A Novel GDP-Mannose Mannosyl Hydrolase Shares Homology with the MutT Family of Enzymes*

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The product of the Escherichia coli orf1.9, or yecf, gene (GenBank accession number L11721) has been expressed under the control of a T7 promoter, purified to apparent homogeneity, and identified as a novel enzyme that hydrolyzes GDP-mannose or GDP-glucose to GDP and the respective hexose. The enzyme has little or no activity on other nucleotides, dinucleotides, nucleotide sugars, or sugar phosphates. It has a pH optimum between 9.0 and 9.5, a Km of 0.3 mM, and a Vmax of 1.6 μmol min⁻¹ mg⁻¹ for GDP-mannose, and it requires divalent cations for activity. This enzyme of 160 amino acids (M, = 18, 405) contains the consensus sequence GX(I/L/V)(E/Q)(X)₂ET(X)₁R(X)₁E(X)₁(I/L), characteristic of the MutT family of proteins and previously shown to form part of the nucleotide-binding site of MutT (Frick, D. N., Weber, D. J., Abeygunawardana, C., Gittis, A. G., Bessman, M. J., and Midlvan, A. S. (1995) Biochemistry 34, 5577-5586). A comparison of the enzymatic reactions catalyzed by the GDP-mannose mannosyl hydrolase and the other enzymes of the MutT family suggests that the consensus signature sequence designates a novel nucleoside diphosphate binding site and catalytic motif.

Many primary amino acid “signature” sequences have been identified that designate protein sites involved in ligand binding or enzymatic catalysis. One of these signature sequences was discovered as part of the Escherichia coli MutT and Strep-tococcus pneumoniae MutX antimutator proteins (1, 2) and independently by computer searches (3). This region of the MutT protein has since been shown by NMR and site-directed mutagenesis to be part of the active site and nucleotide binding site of the MutT protein (2, 4). The structure of the MutT protein, as revealed by heteronuclear multidimensional NMR, shows that this region forms a novel loop-helix-loop nucleotide binding site not previously seen in other proteins (5, 6). In addition, four other proteins containing homology to the MutT family of enzymes (1) have been found to possess various enzymatic and biological activities. These proteins include a MutT homologue (7), a human enzyme that degrades the potential mutagenic nucleoside triphosphate 8-oxoguanosine triphosphate (8-oxo-dGTP) (8-10), the product of the E. coli orf17 gene, a nucleoside triphosphate pyrophosphohydrolase (11, 12), and an E. coli NADH pyrophosphatase (13).

Here we report the discovery and characterization of another such enzyme, a GDP-mannose mannosyl hydrolase. The new enzyme is an E. coli protein coded for by an open reading frame near the GDP-mannose pyrophosphorylase (cspB) and phosphomannomutase (cspG) genes in the 45-min region of the E. coli chromosome. The open reading frame begins 1.9 kilobase pairs from the start of the putative GDP-mannose dehydrogenase gene and is therefore referred to as orf1.9 in GenBank accession number L11721 (14). The protein product of the orf1.9 open reading frame is listed as yecf in the SwissProt data base.

EXPERIMENTAL PROCEDURES

Materials

Media

LB medium was prepared as described by J. H. Miller (15). Bacto tryptone and Bacto yeast extract were purchased from Difco Laboratories (Detroit, MI). Ampicillin was used for selection in media at a concentration of 100 μg/ml, and isopropyl-1-thio-D-galactopyranoside (IPTG) was used as an inducer at a concentration of 1 mM.

Chemicals

Guanosine diphosphate [2-³H]mannose was from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and [α-³2P]thio-dATP for sequencing was from Amersham Corp. (Dexoy)nucleoside diphosphates and triphosphates were from Pharmacia Biotech Inc. NADH, mannose, mannose-1-phosphate, and all nucleotide-sugars were from Sigma. IPTG was from Research Organics (Cleveland, OH). Unless otherwise noted, all other chemicals were from J. T. Baker (Phillipsburg, NJ).

Enzymes

Thermus aquaticus DNA polymerase was from Perkin-Elmer. BamHI, Ndd, calf intestinal alkaline phosphatase, and T4 DNA ligase were from Stratagene (La Jolla, CA). Sequenase T7 DNA polymerase (version 2.0) was from U.S. Biochemical Corp., and unless otherwise noted, all other enzymes were from Sigma.

Nucleic Acids

Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). Plasmid vector pET11b was from Novagen (Madison, WI).

Bacterial Strains

HB101 was from a laboratory stock (M. J. Bessman). HMS174(DE3) was from Novagen (Madison, WI).

