

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 *yefc*, 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 K_m of 0.3 mM, and a V_{max} of 1.6 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ for GDP-mannose, and it requires divalent cations for activity. This enzyme of 160 amino acids ($M_r = 18,405$) contains the consensus sequence $GX(I/L/V)(E/Q)(X)_2ET(X)_6R(X)_4E(X)_2(I/L)$, 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 Mildvan, 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 *Streptococcus 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 active site have been found to possess various enzymatic and biological activities. These proteins include a MutT homologue from *Proteus vulgaris* (7), a human enzyme that degrades the potentially deleterious nucleotide 8-oxo-7-hydro-deoxy-guanosine triphosphate (8-oxo-dGTP)¹ (8–10), the product of the *E. coli* *orf17* gene, a nucleoside triphosphate pyrophospho-

hydrolase (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 (*cpsB*) and phosphomannomutase (*cpsG*) 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 *yefc* 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 $\mu\text{g/ml}$, and isopropyl-1-thio- β -D-galactopyranoside (IPTG) was used as an inducer at a concentration of 1 mM.

Chemicals

Guanosine diphosphate [$2\text{-}^3\text{H}$]mannose was from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and [$\alpha\text{-}^{35}\text{S}$]dATP for sequencing was from Amersham Corp. (Deoxy)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 (Phillipsburgh, NJ).

Enzymes

Thermus aquaticus DNA polymerase was from Perkin-Elmer. BamHI, NdeI, 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).

Resins

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

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¹ The abbreviations used are: 8-oxo-dGTP, 8-oxo-7-hydro-deoxy-guanosine triphosphate; HPAEC, high performance anion exchange chromatography; IPTG, isopropyl-1-thio- β -D-galactopyranoside.

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 Kraft *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 Mandel 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 Bio-LC (Dionex Corp., Sunnyvale, CA) and a CarboPac PA-1 column (4 × 250 mm) and a pulsed amperometric detector (PAD-II) (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 Silhavy *et al.* (24). Oligonucleotide primers orf1.9Nde (5' CG CGC GCG CAT ATG ATG TTT TTA CGT CAG GAA GAC 3') and orf1.9Bam (5' CG CGC GGA TCC TCA TAA TCC GGG TAC TCC GGT ACG-3') 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 *NdeI* and *BamHI*, purified again, and ligated into the *NdeI* and *BamHI* 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 and 1 mM EDTA, pH 7.5). The crude extract was clarified by centrifugation at 15,000 × g for 30 min (Fraction I).

Ammonium Sulfate Fractionation—Solid ammonium sulfate (6.55 g) was added to 25.4 ml of Fraction I, and the precipitate was collected by centrifugation and discarded. Ammonium sulfate (5.74 g) was added to the supernatant. The precipitate (45–80%) contained the Orf1.9 protein and was dissolved in 2.5 ml of buffer A (Fraction II).

Sephadex G-100—Fraction II (2 ml) was loaded onto a gel filtration column (Sephadex G-100; 2.5 cm × 60 cm) and eluted with buffer A containing 50 mM NaCl. The fractions containing the Orf1.9 protein were combined (30 ml). 15.5 g of ammonium sulfate were added, and the precipitate was collected by centrifugation, dissolved in 5 ml buffer A, and dialyzed against this same buffer (Fraction III).

DEAE-Sephacel—Fraction III (5 ml) was loaded onto a 1.5 cm × 5.0-cm DEAE-Sephacel column, washed with 100 ml of 50 mM Tris-Cl, pH 7.5, and then washed with 100 ml of 50 mM Tris-Cl, 50 mM NaCl, pH 7.5. The Orf1.9 protein was eluted with 50 mM Tris-Cl, pH 7.5, containing 200 mM NaCl. The fractions containing the Orf1.9 protein were pooled (40 ml). 20.6 g of ammonium sulfate were added, and the precipitate was collected by centrifugation, dissolved in 1.5 ml buffer A, and dialyzed as above. Glycerol was added to a final concentration of 20%, and the protein (Fraction IV) was stored at -80 °C.

Enzyme Assays

Kinetic experiments were performed at 37 °C, and in all assays, a unit of activity was defined as 1 µmol of substrate hydrolyzed per min.

