Orf135 from *Escherichia coli* Is a Nudix Hydrolase Specific for CTP, dCTP, and 5-Methyl-dCTP*

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Orf135 from *Escherichia coli* is a new member of the Nudix (nucleoside diphosphate linked to some other moiety, χ) hydrolase family of enzymes with substrate specificity for CTP, dCTP, and 5-methyl-dCTP. The gene has been cloned for overexpression, and the protein has been overproduced, purified, and characterized. Orf135 is most active on 5-methyl-dCTP (k_{cat}/K_{m} = 301,000 M^{-1} s^{-1}), followed by CTP (k_{cat}/K_{m} = 47,000 M^{-1} s^{-1}) and dCTP (k_{cat}/K_{m} = 18,000 M^{-1} s^{-1}). Unlike other nucleoside triphosphate pyrophosphohydrolases of the Nudix hydrolase family discovered thus far, Orf135 is highly specific for pyrimidine (deoxy)nucleoside triphosphates. Like other Nudix hydrolases, the enzyme cleaves its substrates to produce a nucleoside monophosphate and inorganic pyrophosphate, has an alkaline pH optimum, and requires a divalent metal cation for catalysis, with magnesium yielding optimal activity. Because of the nature of its substrate specificity, Orf135 may play a role in pyrimidine biosynthesis, lipid biosynthesis, and in controlling levels of 5-methyl-dCTP in the cell.

The Nudix hydrolases are a family of enzymes catalyzing the hydrolysis of substrates consisting of a nucleoside diphosphate linked to some other moiety, χ (hence, the acronym Nudix), and are defined by the signature sequence GX,EX,REUXEX GU where X is a bulky aliphatic amino acid, Ile, Leu, or Val (1). The family consists of enzymes that hydrolyze (d)NTPs (2–8), NADH (9, 10), GDP-mannose (11), ADP-ribose (10, 12–14), diadenosine polyphosphates (10, 15–21), and diphosphoinositol polyphosphates in addition to diadenosine polyphosphates (22–24). The Nudix hydrolases were discovered first through a comparison of *Escherichia coli* MutT and *Streptococcus pneumoniae* MutX and from BLAST (25) searches of MutT, which revealed the signature sequence, common in a number of open reading frames (26, 27). The family has grown to include over 450 open reading frames in 85 species as well as a variety of enzymes as indicated above. Nudix hydrolases are ubiquitous throughout nature, existing in eukaryotes, prokaryotes, and archaea (12) and appear to control the level of potentially toxic substances that would be detrimental to the cell at elevated levels (1, 23) and to regulate the accumulation of metabolic intermediates (1, 23).

Orf135 is a true member of the Nudix hydrolase family; it contains the signature sequence GX,EX,REUXEXGU and it cleaves the nucleoside diphosphate derivatives CTP, dCTP, and 5-methyl-dCTP. Yet, it is unique in its substrate specificity, because it is the first Nudix hydrolase highly specific toward pyrimidine substrates. In this paper, we describe the cloning and expression of the orf135 gene and the purification and characterization of the Orf135 enzyme, and we discuss its possible role in intermediary metabolism.

EXPERIMENTAL PROCEDURES

Materials

Nucleic Acids—Oligodeoxynucleotides were obtained from Integrated DNA Technologies, the plasmid pET11b was from Novagen, and chromosomal DNA from *E. coli* strain MG1655 was kindly provided by Dr. Frederick R. Blattner (University of Wisconsin).

Bacterial Strains—Competent cells of *E. coli* DH5a were obtained from Life Technologies, Inc., and *E. coli* HMS174(DE3) was from Novagen.

Enzymes—Pfu DNA polymerase was from Stratagene, restriction enzymes NdeI and BamHI and T4 DNA ligase were obtained from Life Technologies, Inc., and inorganic pyrophosphatase came from Sigma.

Chemicals—Isopropyl-β-D-thiogalactopyranoside was from Research Organics and Sephadex G50 from Pharmacia Biotech, nucleotide substrates were from Sigma, and other general chemicals were from Sigma or J. T. Baker, Inc.