Reins

Sephadex G-100 and DEAE-Sepharose (fast flow) were from Pharmacia Biotech Inc.

Methods

General Techniques

The polymerase chain reaction was performed using a GeneAmp kit from Perkin-Elmer. DNA was analyzed by agarose gel electrophoresis and visualized by staining with ethidium bromide as described by Sambrook et al. (16). DNA was purified from agarose gels using either a GeneClean kit (Bio101, La Jolla, CA) for DNAs longer than 1000 bp or phenol/chloroform.
base pairs or the "freeze-squeeze" method (17) for DNAs shorter than 1000 base pairs. Plasmids were isolated and purified using techniques from Sambrook et al. (16). Plasmid DNA was purified for sequencing by polyethylene glycol precipitation followed by alkali denaturation as described by Kraf t et al. (18). DNA was sequenced using the Sequenase T7 DNA polymerase kit (version 2.0) from U.S. Biochemical Corp. Bacterial cells were made competent for transformation by treatment with CaCl₂ as described by Mand el and Higa (19).

Protein extracts were analyzed using native and denaturing polyacrylamide gel electrophoresis as described by Laemmli (20), and proteins in polyacrylamide gels were stained using Coomassie Brilliant Blue R.

Paper electrophoresis of nucleotides was performed in 25 mm citrate buffer, pH 4.9, as described by Markham and Smith (21). Nucleotides were visualized with ultraviolet light.

High performance anion exchange chromatography (HPAEC) was done using a BioLC (Dionex Corp., Sunnyvale, CA) and a CarboPac PA-1 column (4 × 250 mm) and a pulsed amperometric detector (PAD) (22). The HPAEC data were analyzed with A1-450 chromatography software (Dionex).

Protein concentrations were determined using a reagent from Bio-Rad (Hercules, CA) based on the method of Bradford (23). Bovine serum albumin was used as a standard.

Cloning of E. coli orf1.9

Chromosomal DNA was prepared from strain HB101 using the method of Stillman et al. (24). Oligonucleotide primers orf1.9Bam (5'-CGCGCATATGATGGTTTTCCGAGGAC3') and orf1.9Sal (5'-GGGGGAATTCCTTAATCCCGGGGATCCGAC3') were used to amplify and attach restriction sites (underlined) to the orf1.9 gene from strain HB101 chromosomal DNA in a polymerase chain reaction. The 491-base pair polymerase chain reaction product was purified from an agarose gel, digested with Ndel and BamH1, purified again, and ligated into the NdeI and BamH1 sites of the pET11b plasmid. In the resulting plasmid, the orf1.9 gene was under the control of a T7 promoter. The plasmid, pETorf1.9, was transformed into strain HB101 for storage and into strain HMS174(DE3) for protein expression.

Purification of the Orf1.9 protein

Growth and Expression—Single colonies of strain HMS174(DE3) containing pETorf1.9 were inoculated into 50 ml of LB medium containing 100 µg/ml ampicillin. After the cells grew to saturation, they were transferred to 2 liters of fresh medium containing ampicillin. When the cells reached an A600 of 1.0, they were induced with 1 mM IPTG and grown for an additional 2 h.

Crude Extract—Cells were collected by centrifugation, washed with a saline solution (0.5% KCl, 0.5% NaCl), and stored overnight at −80°C. The cell paste (7.6 g) was suspended in 4 volumes of buffer A (50 mM Tris-Cl, pH 7.5) and centrifuged. The crude extract (20 ml) was centrifuged for 10,000 g for 30 min at 4°C. The supernatant was used as source of Orf1.9 protein as described under "Methods." and the region containing the insert was transferred to GenBank (accession number L11721) and designates a 160-amino acid protein (M_r = 18,405) (14). Sequence similarity of Orf1.9 to the MutT protein was reported by Méjean et al. (2). The orf1.9 gene was cloned into the vector pET11b directly from chromosomal DNA as described under "Methods," and the region containing the insert was sequenced. The nucleotide sequence agreed with that submitted to GenBank (accession number L11721). The region of homology with the MutT protein and other MutT-like proteins spans amino acids 49–73.

Orf1.9 Protein Expression and Purification— Cultures of strain HMS174(DE3) containing pETorf1.9 were grown, induced, harvested, and extracted as described under "Methods." The expression of the Orf1.9 protein in this system is shown in Fig. 1 by the appearance of a new band at approximately 18 kDa after induction with IPTG. Also shown, are aliquots from each stage of the purification, the final stage appearing over 99% pure.