Both radioactive (assay 1) and colorimetric (assay 2) assays were used to measure the activity of the Orf1.9 *E. coli* GDP-mannose hydro-

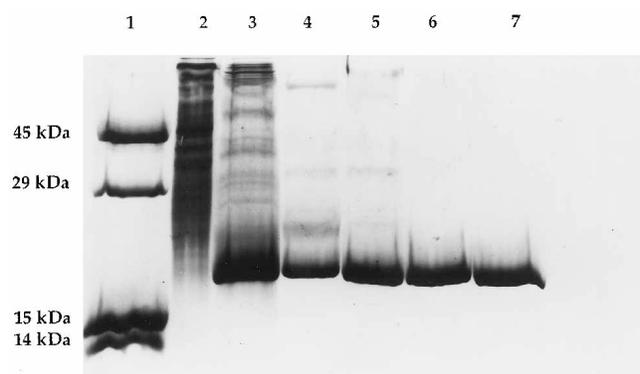


Fig. 1. Expression and purification of the Orf1.9 protein. A 15% SDS-polyacrylamide gel stained with Coomassie Blue. Lane 1 contains 4 µg each of markers ovalbumin (45 kDa), carbonic anhydrase (29 kDa), MutT (15 kDa), and α -lactalbumin (14 kDa). Lanes 2 and 3 show the expression of the Orf1.9 protein. HMS174(DE3) cells containing the plasmid pETorf1.9 were grown in LB-ampicillin (100 µg/ml) medium. Cells (1.5 ml) were harvested before (lane 2) and 2 h after (lane 3) IPTG addition. The A₆₀₀/ml was determined for each aliquot, and the cells were boiled in 400 µl of SDS loading buffer/A₆₀₀ unit. Lane 2 contains 20 µl of the preinduced cell extract, and lane 3 contains 20 µl of the induced cell extract. Lanes 4–7 show stages in the purification of the Orf1.9 protein and contain 6 µg of fractions I–IV, respectively.

lase, each based on the following reaction: GDP-mannose → GDP + mannose.

Assay 1—The reaction mixtures (25 µl) contained 80 mM glycine (pH 8.5), 20 mM MgCl₂, 2 mM GDP-[2-³H]mannose (200–2,000 cpm/nmol), and 0.1–1.0 milliunit of enzyme. After 10–30 min at 37 °C, the reactions were terminated by the addition of 25 µl of a 4:1 mixture of Norit (20% packed volume), perchloric acid (7%). After 5 min on ice, the samples were centrifuged, and [2-³H]mannose in the Norit supernatants was measured using a Packard Tri-Carb liquid scintillation counter.

Assay 2—In a coupled assay, the GDP product was hydrolyzed to deoxyguanosine and inorganic phosphate by the addition of calf intestinal alkaline phosphatase (EC 3.1.3.1). The reaction mixtures (50 µl) contained 80 mM glycine (pH 8.5), 20 mM MgCl₂, 2 mM GDP-mannose, 1–2 units of calf intestinal alkaline phosphatase, and 0.1–1.0 milliunit of enzyme. After 10–30 min at 37 °C, the reactions were terminated by boiling, and phosphate was determined by the method of Ames and Dubin (25).

RESULTS

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 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-

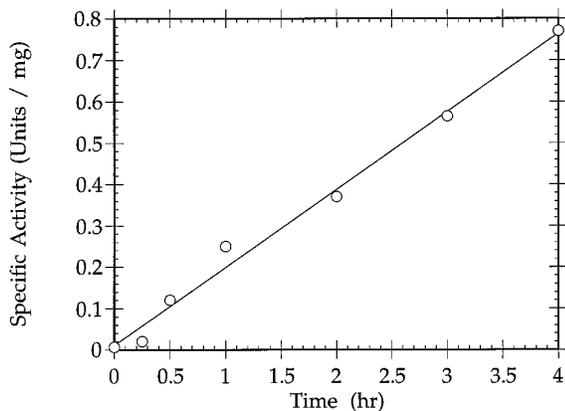


FIG. 2. The induction of the GDP-mannose hydrolase in crude extracts of HMS174(DE3) cells containing the plasmid pET-orf1.9. Cells were grown to an A_{600} of 0.6, and IPTG was added to a final concentration of 1 mM. Cells were collected by centrifugation, resuspended in 4 volumes of buffer A, and sonicated using a Branson Sonifier cell disrupter. After centrifugation to remove cell debris, enzyme activity in the supernatants was measured using assay 1 (see "Methods").