Methods

Cloning—The orf135 gene was amplified from *E. coli* strain MG1655 chromosomal DNA using polymerase chain reaction. An NdeI restriction site was incorporated at the start of the gene and a BamHI site at its end using oligodeoxynucleotide primers containing these sites. The amplified gene was purified, digested with NdeI and BamHI, and ligated into the respective restriction sites of plasmid pET11b to place the orf135 gene under control of a T7 lac promoter for expression. The resultant plasmid, pETorf135, was used to transform *E. coli* strain DH5a for storage and *E. coli* strain HMS174(DE3) for expression. The sequence of orf135 in the resultant plasmid was confirmed using the fluorescent dideoxy terminator method on a PerkinElmer ABI 377 automated DNA sequencer by the DNA analysis facility at The Johns Hopkins University.

Expression and Enzyme Purification—*E. coli* strain HMS174(DE3) containing pETorf135 was grown at 37 °C in 2 liters of broth containing 100 µg/ml ampicillin. When growth reached an A_{600} of 0.8, the culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside and grown for an additional 2 h, and the cells (3.5 g) were harvested, washed in buffered isonic saline solution, recentrifuged, and stored at −80 °C. Orf135 was extracted from the frozen cells by resuspending them in 2 volumes of buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol). Prior freezing was necessary to render the protein extractable in this manner. The suspension was centrifuged, and the supernatant (Fraction I) containing ~80 mg of protein was concentrated by precipitation with 65% ammonium sulfate and dissolved in 1.2 ml of buffer A (Fraction II). Fraction II (containing ~65 mg of protein) was loaded onto a 1.5 × 52-cm Sephadex G50 gel filtration column (calibrated with molecular mass standards of 14, 20, and 29 kDa) and eluted with buffer A containing 200 mM NaCl. The fractions containing Orf135 and essentially free of other proteins were combined (Fraction III) and concentrated by precipitation with 80% ammonium sulfate.

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sulfate, dissolved in buffer A to a final volume of 2 ml (Fraction IV), and stored at −80 °C where Orf135 was stable indefinitely. Fraction IV contained 25 mg of protein with 2300 units of CTPase activity.

Enzyme Assays—A standard reaction mixture of 50 μl contained 50 mM Tris-HCl, pH 9, 1 mM dithiothreitol, 5 mM MgCl₂, 4 mM substrate (CTP), 500 milliunits of inorganic pyrophosphatase, and 0.3–5 milliunits of Orf135. The mixture was incubated at 37 °C for 15 min, stopped by the addition of 50 μl of 4 parts Norit (20% packed volume) and 1 part 7% perchloric acid, and centrifuged, and 50 μl of the supernatant was analyzed for inorganic orthophosphate by the colorimetric procedure of Ames and Dubin (28). One unit of Orf135 hydrolyzes 1 μmol of substrate/min.

To identify the products and stoichiometry of the reaction, the standard reaction mixture was scaled up 20-fold, inorganic pyrophosphatase was omitted, and the reactions were quenched with excess EDTA (relative to magnesium). The substrate and nucleotide product were quantitated using a high performance liquid chromatography system with a YMC ODS-AM column, and an isocratic mobile phase of 12.5 mM citric acid, 25 mM sodium acetate, 10 mM acetic acid, adjusted to pH 6.3 with sodium hydroxide. The substrate and nucleotide product were detected at 254 nm and identified with standards of CTP, CDP, and CMP. Inorganic pyrophosphate was quantitated by the colorimetric assay after hydrolysis to inorganic orthophosphate by boiling for 15 min in 0.5 M HCl.

Kinetic studies were carried out using a 0.1–4 mM substrate, 5 mM MgCl₂, and 0.1–5 milliunits of Orf135.

Assay for Mutator Phenotype—orf135 was subcloned from pETorf135 and into a pT7C99A vector containing an E. coli lac promoter, transformed into E. coli strain SB3 lacking a functional mutT gene, and mutation frequencies were determined as described in O’Handley et al. (8).