R E S U L T S

Cloning of orf1.9—The orf1.9 gene was first sequenced by K. M. Aoyama and P. R. Reeves (GenBank accession number L11721) and designates a 160-amino acid protein (M_r = 18,405) (14). Sequence similarity of Orf1.9 to the MutT protein was reported by Méjean et al. (2). The orf1.9 gene was cloned into the vector pET11b directly from chromosomal DNA as described under "Methods," and the region containing the insert was sequenced. The nucleotide sequence agreed with that submitted to GenBank (accession number L11721). The region of homology with the MutT protein and other MutT-like proteins spans amino acids 49–73.

Orf1.9 Protein Expression and Purification— Cultures of strain HMS174(DE3) cells containing pETorf1.9 were grown, induced, harvested, and extracted as described under "Methods." The expression of the Orf1.9 protein in this system is shown in Fig. 1 by the appearance of a new band at approximately 18 kDa after induction with IPTG. Also shown, are aliquots from each stage of the purification, the final stage appearing over 99% pure. One interesting aspect of this purification procedure is that the overproduced Orf1.9 protein can be extracted from E. coli simply by freezing and thawing the cells and suspending the cells in a low ionic strength buffer (i.e. 50 mM Tris-Cl). This phenomenon was also observed in our laboratory by L. C. Bullions while purifying the E. coli Orf17 nucleoside triphos-
phosphate pyrophosphohydrolase protein\(^2\) and could result from the accumulation of the overproduced protein in the periplasmic space. Alternatively, the overexpression of the Orf1.9 E. coli GDP-mannose hydrolase could destabilize the outer membrane of E. coli by hydrolyzing an important precursor of cell wall disaccharides. The induction of a GDP-mannose degrading activity after IPTG addition is shown in Fig. 2. Four hours after induction, the specific activity of the extracts (units of enzyme/mg of total protein) increases over 800-fold. This strongly suggests that the new band appearing at 18 kDa is a GDP-mannose hydrolase. Also, the hydrolase activity co-purifies with the 18-kDa protein (data not shown).

Products of the E. coli Orf1.9-catalyzed Reaction—Because orf1.9 is in a region of the E. coli chromosome containing several genes involved in the production of GDP-mannose and because all other known enzymes containing the MutT-like consensus region is its pH characteristics, which are identical to those of authentic mannose (22). Fig. 5B shows further that the accumulation of free mannose is dependent on time of incubation. The areas under each peak were used to determine the moles of mannose released. The moles of GDP at each of the time points in Fig. 4B were also determined using assay 2 to measure alkaline phosphatase activity. At each time, 2 mol of phosphate, and hence 1 mol of GDP, were detected for each mole of mannose produced (data not shown). This agrees with the data presented in Fig. 3. Thus the equation for the reaction may be written as follows: GDP-mannose → GDP + mannose.

Properties of the E. coli Orf1.9 GDP-Mannose Mannosyl Hydrolase—As summarized in Table I, the enzyme is active only on GDP-mannose or GDP-glucose, with very little or no detectable activity on other nucleotide sugars. In addition to the nucleotide sugars listed in Table I, the purified Orf1.9 protein was also tested for activity on all 16 (deoxy)nucleoside diphosphates and triphosphates, NADH, and mannose-1-phosphate. None of these compounds were hydrolyzed at any measurable rates. The Orf1.9 enzyme’s restricted substrate specificity is notably different from that of other MutT-like enzymes. For example, the MutT, MutX, and Orf17 nucleotide triphosphohydrolases degrade all eight canonical nucleoside triphosphates (11, 12, 26), and the E. coli NADH pyrophosphatase also hydrolyzes a variety of dinucleotide pyrophosphates and nucleotide sugars (13).

A kinetic analysis of substrate titrations with GDP-mannose and GDP-glucose is presented in Table II. Although the enzyme has a 4.7-fold higher V\(_{\text{max}}\) for GDP-glucose than for GDP-mannose, the K\(_m\) for GDP-mannose is 6.3-fold lower, resulting in a somewhat higher overall catalytic efficiency (V\(_{\text{max}}\)/K\(_m\)) for GDP-mannose.

Like other enzymes in this class, the E. coli GDP-mannose mannosyl hydrolase absolutely requires divalent metal cations for activity as shown in Fig. 4, lane 5, where MgCl\(_2\) is absent from the reaction mixture. The only metals tested that effectively activated the enzyme were Mg\(^{2+}\) and Mn\(^{2+}\). Ca\(^{2+}\), Zn\(^{2+}\), and Co\(^{2+}\) supported 4, 3, and <1%, respectively, of the activity of Mg\(^{2+}\).