phate pyrophosphohydrolase protein² and could result from the accumulation of the overproduced protein in the periplasmic space. Alternatively, the overexpression of the Orf1.9 GDP-mannose hydrolase could destabilize the outer membrane of *E. coli* by hydrolyzing an important precursor of cell wall oligosaccharides. 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 conserved region hydrolyze phosphoanhydride bonds linked to nucleotides (13), we suspected that the product of *orf1.9* might be a guanine nucleotide sugar pyrophosphatase analogous to ADP-sugar pyrophosphatase (EC 3.6.1.21). Indeed, as shown in Fig. 3, the purified Orf1.9 protein does release mannose from the sugar nucleotide linkage. This is revealed by the detection of a radiolabeled, Norit nonadsorbable product when GDP-[2-³H]mannose is used as a substrate. Fig. 3 also shows that no free orthophosphate is produced upon digestion with the enzyme. However, after incubation of the products with calf intestinal alkaline phosphatase, 2 mol of inorganic orthophosphate are detected for each mole of sugar released. This experiment shows that the enzyme catalyzes the release of mannose from GDP-mannose with the formation of 2 mol of phosphatase labile phosphate for each mole of mannose. This stoichiometry is reminiscent of our recently discovered *E. coli* NADH pyrophosphatase (13), which cleaves NADH to produce AMP and nicotinamide mononucleotide; therefore, it seemed likely that the Orf1.9 enzyme was catalyzing the hydrolysis of the pyrophosphate bond joining GMP to mannose-1-phosphate.

Surprisingly, however, the products of the reaction were not GMP and mannose-1-phosphate. One of the products was identified as GDP by paper electrophoresis (Fig. 4). The other product, mannose, was identified and quantified using HPAEC (Fig. 5). Fig. 5A identifies the Norit nonadsorbable product of

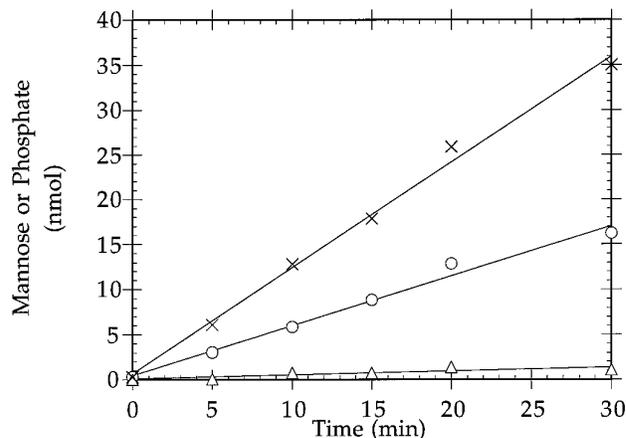


FIG. 3. The stoichiometry of the Orf1.9 catalyzed reaction. Purified Orf1.9 protein (0.5 milliunit) was incubated with 2 mM GDP-[³H]mannose, 20 mM MgCl₂, 80 mM glycine, pH 9.3. The reactions were terminated by boiling, and aliquots were used to determine free [³H]mannose (○) using assay 1 (see "Methods"), inorganic orthophosphate (△) using the method of Ames and Dubin (25), and inorganic orthophosphate after digestion of the products with alkaline phosphatase (×) using assay 2 (see "Methods").

the reaction as mannose based on its chromatographic 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 labile phosphate. 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 nucleoside triphosphate pyrophosphohydrolases 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_{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_{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, *Jane 5*, where MgCl₂ is absent from the reaction mixture. The only metals tested that effectively activated the enzyme were Mg²⁺ and Mn²⁺. Ca²⁺, Zn²⁺, and Co²⁺ supported 4, 3, and <1%, respectively, of the activity of Mg²⁺.

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

² Linda C. Bullions and Maurice J. Bessman, unpublished observation.