RESULTS

Subcloning, Expression, and Purification

We identified the orf135 gene, which had been sequenced and deposited as part of the E. coli sequencing project (29), from a BLAST search (30) of the Nudix signature sequence in MutT. The gene was cloned directly from chromosomal DNA as described under “Methods,” and its sequence agrees with that reported in the Genbank™. Expression of orf135 results in the appearance of a major band on a denaturing polyacrylamide gel corresponding to a 15-kDa protein, not readily detectable by eye in the same strain of E. coli containing pET11b without the insert (Fig. 1). As has been reported for other Nudix hydrolase enzymes (8, 11, 14, 18), a majority of the Orf135 is released into solution simply by freezing and thawing the cells, leaving most of the other proteins behind in the cell. This results in an extract much more highly enriched in Orf135 than would ensue from a more complete disruption of the cells, such as that caused by sonication, and it greatly simplifies purification, requiring only an additional ammonium sulfate fractionation and gel filtration step. This procedure yields ~25 mg of essentially pure enzyme from a 2-liter preparation (Fig. 1).

Orf135 migrates on a denaturing polyacrylamide gel as expected for the 15-kDa polypeptide predicted from its amino acid composition, and it elutes from a G50 gel filtration column as expected for a monomer of this size.

Substrate Specificity

Like MutT and Orf17, Orf135 is a (deoxy)ribonucleoside triphosphatase. However, the substrate specificity is markedly different for these three enzymes. Whereas MutT and Orf17 hydrolyze all 8 canonical nucleoside triphosphates with preference for dGTP and dATP, respectively (2, 8), Orf135 is very specific for CTP, dCTP, and 5-methyl-dCTP (Table I). This is the first (deoxy)ribonucleoside triphosphatase of the Nudix hydrolase family that is base-specific and the first Nudix hydrolase in general that is specific for pyrimidine substrates (1). Orf135 hydrolyzes UTP and dTTP at less than 5% of the rate of CTP, and there is no significant hydrolysis of ATP, dATP, GTP, or dGTP. Also, as we have observed with MutT and Orf17, cleavage of the pyrophosphate linkage in the nucleoside diphosphates by Orf135 is minimal, and cleavage of the nucleoside monophosphates by Orf135 is undetectable.

The influence of the various functional groups of the substrates on the rate of hydrolysis is compared in Fig. 2. 5-Methyl-dCTP and CTP have an amino group at the C4 position,
which is replaced in dTTP and UTP by a keto group. 5-Methyl-dCTP, CTP, and dCTP are hydrolyzed at rates 50 times greater than dCTP and UTP, respectively, indicating the importance of the C4 amino group for recognition and catalysis. Likewise, 5-methyl-dCTP is hydrolyzed more rapidly than dCTP and dTTP at a higher rate than dUTP, both by a factor of ~5, indicating that the 5-methyl group also enhances the overall rate, although not as dramatically as the influence of the C4 amino group. As for the sugar, the presence of the hydroxyl group at the C2 position does not have that great of an effect on activity (the rate of hydrolysis of CTP is double that of dCTP), but epimerization of the hydroxyl group at the C2 position decreases activity by ~20-fold as indicated by a comparison of cleavage of CTP versus ara-CTP. It will be interesting to examine whether these important functional groups on the substrates make vital contacts with amino acids in the protein by analyzing a three-dimensional structure of the enzyme complexed with the substrate.

A kinetic analysis of the preferred substrates (Table II) shows that Orf135 cleaves 5-methyl-dCTP with a catalytic efficiency 6 times greater than that for CTP and 17 times greater than that for dCTP. The possible biological significance of these differences will be discussed below.

**Requirements of the Enzyme**

**pH Optimum**—Orf135 has optimal activity between pH 8.5 and 9.0 in Tris-HCl or glycine buffers with the rate dropping to 50% at pH 7.8 and 9.8. This alkaline pH optimum is typical of the Nudix hydrolase family of enzymes (with the pH optima for MutT, Orf17, Orf257, Orf186, and Orf1.9 being pH 9.0, 8.6, 8.5, 9.3, and 9.0, respectively (2, 8–11)), indicating a common mechanism of hydrolysis as discussed previously by O’Handley et al. (10).