Another property that this enzyme shares in common with several enzymes containing the consensus region is its pH versus rate profile. The enzyme has an alkaline pH optimum at

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\(^2\) Linda C. Bullions and Maurice J. Bessman, unpublished observation.
sequence shared by Orf1.9-like proteins. The signature sequence differs from the one published by Koonin's (3). Koonin's signature was based solely on a computerized comparison of all sequences deposited in the data bank, whereas the signature sequence shared by Orf1.9-like proteins can be summarized as follows: (6).

**Table I**

| Specific activity | unit/mg | GDP-α-D-glucose | 4.4 |
|-------------------|---------|-----------------|-----|
| GDP-α-D-mannose   | Glucose | 1.3             |     |
| GDP-β-D-fucose    | Fucose  | 0.020           |     |
| GDP-γ-D-fucose    | Fucose  | <0.01           |     |
| ADP-α-D-glucose   | Glucose | <0.01           |     |
| ADP-β-D-ribose    | Ribose  | <0.01           |     |
| UDP-α-D-mannose   | Mannose | <0.01           |     |
| UDP-β-D-glucose   | Glucose | <0.01           |     |
| NADH              | Adenosine | <0.001       |     |
| Mannose-1-phosphate | NA^a | <0.001       |     |
| (d)NDP<sup>b</sup>,<sup>c</sup> | PO<sub>4</sub>H<sub>2</sub> | A, T, G, or C | <0.001 |
| (d)NDP<sup>d</sup> | H      | A, T, G, or C | <0.001 |

^a A unit is defined as 1 μmol of substrate cleaved per min.
^b Alkaline phosphatase was omitted from the reaction mixture.
^c Not applicable.
^d (d)NTP, (deoxy)nucleoside triphosphate.
^e To detect the possible formation of pyrophosphate, the reactions were repeated in the presence of 0.5 unit of inorganic phosphatase (EC 3.6.1.1).
^f (d)NDP, (deoxy)nucleoside diphosphate.

**Table II**

| Specific activity | unit/mg | GDP-α-D-glucose | 4.4 |
|-------------------|---------|-----------------|-----|
| GDP-α-D-mannose   | Glucose | 1.3             |     |
| GDP-β-D-fucose    | Fucose  | 0.020           |     |
| GDP-γ-D-fucose    | Fucose  | <0.01           |     |
| ADP-α-D-glucose   | Glucose | <0.01           |     |
| ADP-β-D-ribose    | Ribose  | <0.01           |     |
| UDP-α-D-mannose   | Mannose | <0.01           |     |
| UDP-β-D-glucose   | Glucose | <0.01           |     |
| NADH              | Adenosine | <0.001       |     |
| Mannose-1-phosphate | NA^a | <0.001       |     |
| (d)NDP<sup>b</sup>,<sup>c</sup> | PO<sub>4</sub>H<sub>2</sub> | A, T, G, or C | <0.001 |
| (d)NDP<sup>d</sup> | H      | A, T, G, or C | <0.001 |

^a A unit is defined as 1 μmol of substrate cleaved per min.
^b Alkaline phosphatase was omitted from the reaction mixture.
^c Not applicable.
^d (d)NTP, (deoxy)nucleoside triphosphate.
^e To detect the possible formation of pyrophosphate, the reactions were repeated in the presence of 0.5 unit of inorganic phosphatase (EC 3.6.1.1).
^f (d)NDP, (deoxy)nucleoside diphosphate.

**Fig. 4. Identification of GDP product.** Reactions were incubated for 15 min at 37 °C in 50 mM Tris-Cl, pH 8.9, 10 mM MgCl<sub>2</sub>, 4 mM GDP-mannose, and terminated by boiling. 20 μl of supernatant were spotted for electrophoresis. Paper electrophoresis was done according to Markham and Smith (21), in 25 mM citrate buffer, pH 4.9, at 1400 volts for 2 h. Lanes 1 and 6 contained 100 nmoles of markers GTP, GDP, and GMP. The reaction in lane 2 contained no Orf1.9 protein; reactions in lanes 3 and 4 contained 2 units and 4 units of enzyme, respectively; and the reaction in lane 5 contained the same components as lane 4 without MgCl<sub>2</sub>. Nucleotides were visualized with UV light.