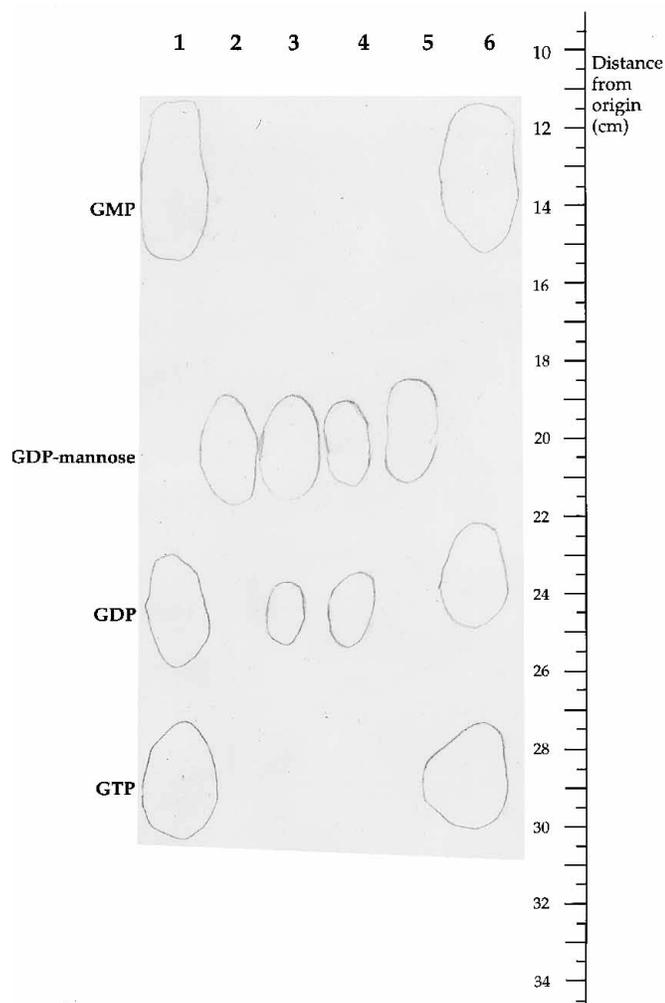


FIG. 4. Identification of GDP product. Reactions were incubated for 15 min at 37 °C in 50 mM Tris-Cl, pH 9.0, 10 mM MgCl₂, 4 mM GDP-mannose and terminated by boiling, and 20 μ l were spotted for electrophoresis. Paper electrophoresis was done according to Markham and Smith (21), in 25 mM citrate buffer, pH 4.9, at 1400 volts for 2 h. Lanes 1 and 6 contained 100 nmol each 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 MgCl₂. Nucleotides were visualized with UV light.

pH 9.3, which is similar to the pH optima of the MutT enzyme (26), the Orf17 enzyme (12), and the NADH pyrophosphatase (6).

Sequence Similarity to Other MutT-like Enzymes—An alignment of the Orf1.9 amino acid sequence with other proteins in the family of MutT-like enzymes using the computer program CLUSTAL W 1.5 (27) is presented in Fig. 6. The amino acids boxed in black are residues that are absolutely conserved, and the amino acids boxed in gray are highly similar (*i.e.* leucine, isoleucine, or valine) in all Orf1.9-like proteins. The signature sequence shared by *E. coli* MutT, *P. vulgaris* MutT, *S. pneumoniae* MutX, human 8-oxo-dGTPase, *E. coli* Orf17, the *E. coli* NADH pyrophosphatase, and the *E. coli* GDP-mannose hydrolase can be summarized as follows: GX(I/L/V)(E/Q)(X)₂ET(X)₆R(X)₄E(X)₂(I/L). Any one of the amino acids in parentheses is allowed, and X represents any amino acid. It should also be noted in Fig. 6 that the six proteins other than Orf1.9 share additional similarities, and a consensus sequence summarizing these conserved residues has been previously reported (13). For example, the Orf1.9 protein lacks two glutamic acid residues and also a bulky aliphatic residue (I, V, or

