Available online at www.sciencedirect.com
ScienceDirect
Archives of Biochemistry and Biophysics 470 (2008) 20–26
ABB
www.elsevier.com/locate/yabbi

Mutational analysis of the nucleotide binding site of *Escherichia coli* dCTP deaminase

Majbritt Thymark [a], Eva Johansson [a,b,1], Sine Larsen [a,b], Martin Willemoës [c,*]

[a] *Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark*
[b] *European Synchrotron Radiation Facility, BP220 F38043 Grenoble Cedex, France*
[c] *Department of Molecular Biology, University of Copenhagen, Ole Maaløes vej 5, DK-2200 Copenhagen N, Denmark*

Received 4 September 2007, and in revised form 12 October 2007
Available online 30 October 2007

Abstract

In *Escherichia coli* and *Salmonella typhimurium* about 80% of the dUMP used for dTMP synthesis is derived from deamination of dCTP. The dCTP deaminase produces dUTP that subsequently is hydrolyzed by dUTPase to dUMP and diphosphate. The dCTP deaminase is regulated by dTTP that inhibits the enzyme by binding to the active site and induces an inactive conformation of the trimeric enzyme. We have analyzed the role of residues previously suggested to play a role in catalysis. The mutant enzymes R115Q, S111C, S111T and E138D were all purified and analyzed for activity. Only S111T and E138D displayed detectable activity with a 30- and 140-fold reduction in k_{cat}, respectively. Furthermore, S111T and E138D both showed altered dTTP inhibition compared to wild-type enzyme. S111T was almost insensitive to the presence of dTTP. With the E138D enzyme the dTTP dependent increase in cooperativity of dCTP saturation was absent, although the dTTP inhibition itself was still cooperative. Modeling of the active site of the S111T enzyme indicated that this enzyme is restricted in forming the inactive dTTP binding conformer due to steric hindrance by the additional methyl group in threonine. The crystal structure of E138D in complex with dUTP showed a hydrogen bonding network in the active site similar to wild-type enzyme. However, changes in the hydrogen bond lengths between the carboxylate and a catalytic water molecule as well as a slightly different orientation of the pyrimidine ring of the bound nucleotide may provide an explanation for the reduced activity.

Keywords: Deoxynucleotide metabolism; Deamination; dUTP; Enzyme regulation; Catalytic mechanism

The dUDP supplied by the ribonucleotide reductase constitutes only a minor source of dUMP required for dTMP synthesis in both eukaryotes and prokaryotes. In both types of organisms the main supply of dUMP appears to come from the deamination of a cytosine deoxyribonucleotide [1–3]. In eukaryotes and most Gram-positive bacteria the deamination of dCMP is catalyzed by dCMP deaminase, an enzyme allosterically regulated by dCTP (activator) and dTTP (inhibitor) [4–6]. In other prokaryotes, dCTP deaminase catalyses the conversion of dCTP to dUTP [7,8], which is subsequently hydrolyzed to dUMP and diphosphate by dUTPase, or as shown in archaea, dUMP may be formed directly by the bifunctional dCTP deaminase:dUTPase [9,10]. The dCTP deaminase, the trimeric dUTPase and the bifunctional dCTP deaminase:dUTPase constitute a family of structurally closely related enzymes [11]. They all form homotrimers where the core structure of the subunits may be superimposed and the bifunctional enzyme shares active site residues with both of the monofunctional family members. However, only the dCTP deaminase and the bifunctional enzyme are subjected to regulation by dTTP [8,9,12].

We have previously published the crystal structure of the dCTP deaminase from *Escherichia coli* and a mutant enzyme, E138A, in complex with dUTP and dCTP [11]. Recently, we have also shown that dTTP inhibits the enzyme by binding to the active site but also by stabilizing

* Corresponding author.
E-mail address: willemoes@mermaid.molbio.ku.dk (M. Willemoës).
[1] Present address: Diabetes Protein Engineering, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv, Denmark.

0003-9861/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2007.10.013

an inactive form of dCTP deaminase through a concerted mechanism that coordinates the conformation of all three active sites in the trimer [12].

In this work we have addressed the role of selected active site residues in dCTP deaminase by mutational analysis, residues that we have previously suggested to play a role in catalyzing the deamination of dCTP [11]. The dCTP deaminase active site is quite unique in that no metal ion participates in the reaction to activate the hydrolyzing water molecule in contrast to the dCMP- [2], cytidine- [13,14] and cytosine-deaminase [15,16] that are all structurally closely related [17]. In the active site of dCTP deaminase a glutamate residue, Glu138, serves as the catalytic acid/base. The change of Glu138 to an alanine residue renders the enzyme inactive, but the binding of nucleotide (dCTP or dUTP) is very similar to what is observed for the wild-type enzyme. Therefore, the E138A mutant enzyme has served as a model for structural studies of protein–ligand interactions [11].

The side chains of Ser111 and Arg115 in the active site of *E. coli* dCTP deaminase interact via hydrogen bonds. These two amino acid residues are together with the main chain carbonyl of Ala124 suggested to stabilize the hydroxide formed by extraction of a proton from the catalytic water molecule by Glu138 [11]. Here we report an analysis of the mutant enzymes S111C, S111T, R115Q and E138D that aimed to establish the role of these residues in the active site of dCTP deaminase by investigating the structural and reaction kinetic effects of these side chain replacements. Interestingly, this analysis also provided additional evidence for the recently suggested mechanism of dTTP inhibition [12].