**Fig. 5. Identification of mannoside product.** A, reactions were done at 37 °C in 40 mM glycine, pH 9.3, 10 mM MgCl<sub>2</sub>, and were terminated by the addition of 1 volume of 18% Norit in 0.1 N HCl before (C) and 20 min after (△) addition of purified Orf1.9 protein (1.1 milliunits). After centrifugation, 50 μl of the supernatant was evaporated to dryness and dissolved in 500 μl of H<sub>2</sub>O. 50 μl were analyzed by HPAEC on a CarboPac PA1 column (4 × 250 mm) eluting with 16 mM NaOH (22). The Norit nonadsorbable product eluted at the same time as a mannoside standard (×) (1 nmol). B, reactions were done as described above and were terminated by boiling after 10 min (——), 20 min (— — —) or 30 min (— — —), evaporated to dryness, and dissolved in 2.5 ml of H<sub>2</sub>O. 50 μl were chromatographed as described above. (Mannose-1-phosphate is not eluted from the column under these conditions.)
For each substrate, initial velocities of nucleotide sugar hydrolysis were measured in the presence of 20 mM MgCl₂ and 80 mM glycine, pH 9.3, using assay 2 described under "Methods." Initial velocities were measured at six substrate concentrations ranging from 0.1 to 4.0 mM. Kₘ and Vₘax values were obtained by non-linear regression weighted to substrate concentrations (40) using initial estimates obtained from Lineweaver-Burk analyses (41).

| Substrate     | Vₘax (units mg⁻¹) | Kₘ (mM) | Vₘax/Kₘ (units mg⁻¹ m⁻¹) |
|---------------|-------------------|--------|--------------------------|
| GDP-glucose   | 7.5 ± 0.6         | 1.9 ± 0.3 | 4.0 ± 0.6                |
| GDP-mannose   | 1.6 ± 0.1         | 0.30 ± 0.8 | 5.7 ± 1.5                |

Fig. 6. Alignment of the Orf1.9 amino acid sequence with other enzymes containing homology to the MutT active site. The amino acid sequences of the MutT and Orf1.9 proteins were aligned using the computer program CLUSTAL W 1.5 (27). Identical amino acids are boxed in black, and similar amino acids are noted with gray boxes. The sequences aligned with the Orf1.9 GDP-mannose hydrolase are as follows: MutT, the MutT protein from E. coli (42); Orf17, the MutT-like protein from P. vulgaris (7), the MutX protein from S. pneumoniae (11), NADHase, the NADH pyrophosphatase from E. coli (33), and similar roles are conceivable for the Orf1.9 protein (13) and the GDP-mannose hydrolase reported herein. We suggest the name GDP-mannose mannosyl hydrolase (EC 3.2.1.42) isolated from yeast (33). We suggest the name GDP-mannose mannosyl hydrolase (EC 3.2.1.42) isolated from yeast (33).
In addition to the enzymes mentioned here, recent computer searches of nucleic acid and protein data bases have revealed that several viral, prokaryotic, and eukaryotic proteins also share homology to this motif. These include proteins from African swine fever virus, vaccinia virus, fowlpox virus, variola virus, Streptomyces ambofaciens, proteins coded for by the antisense RNA of Xenopus laevis and human basic fibroblast growth factors (2, 3), and a protein from Chilo iridescens virus (39). The results presented here should be considered when speculating about the cellular functions of these proteins. The observations that both an NADH pyrophosphatase and a GDP-mannose hydrolase contain this consensus sequence indicate that the catalytic region designated by this motif is not confined to enzymes having nucleoside triphosphatase activity. Instead, it has been conserved during evolution and adapted to participate in diverse metabolic reactions involving the cleavage of substrates containing a pyrophosphate group linked to a nucleoside.

Structural analysis of the MutT protein has already revealed that the conserved region folds to form a unique nucleotide binding motif (5, 6) and that amino acids in the signature sequence are in intimate contact with bound nucleotides (4). Further studies are designed to uncover the roles of specific amino acids in this domain. Detailed structural and enzymatic analyses of these other MutT-like proteins would likewise be of interest to determine whether or not the consensus sequence GX((I/L)V)/E(Q)/(X)_{3}ET(X)_{3}R(X)_{3}/E(X)_{3}/(I/L) forms a similar nucleotide binding site in these proteins as well. Thus far, all seven of the characterized proteins containing the above signature sequence hydrolyze nucleoside pyrophosphate compounds, suggesting that the other uncharacterized proteins sharing this signature sequence have similar activities. These include proteins of unknown function from a wide variety of organisms, ranging from viruses to humans. It is tempting to speculate that the identification and biochemical characterization of consensus sequences such as these in the rapidly expanding data banks will, in the future, facilitate the determination of protein function from sequence data alone.

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