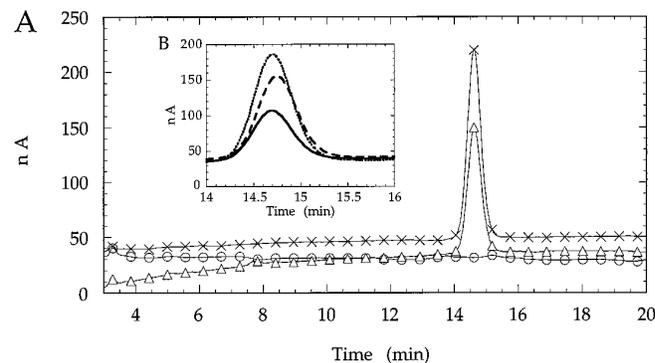
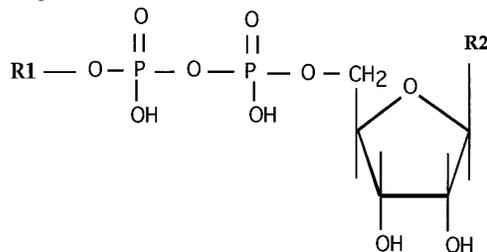


FIG. 5. Identification of mannose product. A, reactions were done at 37 °C in 40 mM glycine, pH 9.3, 10 mM MgCl₂, and were terminated by the addition of 1 volume of 18% Norit in 0.1 N HCl before (○) and 20 min after (Δ) addition of purified Orf1.9 protein (1.1 milliunits). After centrifugation, 50 μ l of the supernatant was evaporated to dryness and dissolved in 500 μ l of H₂O. 50 μ l were analyzed by HPAEC on a CarboPac PA1 column (4 \times 250 mm) eluting with 16 mM NaOH (22). The Norit nonadsorbable product eluted at the same time as a mannose 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 H₂O. 50 μ l were chromatographed as described above. (Mannose-1-phosphate is not eluted from the column under these conditions.)

TABLE I
Specificity of *E. coli* Orf1.9 nucleotide sugar hydrolase

The specific activity of the enzyme on each of the nucleotides listed below was measured using assay 2 described under "Methods." Reactions were done at 37 °C in 80 mM Glycine, pH 9.3, 20 mM MgCl₂, 2 mM each substrate, 1 unit of alkaline phosphatase, and 0.1–170 milliunits of the Orf1.9 protein (Fraction IV).



	R1	R2	Specific activity
			units ^a /mg
GDP- α -D-glucose	Glucose	Guanine	4.4
GDP- α -D-mannose	Mannose	Guanine	1.3
GDP- β -L-fucose	Fucose	Guanine	0.020
GDP- α -L-fucose	Fucose	Guanine	<0.01
ADP- α -D-glucose	Glucose	Adenine	<0.01
ADP- β -D-ribose	Ribose	Adenine	<0.01
UDP- α -D-mannose	Mannose	Uracil	<0.01
UDP- α -D-glucose	Glucose	Uracil	<0.01
NADH	Adenosine	Nicotinamide	<0.001
Mannose-1-phosphate ^b	Mannose	NA ^c	<0.001
(d)NTP ^{b,d,e}	PO ₃ H ₂	A, T, G, or C	<0.001
(d)NDP ^{b,f}	H	A, T, G, or C	<0.001

^a A unit is defined as 1 μ mol 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 pyrophosphatase (EC 3.6.1.1).

^f (d)NDP, (deoxy)nucleoside diphosphate.

L). This signature sequence differs from the one published by Koonin, G(X)₅E(X)₅(U/A)XRE&XEE(X)₉& (where U represents the bulky aliphatic residue I, L, V, or M and & represents the bulky hydrophobic residue I, L, V, M, F, Y, or W) (3). Koonin's signature was based solely on a computerized comparison of all sequences deposited in the data bank, whereas the signature

TABLE II
Kinetic analysis of the nucleotide sugar hydrolase

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. K_m and V_{max} values were obtained by non-linear regression weighted to substrate concentrations (40) using initial estimates obtained from Lineweaver-Burk analyses (41).

Substrate	V_{max}	K_m	V_{max}/K_m
	units mg ⁻¹	mM	units mg ⁻¹ mM ⁻¹
GDP-glucose	7.5 ± 0.6	1.9 ± 0.3	4.0 ± 0.6
GDP-mannose	1.6 ± 0.1	0.30 ± 0.08	5.7 ± 1.5

