of the mutant dCTP deaminases prepared displayed any detectable activity (less than 1000-fold the wild-type activity) indicating that both side chains Arg134 and Glu138 are obligatory for the deamination reaction. As it will be discussed below an important function in catalysis of both residues can be deduced from the structure of the enzyme. Glu138 corresponds to Glu145 in the bifunctional Archaean enzyme for which it was also shown that the E145Q enzyme had lost the deaminase activity (3). The structural integrity of the mutant enzymes was verified by comparing the CD spectra of wild-type and mutant enzymes. The CD spectra of wild-type enzyme and the R115A and E138A enzymes were virtually superimposable (data not shown).

Crystal Structure Determination—The structure of the mutant enzyme E138A in complex with dCTP-Mg\(^{2+}\) was determined by a combination of molecular replacement, using the bifunctional dCTP deaminase-dUTPase from *M. jannaschii* as search model, and single wavelength anomalous dispersion using selenomethionine-substituted enzyme. The experimental phases obtained from the single wavelength anomalous dispersion experiment gave a high quality electron density map sufficient for automatic model building of a large part of the final model. The structure of the wild-type enzyme in complex with dUTP-Mg\(^{2+}\) was determined by difference Fourier methods, and the structure of the mutant enzyme E138A in complex with dUTP-Mg\(^{2+}\) was determined using the molecular replacement method. The statistics of the diffraction data, and the refinement for the three structures are summarized in Table I.

The subunit of *E. coli* dCTP deaminase is composed of 193 amino acid residues with a molecular mass of 21.2 kDa. The asymmetric unit in the crystals contains six subunits (chains A–F) that form two independent homotramers (chains A–C and D–E, respectively), and has clear electron density for all amino acid residues. The Ca atoms of the subunits may be superimposed with root mean square deviation values ranging from 0.128 Å (chains A and E, E138A-dCTP-Mg\(^{2+}\)) to 0.296 Å (chains A and C, E138A-dCTP-Mg\(^{2+}\)) using default parameters in the program O (26). In the Ramachandran plots there are no non-glycine residues in disfavored regions except for residues Asn20 and Pro21, which form a cis-peptide bond. In the three structures, every active site (three per trimer) binds a well defined dCTP-Mg\(^{2+}\) or dUTP-Mg\(^{2+}\) complex.

Overall Fold—The dCTP deaminase subunit is composed of 14 \( \beta \)-strands (\( \beta1–14 \)), three \( \alpha \)-helices (\( \alpha1–3 \)), and four \( \beta \)-strands forming two \( \beta \)-sheets: S1 (\( \beta1, \beta8, \beta12, and \beta10 \)), S2 (\( \beta2, \beta3, \beta14, \beta9, and \beta11 \)), and S3 (\( \beta4 and \beta6 \)), and one mixed \( \beta \)-sheet, S4 (\( \beta5 and \beta13 \) from one subunit of the homotrimer and \( \beta7 \) from another subunit), illustrated with different shades of blue in Fig. 2A. S2 pack on top of S1 in this manner a distorted \( \beta \)-barrel is created. The subunits assemble, forming a compact homotrimer related by a 3-fold rotational NCS with one nucleotide-Mg\(^{2+}\) complex bound between pairs of subunits, resulting in three active sites per trimer as illustrated in Fig. 2B. The homotrimer has an equilateral (\( \sim 55 \) Å long side) triangular face perpendicular to the 3-fold axis. The height along the 3-fold axis is \( \sim 45 \) Å. An analysis of the interactions of two of the subunits (chains A and C, E138A-dCTP-Mg\(^{2+}\)) in the trimer with the Protein-Protein Interaction Server (31) gives a value of 2,006 Å\(^2\) for the interface accessible surface area with residues from eight different segments of chain A and five segments from chain C. Of the residues in the surface, 64% are non-polar and 36% polar.

Active Site Interactions with the Nucleotide-Mg\(^{2+}\) Complex—The wild-type dCTP deaminase from *E. coli* was co-crystallized with the reaction product, dUTP, and the mutant enzyme E138A was co-crystallized with both the substrate, dCTP, and the product, dUTP. In all three crystal structures a magnesium ion is octahedrally coordinated to the \( \alpha \)-, \( \beta \)-, and
γ-phosphate of the nucleotide and to three water molecules. At the base of the formed bipyramid, O2B and O3G from the nucleotide are found next to each other, and in one of the pyramidal tips O1A from the nucleotide is positioned. The other positions are filled with water molecules. Even though the magnesium ion in dCTP deaminase plays no catalytic role, the observation that dCTP is bound in complex with Mg$^{2+}$ is in agreement with kinetic studies showing that the dCTP-Mg$^{2+}$ complex is the true substrate (30). There is clear electron density for the nucleotide and the magnesium ion in all active sites.