**Divalent Cation Requirement**—As with all of the other Nudix hydrolases discovered thus far, Orf135 absolutely requires a divalent metal cation for activity. Magnesium is most effective with optimal activity at 5 mM Mg2+ in the presence of 4 mM substrate. Mn2+ can partially substitute for Mg2+ with ~10% activity at optimal concentration, whereas no activity was observed in the presence of Zn2+, Co2+, or Cu2+.

**Product Formation**

The products of the hydrolysis of CTP were determined from a scaled-up, standard reaction excluding inorganic pyrophosphatase. The decrease of CTP and increase of the nucleotide product, CMP, during the course of the reaction were monitored over time by chromatographic analysis as shown in Fig. 3. The other product formed, inorganic pyrophosphate, was quantified by the colorimetric assay of Ames and Dubin (28) after hydrolysis to inorganic orthophosphate, as described under “Methods”; this is also shown in Fig. 3. The CTP hydrolyzed corresponds to the CMP and inorganic pyrophosphate produced in a 1:1 molar ratio at each time point. No CDP was detected throughout the reaction, and no inorganic orthophosphate appeared, as analyzed by the method of Fiske and Subbarow (31), which is specific for inorganic orthophosphate and will not detect pyrophosphate. Thus, the hydrolysis of CTP catalyzed by Orf135 may be written as follows (Reaction 1).

\[
\text{CTP} + \text{H}_2\text{O} \rightarrow \text{CMP} + \text{PP}_i
\]

**REACTION 1**

The reaction products are similar to those generated by the other nucleoside triphosphate pyrophosphohydrolases of the Nudix hydrolase family. MutT and MutX hydrolyze dGTP to dGMP and pyrophosphate (2, 7), and Orf17 hydrolyzes dATP to dAMP and pyrophosphate (8). Using H218O, we have shown that both MutT and Orf17 hydrolyze their respective substrates by nucleophylic attack at the β phosphorus (8, 32). Because Orf135 yields similar products, it most likely uses a similar mechanism to hydrolyze CTP.

**DISCUSSION**

As can be seen from a list of the known Nudix hydrolase enzymes, Orf135 fits in well as a member of the Nudix hydrolase family (Fig. 4). All of the other enzymes are distinct from Orf135, but they all share the common signature sequence...
(GX\textsubscript{2}EX\textsubscript{2}REUXEEXGU where U is Ile, Leu, or Val) characteristic of this family (1), and they all hydrolyze nucleoside diphosphate compounds.

A BLAST search using Orf135 as the query identifying many of these previously discovered Nudix hydrolases, as well as a number of unknown open reading frames, is shown in Fig. 5. The closest matches to identified enzymes are to MutT from Proteus vulgaris, Haemophilus influenzae, and E. coli with E values of 5 \times 10^{-10}, 3 \times 10^{-9}, and 8 \times 10^{-8}, respectively. Also, when threading programs were used to compare the predicted structures of Orf135 and the other Nudix hydrolases to the solved NMR solution structure of MutT, Orf135 had the highest match of any of the known, characterized Nudix hydrolases (results not shown). However, as described throughout this paper, Orf135 is distinct from MutT in its substrate specificity, preferentially hydrolyzing CTP, dCTP, and 5-methyl-dCTP as opposed to dGTP. Because Orf135 appears more similar to MutT in primary and predicted secondary structure than other Nudix hydrolases, it was important to ascertain whether the cellular roles of Orf135 and MutT are distinctly different from one another. Accordingly, a plasmid carrying the orf135 gene was transformed into a mutT strain of E. coli and analyzed for a decrease in mutation frequency. Orf135 did not complement MutT (results not shown). This result was not surprising to us, because no other Nudix hydrolases except MutT orthologs active on dGTP have been shown to complement MutT (8, 12), and it demonstrates that even open reading frames with similar primary and (predicted) secondary structures can have different enzymatic activities and cellular functions. Thus, as