Orf1.9	-----MMFLR-----QEDFATVVRSTPLVSLD-----FIVENS
MutT	-----MKKLOIAYG-----LIRNE
Orf17	-----MKDKYKRPFVSLVV-----IYAQD
NADHase	MDRIIEKLDHCWVWVSHQKQLWLPKGELPYGERANFDLVGQRALQIGEMQGEVWVWVQOQ
MutT (PV)	-----MMDKKKLIHAAG-----VICDK
MutX	-----MPQLATICY-----EDNG
8oxodGTPase	-----MGASRLRYTLVL-----VLQP
Orf1.9	--RGE-----
MutT	-----NNE-----
Orf17	-----TKR-----
NADHase	RRHDMGSRQVILDLVGLFQLAGRGVQLAEFYRSHKYCYGCGHEMPSKTEWAMLCSHCR
MutT (PV)	-----HNN-----
MutX	-----KE-----
8oxodGTPase	-----QR-----
Orf1.9	-----FELGKRTN-----RPAQGYWVPCGRVQKLEDEEAFFRITMABELG
MutT	-----IFITRRAAD-----AHMANKLEFPCKTEEMCEPPEQAVVREIQEPEVVG
Orf17	-----VLMQRRDD-----PDFWQSVLISVSEVGEFPAQAMREVKPEEVT
NADHase	ERYYPQIAPCLITVAIRRDDSLLLAQHTRRHNGVHTVLAQFVEVGEDELEQAVAREVPEESG
MutT (PV)	-----VFIAORPKL-----SHMGFWPEFPCGKEDENDEPQALIREIQEPIG
MutX	-----LMLHRNKK-----PNDVHEGKWIQVCGKCEPPEQCAVRELEBETG
8oxodGTPase	-----VLEGMKRG-----FGAGRWNGFCRKYQEGEITLEDGAREPPEESG
Orf1.9	ERLPITAGQYGVWQHFFYDDNFS-GTET-----THYVVLGFRFRVSEE---ELLLPDE-
MutT	ETP-----QHSFLFKL-EYFP-----DRHTLWFWLVERWE--GEPWKE-
Orf17	EDVA-----EOLTLIDCQRTVPELIFSHLRHRYPAGVTRNTEFSW--GLALPHER
NADHase	EKV-----KNLRYVTSQ-PWFHP-----QSLMTAFMAEYDSDG--IVLDPKE-
MutT (PV)	EDV-----TQCTLLDTV-AHFP-----DRHTLTSFFLVTEWK--NELTEKK-
MutX	EKAKFVLKGVITPPEFTPDLWY-TYVFKVT--EFEGDLIDCNEGMLWVVPYDEVLSKPT
8oxodGTPase	ETVDALHKVGVIVFPEVGEPELMDVHFCETDS--IQGTFVSEDEMPKCFWQLDQIPFKD-
Orf1.9	-----QHDDYRWLTSALL--ASDNVHANSRAYPLAEKRTGVGFL-
MutT	-----GQGE-WMSLVGLN--ADDFPANEFPVIAKRLK-----
Orf17	QIVPTEHLAYKWLDAFAA--ALTKSWSN-RQATEQPVINAA--
NADHase	-----LLEAN-WRYDDLP--LPPFGTVARRLIEDIVAMCRAEYE
MutT (PV)	-----GS-----CRVGHLLCL-
MutX	WEG--DHTEVWEWLEDKPF--FSAKFVYDGDKLLDQVDFYE----
8oxodGTPase	-----MWEDDSYWFPLLLQKKFKHGYFKFQOQDTILDYTLREVDIV--

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); Orf1.7, nucleoside triphosphate pyrophosphohydrolase from *E. coli* (11), NADHase, the NADH pyrophosphatase from *E. coli* (13); MutT(PV), the MutT homologue from *P. vulgaris* (7); MutX, the MutT homologue from *S. pneumoniae* (2); and 8-oxo-dGTPase, an enzyme from human cells that degrades 8-oxo-dGTP (9). Gaps in the protein sequences required to optimize the alignment are represented by hyphens.

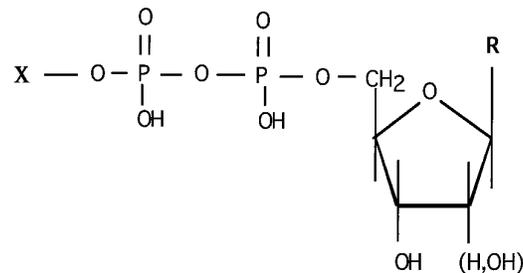
reported above represents the conserved residues in only those proteins shown to possess enzymatic activities.