Experimental procedures

Materials

Unless otherwise is stated, all buffers, nucleotides and salts were obtained from Sigma–Aldrich. Radioactive nucleotides were obtained as ammonium salts from Amersham Biosciences. Thin-layer chromatography was performed with poly-ethylene-imine coated cellulose plates from Merck.

Molecular biology and protein methods

The alleles of the *dcd* gene encoding the mutant *E. coli* dCTP deaminases E138D, S111T, S111C and R115Q, were constructed using the QuikChange method (Stratagene) and the primers: E138D5-3, 5′-ATTG TGCTGGA*C*TTCTACAAC-3′; E138D3-5, 5′-GTTGTAGAA*G*TCCA GCACAAT-3′; S111T5-3, 5′-GACGGGCGT*A*CCTCACTGGCG-3′; S111T3-5, 5′-CGCCAGTGAGG*T*ACGCCCGTC-3′; S111C5-3, 5′-GAC GGGCGTT*G*CTCACTGGCG-3′; S111C3-5, 5′-CGCCAGTGAG*C*AA CGCCCGTC-3′; R115Q5-3, 5′-TCACTGGCGC*AG*CTGGGGCTG-3′; R115Q3-5, 5′-CAGCCCCAG*CT*GCGCCAGTGA-3′, where letters in italics indicate the resultant base changes. The previously described plasmid pETDCD [11] was used as a template for the mutagenesis reaction. pETDCD contains the reading frame of the *E. coli dcd* gene under control of the late T7 promoter in the vector pET11a (Novagen). All mutations were verified by sequencing of the entire *dcd* open reading frame on an ABI PRISM 310 sequencer according to the supplier's manual. The wild-type and mutant proteins were produced and purified as previously described [11].

Enzyme kinetics and equilibrium ligand binding

Initial velocities were recorded at 37 °C as described in detail previously [9,12]. Thin-layer chromatography and subsequent liquid scintillation counting was used to first separate and thereafter quantify [5-^{3}H] dUTP produced from [5-^{3}H] dCTP. Assays were performed over a time period of 5 minutes at two enzyme concentrations. In addition to varying concentrations of the nucleotides dCTP and dTTP as shown under results, the incubations contained in 50 mM Hepes, pH 6.8, 2 mM $MgCl_2$ and 2 mM DTT.

Incubations for equilibrium binding experiments contained 50–100 μM protein, 50 mM Hepes, pH 6.8, 2 mM $MgCl_2$ and between 0 and 320 μM of [methyl-^{3}H] dTTP or [5-^{3}H] dCTP. Free nucleotide was separated from bound using Amicon Ultrafree-MC 30.000 NMWL centrifugal filter devices as described previously [12,18,19]. Samples representing free and total radioactive nucleotide were applied to thin-layer chromatography plates and washed by chromatographing in 1 M acetic acid. Finally, radioactivity in the samples was quantified by liquid scintillation as above.

Data from steady state kinetic- and equilibrium binding experiments were analyzed using the computer program Ultrafit from BioSoft (version 3.0). The equations used were Eq. (1), the Hill equation, for sigmoid saturation curves, Eq. (2) for sigmoid substrate saturation curves and sigmoid competitive inhibition and Eq. (3) for hyperbolic binding of ligands to the enzyme;

$$\text{rate} = k_{\text{cat}}[\text{S}]^n/(S_{0.5}^n + [\text{S}]^n) \quad (1)$$

$$\text{rate} = k_{\text{cat}}[\text{S}]^n/(S_{0.5}^n(1 + ([I]/I_{0.5})^{ni}) + [\text{S}]^n) \quad (2)$$

$$N = N_{\max}[\text{L}]/(K_{\text{d}} + [\text{L}]) \quad (3)$$

where rate is the initial turnover of the enzyme with a maximum of k_{cat}, $S_{0.5}$ is the concentration of substrate S at half-maximal saturation of the enzyme, $I_{0.5}$ is the concentration of inhibitor I for half maximal inhibition, n and ni are the Hill coefficients associated with substrate saturation and inhibition, respectively. N is the degree of binding with the dissociation constant K_{d} of ligand L to the enzyme with the maximal number of binding sites, $N_{\max}$.

Crystallization of the E138D mutant protein

A protein solution of 2 mg/ml protein mixed with 5 mM dUTP and 20 mM $MgSO_4$ were used in vapor diffusion hanging drop experiments. Small plates appeared after 24 h in drops of 2 μl protein with ligand mixed with 2 μl of reservoir solution (27.5% polyethylene glycol 400, 50 mM $MgSO_4$ and 0.1 M Hepes, pH 7.5) equilibrated against 500 μl of the reservoir solution at room temperature.

Diffraction data collection

The crystal was flash-cooled in liquid nitrogen. Diffraction data to 2.2 Å resolution were collected at 100 K at beamline I911-3, Max-lab, Lund University Sweden on a MAR Research CCD detector. The crystal belongs to the orthorhombic space group $P2_12_12_1$ with the following cell dimensions: $a = 94.2$ Å, $b = 71.3$ Å, $c = 319.9$ Å. There were 12 polypeptide chains corresponding to four trimers in the asymmetric unit and approximately 41% solvent (Matthews coefficient 2.10 Å^3/Da). Auto indexing, data integration and scaling were performed using programs from the HKL suite [20]. Diffraction data statistics are summarized in Table 1.

Structure determination and refinement

The structure of *E. coli* E138D dCTP deaminase was determined using the molecular replacement technique as implemented in the program Molrep [21] with the crystal structure of *E. coli* wild-type dCTP deaminase