FIG. 3. Views of the active site in E. coli dCTP deaminase. A, close up stereo and B, schematic view of the wild-type enzyme in complex with dUTP. C, close up and D, schematic view of the E138A mutant enzyme in complex with dCTP. E, close up and F, schematic view of the E138A mutant enzyme in complex with dUTP. Hydrogen bonds shorter than 3.4 Å are shown as broken lines. The displayed 2Fo − Fc difference electron density maps are contoured at 1.5 σ level at a distance of 1.2 Å around the nucleotide and the magnesium ion. Residues from different subunits are colored in yellow or displayed in plain face letters for chain A and colored dark blue or displayed in boldface letters for chain C. The A, C, and E panels were prepared with MOLESCRIPT (41) and Raster3D (42).
sites of the three structures described in this work. A close up on the nucleotides and Mg\(^{2+}\) binding between two subunits in the trimer (chains A and C) for the three determined structures are shown in Fig. 3. Fig. 3B gives a schematic representation of the residues forming hydrogen bonds to the respective nucleotides and the Mg\(^{2+}\) ion. The magnesium ion clearly serves to shield the negative charge of the phosphates of the nucleotide so that the C-terminal fold can close over the active site nucleotide-Mg\(^{2+}\) complex (Fig. 4). Interactions with the triphosphate moiety of the nucleotide are mediated by Arg\(^{126}\) and Ser\(^{111}\) (α-phosphate), Ser\(^{112}\), and Arg\(^{110}\) (β-phosphate) and from the C-terminal fold, Lys\(^{178}\) and Tyr\(^{171}\) (γ-phosphate).

Trp\(^{131}\) is stacked with the deoxycytosine ring in an equivalent way to the tyrosine or phenylalanine residues found in dUTPase structures (32–35) and the bifunctional dCTP deaminase-dUTPase from \(M. \ jannaschii\) (5, 36). The enzyme’s discrimination against CTP appears to be a result of the large, hydrophobic side chain of Trp\(^{131}\) that leaves no room for a hydroxyl group at position 2 on the ribose ring. The side chain of Ile\(^{35}\) makes hydrophobic interactions with the substrate, stacking with the pyrimidine ring. The corresponding residue in a dCTP deaminase amino acid sequence alignment (5) is always a hydrophilic residue with matching stacking properties (Ile, Val, Leu, or Trp). O\(_2\) of dCTP and dUTP (Fig. 3, B, D, and F) forms hydrogen bonds to Gln\(^{132}\) and the backbone amino group of Val\(^{136}\) as well as to the invariant Arg\(^{115}\) from the other subunit. The hydrogen bond network, including hydrogen bonds between Arg\(^{115}\) and Gln\(^{132}\), locks the two subunits together on one side of the pyrimidine ring (Fig. 3A). The backbone carbonyl of Val\(^{136}\) forms a hydrogen bond to N3 and in dCTP, also to N4 (Fig. 3D). OD2 from the carboxylate of Asp\(^{128}\) and the backbone amino group of the same residue both form hydrogen bonds to O\(_\gamma^1\) of the deoxyribose ring.

Structural Similarity—The structure of \(E. \ coli\) dCTP deaminase show the greatest similarity to the previously determined structure of the bifunctional dCTP deaminase-dUTPase from \(M. \ jannaschii\) (5, 36), as demonstrated by the possibility to use parts of this structure as a search model and obtaining a correct molecular replacement solution. One subunit of \(E. \ coli\) dCTP deaminase (chain A, E138A-dCTP-Mg\(^{2+}\)) and the bifunctional dCTP deaminase-dUTPase from \(M. \ jannaschii\) (PDB code 1OGH; chain A) superimpose with a root mean square deviation of 1.30 Å for 140 Ca atoms as determined using default parameters in the program O (26) (Fig. 5A). The amino acid sequence identity between the superimposed residues is 31% (Fig. 5B). The central distorted β-barrel is conserved between the structures and the main differences are additional secondary structure elements (γ2, β3, and α2) in \(E. \ coli\) dCTP deaminase, while there is an α-helix and a β-strand in the bifunctional enzyme (residues 71–93), which are not present in the \(E. \ coli\) enzyme (Fig. 5, A and B).