DISCUSSION

The majority of the enzymes that hydrolyze nucleotide sugars are pyrophosphatases (28, 29), phosphorylases (30, 31), or pyrophosphorylases (32). Unlike the enzyme described here, these enzymes release sugar-1-phosphates rather than free sugar. Only one other enzyme has been reported that releases a free sugar from the nucleotide sugar, a GDP-glucose glucosylhydrolase (EC 3.2.1.42) isolated from yeast (33). This enzyme differs from *E. coli* Orf1.9 in that it has no activity on GDP-mannose and does not require divalent metal ions for activity (33). We suggest the name GDP-mannose mannosyl hydrolase for the *E. coli* enzyme to point out this difference. This name also conforms to the standard practice of naming the enzyme after the substrate with the lowest K_m . The possible biological

TABLE III
Summary of the reactions catalyzed by proteins containing the nucleoside diphosphate binding motif

Substrate:



Protein	Source	X	R	Products
MutT	<i>E. coli</i>	PO ₃ H ₂	Pur, Pyr	NMP + PP _i
MutT	<i>P. vulgaris</i>	PO ₃ H ₂	Pur, Pyr	NMP + PP _i
MutX	<i>S. pneumoniae</i>	PO ₃ H ₂	Pur, Pyr	NMP + PP _i
8-oxo-dGTPase	<i>Homo sapiens</i>	PO ₃ H ₂	8-oxo-guanine	8-oxo-dGMP + ?
Orf17	<i>E. coli</i>	PO ₄	Pur, Pyr	NMP + PP _i
NADHase	<i>E. coli</i>	Adenosine	Nicotinamide	NMN + AMP
Orf1.9	<i>E. coli</i>	Glucose or Mannose	Guanine	GDP + Sugar

roles of nucleotide sugar hydrolases were discussed by Sonnino *et al.* (34), and similar roles are conceivable for the *E. coli* enzyme. The enzyme could participate in the regulation of cell wall biosynthesis by influencing the concentration of GDP-mannose or GDP-glucose in the cell. Because the enzyme shows little activity on GDP-fucose and is present in the *cps* gene cluster that may be involved in the synthesis of GDP-fucose (14), another role of the Orf1.9 protein could be to degrade GDP-mannose and GDP-glucose, diverting the GDP to the synthesis of GDP-fucose as required.

Our interest in the enzyme, however, is mainly focused on its similarity to the MutT protein which, when defective, increases the simultaneous rate on the order of 1000-fold (35). Since we noticed that the MutT catalytic core was widely distributed throughout nature (2), we have begun to characterize other proteins that share this common motif in order to uncover its biochemical mechanism and to catalogue the physiological function of proteins harboring the signature sequence. These MutT-like proteins may be of special interest, because there have been reports of suppressors of the *mutT* phenotype in *E. coli* (36, 37). Such genes could code for proteins with similar functions to MutT that could also be involved in the maintenance of the fidelity of DNA replication.

To date, the enzymatic activities of seven proteins containing the GX(I/L/V)(E/Q)(X)₂ET(X)₆R(X)₄E(X)₂(I/L) signature have been identified, namely those of MutT from *E. coli* (38), the homologous MutT from *P. vulgaris* (7), the MutX protein from *S. pneumoniae* (2), the human 8-oxo-dGTPase (9), the Orf17 protein (11), the *E. coli* NADH pyrophosphatase (13), and the *E. coli* GDP-mannose hydrolase. Table III summarizes the reactions catalyzed by these proteins. The first five of these enzymes (listed in the order of their discovery) are nucleoside triphosphatases. The four that have been characterized have been shown to hydrolyze the linkage between the α and β phosphates forming a nucleoside monophosphate and inorganic pyrophosphate. Based on our recent discovery of the NADH pyrophosphatase (13) and the GDP-mannose hydrolase reported herein, we can narrow down the features of the substrate required for recognition by the catalytic region. All active substrates are derivatives of nucleoside pyrophosphates, and therefore we hypothesize that this signature designates a unique nucleoside diphosphate binding and catalytic domain.

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 iridescent 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 $G\bar{X}(I/L/V)(E/Q)(X)_2ET(X)_6R(X)_4E(X)_2(I/L)$ 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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