3 S. F. O’Handley, C. A. Dunn, and M. J. Bessman, personal observations.
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phate kinase does not appear to recognize 5-methyl-dCMP as a
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gene expression (33). As a result of DNA degradation, 5-meth-
logs in the cell as discussed below.
levels of these compounds or other cytidine triphosphate ana-
and 5-methyl-dCTP. The role of Orf135 may be to regulate the

In lipid biosynthesis, CTP is utilized in the synthesis of
CDP-ethanolamine and CDP-diaciglycerol, both important inter-
mediates in the synthesis of glycerophospholipids, which are
major components of cell membranes (40). There have been
several studies showing a direct correlation between CTP lev-
els and phospholipid biosynthesis. Overexpression of CTP syn-
thease (41) or expression of a CTP synthetase mutant less
sensitive to inhibition by CTP (42) causes an increase in CTP
levels, leading to an increase in phospholipids and neutral
lipids. On the other hand, when cyclopentenylcytosine, a potent

we have pointed out (1), caution must be exercised when ana-
lyzing information from BLAST searches and data bases of
proteins containing the Nudix signature sequence. For exam-
ple, the Japanese E. coli sequencing project lists Orf135 (acces-
sion number BAA15549) as a mutator MutT protein or dTP
pyrophosphohydrolase, whereas the report for Orf135 (acces-
sion number AAC74829), deposited by Blattner’s group (29) as
part of their sequencing of E. coli, simply states that Orf135 is 37%
identical to 125 residues of MutT from H. influenzae.
What then is the possible function of Orf135 in the cell? The
best substrates discovered for Orf135 in vitro are CTP, dCTP,
and 5-methyl-dCTP. The role of Orf135 may be to regulate the
levels of these compounds or other cytidine triphosphate ana-

ectin in the cell as discussed below.
Methylation of the C5 position of cytosine by DNA (cytosine-
5-) methyltransferase is involved in the differential control of
gene expression (33). As a result of DNA degradation, 5-methyl-
dCMP is produced. In human cells, nucleoside monophos-
phate kinase does not appear to recognize 5-methyl-dCMP as a
substrate, and thus phosphorylation of 5-methyl-dCMP to
5-methyl-dCTP does not occur (34, 35). It has been suggested
that this prevents 5-methylcytosine from being randomly in-
corporated into DNA, since 5-methyl-dCTP is an excellent an-
aloge of dCTP and can replace it completely (36). No similar
studies have been reported for E. coli, and so the significance of
5-methyl-dCTPase here is moot. There are, however, bacteri-
ophages that induce kinases that can phosphorylate 5-methyl-
dCMP (37) and 5-hydroxymethyl-dCMP (38), and Orf135 could be
part of a defense mechanism against infection.
Furthermore, Orf135 may monitor the intracellular accumu-
lation of CTP, a key metabolite in both pyrimidine and lipid
biosynthesis. Orf135 may play a role in regulating the synthe-
sis of pyrimidines in E. coli by hydrolyzing CTP. CTP is the
negative regulator of the entire pyrimidine biosynthetic path-
way in E. coli, binding to aspartate transcarbamoylase, the
committed step in the synthesis of UTP and CTP (39). If CTP
accumulates, its inhibition of aspartate transcarbamoylase not
only inhibits its own synthesis but also the synthesis of UMP,
UDP, and UTP. Because dTMP is synthesized from other py-
rimidine deoxyribonucleotides with the common pyrimidine
precursor being UMP, the synthesis of dTMP would also be
inhibited by CTP accumulation. This would ultimately affect
DNA synthesis. Orf135 may circumvent these effects through
hydrolysis of CTP. It is interesting to note that dATP, the major
negative regulator of the deoxynucleotide biosynthetic path-
way, is the substrate of another Nudix hydrolase, Orf17 from E.
coli, which hydrolyzes it to dAMP and pyrophosphate (8).

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