Like the bifunctional enzyme, dCTP deaminase is also similar to homotrimeric dUTPases (Fig. 5, A and B) (5). 98 Ca atoms from \(E. \ coli\) dUTPase (PDB code 1UDU) superimpose, as above, with chain A from \(E. \ coli\) dCTP deaminase (E138A-dCTP-Mg\(^{2+}\)) with a root mean square deviation of 1.80 Å (21% identity). The primary structure of \(E. \ coli\) dUTPase is 40 amino acids shorter than the dCTP deaminase and as a result there is no correspondence in the dUTPase to β4–6, γ2, and α2. The N terminus shows a different fold in the dCTP deaminase compared with dUTPases.

The C-terminal Fold—The 23 C-terminal residues of the bifunctional dCTP deaminase-dUTPase from \(M. \ jannaschii\) are not present in any of the published crystal structures (5, 36) and therefore this part of the structures cannot be compared. The C terminus of one subunit in trimeric dUTPases has on the other hand been shown to interact with the active site in the cleft between two other subunits, resulting in each active site being built from residues contributed by all three subunits (33, 34, 37, 38). This is apparently not the case for \(E. \ coli\) dCTP deaminase, where only two subunits of the trimer contribute to the active site. It is evident from our results that the C-terminal arm in dCTP deaminase folds back on the same subunit from which it is derived and serves to close the active site (Fig. 4). Thereby the C terminus provides interactions with the γ-phosphate of dCTP via Lys\(^{178}\) and Tyr\(^{171}\) and to the O2 of the base moiety via Gln\(^{132}\) (Fig. 3, B, D, and F). Furthermore, the side chain of the C-terminal residue Asp\(^{193}\) forms a salt bridge with Arg\(^{110}\) of the other subunit (Fig. 3A). Since the primary structure of the C-terminal arm of the bifunctional enzyme has higher sequence identity with the dCTP deaminase than with the dUTPases, the dCTP deaminase and the bifunctional enzyme are likely to have a similar structural arrangement of the C terminus. Thus a slightly different arrangement of the active site of the bifunctional enzyme with respect to dUTPase activity can be expected as residues obligatory to dUTP hydrolysis, but not binding of substrate have been identified to reside in the C-terminal arm of dUTPase (38).

Determinants of dUTPase Activity for the Mono- and Bifunctional dCTP Deaminase Family Members—From the recent structures of the \(M. \ tuberculosis\) dUTPase (35) and \(E. \ coli\) dUTPase in complex with dUTP and analogs hereof, a detailed understanding of the role in catalysis of individual conserved amino acid residues is provided. The conformation of dUTP and the magnesium ion coordination is almost identical in the dCTP deaminase and these dUTPase structures. The comparison of \(E. \ coli\) dCTP deaminase and \(M. \ tuberculosis\) dUTPase shows a completely conserved aspartate residue (Asp\(^{129}\) and Asp\(^{85}\), respectively) that in both enzymes interact with the 3′-OH of the bound nucleotide. This residue (Asp\(^{85}\)) also prepares a water molecule for nucleophilic attack on the α-phosphate of dUTP in the \(M. \ tuberculosis\) dUTPase and other dUTPases (Asp\(^{90}\) in the \(E. \ coli\) dUTPase, Fig. 5B). A structural comparison identifies Arg\(^{126}\) as a residue that is crucial for the monofunctionality of the \(E. \ coli\) dCTP deaminase, as a residue of smaller size is found in the equivalent position in the dUTPases. Arg\(^{126}\) occupies the position of the nucleophilic water molecule in dUTPase and forms a salt bridge with Asp\(^{128}\) (Fig. 5C). This exclusion of a potential nucleophilic water molecule is likely to prevent the hydrolysis of the phosphate chain of the bound nucleotide. The bifunctional enzyme from \(M. \ jannaschii\) has a tryptophan residue (Trp\(^{135}\)) replacing Arg\(^{126}\) (Fig. 5B) but this large side chain is in a different conformation turned 180 degrees compared with Arg\(^{126}\). As a
result this tryptophan side chain occupies the same space as Trp106 in the E. coli dCTP deaminase (Fig. 5C).

Catalytic Mechanism—We suggest a mechanism of the reaction catalyzed by dCTP deaminase based on the new structural information (Fig. 6A). Two important interactions necessary for orienting the pyrimidine ring upon binding and anchoring it to the active site during catalysis are the hydrogen bonds between the backbone amino group of Val136 and the Gln182 side chain.
Arg$^{115}$ and both these amino acid residues are invariant among dCTP deaminase amino acid sequences. In the suggested mechanism A (Fig. 6A) the water molecule (S5) is activated by Glu$^{138}$. A tetrahedral reaction intermediate is formed and ammonia is expelled upon extraction of a proton from a second water molecule (S251), which is bifurcatedly coordinated to Glu$^{138}$ and to the carbonyl group of Val$^{136}$. The formed hydroxide ion is thereafter neutralized and Glu$^{138}$ returns to its negatively charged starting position. dUTP can leave the active site and a new dCTP and water molecule may enter. The proposed mechanism A is analogous to previously suggested mechanisms of zinc containing enzymes (40) in that no tautomerization of the pyrimidine takes place. In a previous reaction model (Fig. 6A, mechanism B) suggested initially for the bifunctional dCTP deaminase-dUTPase (36) based on modeling of nucleotides into the active site, it was also proposed that the invariant Glu$^{138}$ (Glu$^{145}$ in M. jannaschii dCTP deaminase-dUTPase) is involved in activation of the nucleophile. Furthermore, tautomerization of the pyrimidine ring was suggested. The distance to Arg$^{115}$ from this oxygen atom is 3.3 Å in the product complex, and hence this arginine residue could help stabilize the negatively charged O$_2$ formed upon tautomerization. Glu$^{145}$ of the bifunctional enzyme was proposed to donate the second proton directly. This we do not find plausible, since the distance from Glu$^{138}$ to O$_4$ is much longer (~4.6 Å) than from Ser$^{251}$ (2.4 Å) in the product complex. Therefore, for the alternative mechanism B water molecule (Ser$^{251}$) most likely donates this second proton to the ammonia-leaving group.

The ammonia molecule that is expelled during the reaction may be harbored in a hydrophobic pocket shielded with the side chains of residues Leu$^{167}$, Ile$^{127}$, and Leu$^{158}$. These amino acid residues are situated on the opposite side of the pyrimidine ring to the attacking water molecule (S5). The mechanism of product release is not obvious, but it must involve opening of the lid assembled of the 20 C-terminal residues that closes over the substrate/product in the active site.

Acknowledgments—We thank Anders Kadziola and Claus Flensburg for help with structure determination, Henning O. Sørensen for discussions regarding twinning and Birthe Kragelund for help with recording the CD spectra. We acknowledge assistance and access to synchrotron radiation at beamline 1711, MAX-laboratory, Lund, Sweden and travel expense support from the EC Access to Research Infrastructure (ARI) program and DANSYNC.

REFERENCES

1. Neuhard, J., and Nygaard, P. (1987) in Escherichia coli and Salmonella typhimurium. Cellular and Molecular Biology. Vol. 1: Purines and Pyrimidines (Neidhart, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaeter, M., and Umbarger, H. E., eds) pp. 445–473, ASM, Washington, D. C.
2. Weiner, K. X., Weiner, R. S., Maley, F., and Maley, G. F. (1993) J. Biol. Chem. 268, 12983–12989
3. Li, H., Xu, H., Graham, D. E., and White, R. H. (2003) J. Biol. Chem. 278, 11100–11106
4. Bjørnberg, O., Neuhard, J., and Nyman, P. O. (2003) J. Biol. Chem. 278, 20667–20672
5. Johansson, E., Bjørnberg, O., Nyman, P. O., and Larsen, S. (2003) J. Biol. Chem. 278, 27916–27922
6. Porter, D. J., and Austin, E. A. (1993) J. Biol. Chem. 268, 24005–24011
7. Ko, T. P., Lin, J. J., Hu, C. Y., Hsu, Y. H., Wang, A. H., and Liaw, S. H. (2003) J. Biol. Chem. 278, 19111–19117
8. Yang, Y., Carlow, D., Wolfenden, R., and Short, S. A. (1992) Biochemistry 31, 4168–4174
9. Cooper, B. F., Sideraki, V., Wilson, D. K., Dominguez, D. Y., Clark, S. W., Quischo, F. A., and Rudolph, F. B. (1997) Protein Sci. 6, 1031–1037
10. Nygaard, P., Duckert, P., and Saxild, H. H. (1996) J. Bacteriol. 178, 846–853
11. Sambrook, J., Fitzch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
12. Wang, L., and Weiss, B. (1992) J. Bacteriol. 174, 5647–5653
13. Laemmli, U. K. (1970) Nature 227, 680–685
14. Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L., and Claridy, J. (1993) J. Mol. Biol. 229, 105–124
15. Stura, E. A., Nemerow, G. R., and Wilson, I. A. (1992) J. Crystallogr. Growth 111, 20667–20672
16. Jancarik, J., and Kim, S. H. (1991) J. Mol. Biol. 226, 273–285
17. Janecek, J., and Kim, S. H. (1991) J. Appl. Crystallogr. 24, 409–411
18. Cerenius, Y., Stahl, K., Svensson, L. A., Urbye, T., Oskarsson, A., Albertsson, J., and Liljas, A. (2000) J. Synchrotron Rad. 7, 203–208
19. Owininwob, Z., and Wilson, W. (1997) Methods Enzymol. 276, 307–326

FIG. 6. Proposed catalytic mechanism for dCTP deaminase. A, two alternative mechanisms are labeled A and B for identification (see text). B, stereo view of the active site of the wild-type enzyme in complex with dUTP with the two water molecules implied in the mechanisms. Only the pyrimidine ring of dUTP is shown for clarity. Panel B was prepared with MOLSCRIPT (41) and Raster3D (42).
19. Navaza, J. (1994) *Acta Crystallogr. Sect. A* 50, 157–163
20. Navaza, J., and Vernoslova, E. (1995) *Acta Crystallogr. Sect. A* 51, 445–449
21. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) *Acta Crystallogr. Sect. D.* 53, 240–255
22. Terwilliger, T. C. (2003) *Acta Crystallogr. Sect. D.* 59, 45–49
23. Bricogne, G., Vonrhein, C., Flensburg, C., Schiltz, M., and Paciorek, W. (2003) *Acta Crystallogr. Sect. D.* 59, 2023–2030
24. Cowtan, K. (1994) *Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography* 31, 34–38
25. Lamzin, V. S., Perrakis, A., and Wilson, K. S. (2001) in *International Tables for Crystallography. Vol. F: Crystallography of Biological Macromolecules* (Rossman, M. G., and Arnols, E. eds) pp. 720–722, Kluwer Academic Publishers, Dordrecht, Netherlands
26. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. Sect. A* 47, 110–119
27. Sheldrick, G. M. (1997) *SHELXL97*, University of Göttingen, Germany
28. Laskowski, R. A., Gwinn, D. M., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr.* 26, 283–291
29. Hoelli, R. W., Friend, G., Sander, C., and Abola, E. E. (1996) *Nature* 381, 272
30. Neuwald, J. F. (1998) *Methods Enzymol.* 31, 418–423
31. Jones, S., and Thornton, J. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 13–20
32. Larsson, G., Svensson, L. A., and Nyman, P. O. (1996) *Nat. Struct. Biol.* 3, 532–538
33. Prasad, G. S., Stura, E. A., Elder, J. H., and Stout, C. D. (2000) *Acta Crystallogr. D. Biol. Crystallogr.* 56, 1190–1199
34. Mol, C. D., Harris, J. M., McIntosh, E. M., and Tainer, J. A. (1996) *Structure* 4, 1077–1092
35. Chan, S., Segelke, B., Lekin, T., Krupka, H., Cho, U. S., Kim, M. Y., So, M., Kim, C. Y., Naranjo, C. M., Rogers, Y. C., Park, M. S., Waldo, G. S., Pashkov, I., Cacchi, D., Perry, J. L., and Sawaya, M. R. (2004) *J. Mol. Biol.* 341, 503–517
36. Huffman, J. L., Li, H., White, R. H., and Tainer, J. A. (2003) *J. Mol. Biol.* 331, 885–896
37. Nord, J., Nyman, P., Larsson, G., and Drakenberg, T. (2001) *FEBS Lett.* 492, 228–232
38. Nord, J., Kiefer, M., Adolph, H. W., Zeppezauer, M. M., and Nyman, P. O. (2000) *FEBS Lett.* 472, 312–316
39. Barabas, O., Pongracz, V., Kovari, J., Wilmans, M., and Vertessy, B. G. (2004) *J. Biol. Chem.* 279, 42907–42915
40. Betts, L., Xiang, S., Short, S. A., Woffenden, R., and Carter, C. W., Jr. (1994) *J. Mol. Biol.* 235, 655–656
41. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* 24, 946–950
42. Merritt, E. A., and Bacon, D. J. (1997) *Methods Enzymol.* 277, 505–524
43. Huang, C. C., Couch, G. S., Petersen, E. F., and Ferrin, T. E. (1996) *Pacific Symp. Bioinform.* 1, 724
Structures of dCTP Deaminase from *Escherichia coli* with Bound Substrate and Product: REACTION MECHANISM AND DETERMINANTS OF MONO- AND BIFUNCTIONALITY FOR A FAMILY OF ENZYMES

Eva Johansson, Mathias Fanø, Julie H. Bynck, Jan Neuhard, Sine Larsen, Bent W. Sigurskjold, Ulla Christensen and Martin Willemoës

*J. Biol. Chem.* 2005, 280:3051-3059.
doi: 10.1074/jbc.M409534200 originally published online November 10, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409534200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 10 of which can be accessed free at http://www.jbc.org/content/280/4/3051.full.html#ref-list-1