Escherichia coli orf17 Codes for a Nucleoside Triphosphate Pyrophosphohydrolase Member of the MutT Family of Proteins

CLONING, PURIFICATION, AND CHARACTERIZATION OF THE ENZYME* (Received for publication, April 8, 1996)

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The product of the Escherichia coli orf17 gene is a novel nucleoside triphosphate pyrophosphohydrolase with a preference for dATP over the other canonical (deoxy)nucleoside triphosphates, and it catalyzes the hydrolysis of dATP through a nucleophilic attack at the β-phosphorus to produce dAMP and inorganic pyrophosphate. It has a pH optimum between 8.5 and 9.0, a divalent metal ion requirement with optimal activity at 5 mM Mg²⁺, a Keq of 0.8 mM and a kcat of 5.2 s⁻¹ at 37 °C for dATP. dAMP is a weak competitive inhibitor with a Ki of approximately 4 mM, while PPi is a much stronger inhibitor with an apparent Ki of approximately 20 μM.

The enzyme contains the highly conserved signature sequence GXXEX3ETGXXVXXE2I designating the MutT family of proteins. However, unlike the other nucleoside triphosphate pyrophosphohydrolases with this conserved sequence, the Orf17 protein does not complement the mutT⁺ mutator phenotype, and thus must serve a different biological role in the cell.

Members of the MutT family of proteins are categorized by a conserved amino acid motif originally identified as an important functional region in the Escherichia coli MutT and the Streptococcus pneumoniae MutX antimutator proteins (1). This conserved amino acid signature sequence was found, by computer search, to be present in a number of open reading frames broadly distributed throughout nature, from viruses to humans (1, 2), and one of these, orf17 (GenBank D10165, 1992), is the subject of this paper. The orf17 gene is located at 41 min on the E. coli chromosome (3) just upstream of the rucC gene (4, 5), which codes for a Holliday junction-specific endonuclease (4).

Both the MutT and MutX proteins are nucleoside triphosphatases (6, 7), as are the corresponding proteins from Proteus vulgaris (8) and from humans (9), and all four are implicated in preventing mutations. On the other hand, two other proteins containing the MutT signature sequence are not nucleoside triphosphatases. One of them, a nucleoside pyrophosphatase, prefers GDP-mannose (11). Nei-triphosphatases. One of them, a nucleoside pyrophosphatase, containing the MutT signature sequence are not nucleoside preventing mutations. On the other hand, two other proteins orf17 subject of this paper. The conserved sequence, the Orf17 protein does not complement the mutT⁺ mutator phenotype, and thus must serve a different biological role in the cell.

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volumes of a saline solution (0.5% KCl, 0.5% NaCl), centrifuged, and frozen at −80°C. Freezing the cells was essential for preparing the extract. The cells were extracted in 2 volumes of buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 0.1 mM DTT), centrifuged, and re-extracted, and the supernatants were combined. A typical extract (Fraction I) yielded approximately 100 mg of protein from 10 g of cells.

Ammonium Sulfate Fractionation—Fraction I was brought to 41% saturation by the addition of 26 ml of saturated ammonium sulfate to 37.5 ml of crude extract. The precipitate was removed by centrifugation and discarded, and an additional 22.5 ml of ammonium sulfate were added to the supernatant to give a 57% saturated solution. This precipitate, containing the Orf17 protein, was collected and dissolved in 1 ml of buffer A (Fraction II).

**Sephacryl G-50**—Fraction II was loaded onto a 1.5 × 55-cm Sephadex G-50F filtration column and eluted with buffer A. The fractions containing the Orf17 protein were combined (19.5 ml), concentrated by precipitation in 60% saturated ammonium sulfate, and dissolved in 1 ml of buffer A, yielding 18 mg of Orf17 protein, which were stored at −80°C (Fraction III).

**Assay for Mutator Phenotype**—The plasmid, pETorfl7, was digested with XhoI and HindIII, and the fragment containing the orf17 gene was ligated into pTrC99A to produce pTRCorf17. The plasmid, pTRmutT, was constructed in a similar way from pETmutT (12) substituting BamHI for XhoI. E. coli strain SB3, lacking a functional MutT protein, was transformed with pTRCorf17, pTRmutT, or pTRcorf17. These strains were grown in LB medium containing 100 μg/ml ampicillin and 1 mM IPTG, and mutation frequencies were determined by plating aliquots on LB plates containing either 150 μg/ml streptomycin or 20 μg/ml nalidixic acid. Similarly, E. coli strains, AB1157 (orf17−) and HRS1024 (orf17−), both gifts from H. Shinagawa (4), were grown in LB medium and mutation frequencies were determined on nalidixic acid plates as above. Mutation frequencies are reported as the average of five separate determinations.

**Enzyme Assays**—Two standard enzyme assays, both based on the production of PP, from dATP, were used.

**Colorimetric Assay**—The standard reaction mixture contained in 50 ml: 4 mM dATP, 50 mM Tris, pH 9, 10 mM MgCl2, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 1% glycerol, 0.5 unit of inorganic pyrophosphatase, and 0.3–3 milliunits of Orf17 enzyme. The reaction was run at 37°C for 15 min, terminated by the addition of 0.5 ml of a mixture of four parts of Norit® (20% packed volume) and one part of 7% HClO4 centrifuged, and an aliquot of the supernatant was analyzed for inorganic orthophosphate by the method of Ames and Dubin (14). A unit of activity is 1 μmol of PP/min under these conditions.

**Radioactive Assay**—This is similar to the standard colorimetric asay with the following modifications. 32P-dATP (about 500 cpm/nmol) was used as the substrate. The reaction was terminated as in the colorimetric assay (except that 5 N HCl was substituted for the HClO4) and an aliquot of the supernatant was analyzed for radioactivity. Since ATP is hydrolyzed at half the rate of dATP (see Table I), a unit of enzyme in this assay is comparable to 2 units in the colorimetric assay.

**Acquisition and Processing of NMR Spectra**—Experiments were done at 32 °C on a Varian Unityplus 600 NMR spectrometer equipped with a 5-mm broad band probe with field/frequency locking on the D,O resonance. The 2H NMR spectra were recorded with a transmitter frequency of 243 MHz, a spectral width of 12,000 Hz, and proton decoupling. Each spectrum was recorded with an acquisition time of 2.75 s and a relaxation delay of 6 s. The enzymatic reaction was monitored continuously in blocks of 64–256 transients for 4 h. The spectrum in this paper was collected with 256 scans between 2.5 and 3 h after the addition of enzyme. Resonances were assigned by comparing reaction spectra to those of standards of dAMP, dADP, dATP, sodium pyrophosphate, and sodium phosphate, and chemical shifts were measured relative to an external standard of 85% phosphoric acid. The data were subjected to Fourier transformation after processing with line broadening of −1 Hz and a shifted sine bell function for resolution enhancement.

**RESULTS AND DISCUSSION**

**Subcloning, Expression, and Purification**

The orf17 gene was cloned as described under “Methods.” Its sequence agrees with Takahagi et al. (4) except for the original GTG start site, which we modified to ATG as a matter of convenience. Expression of the gene from pETorfl7 results in the appearance of a major band on SDS gels corresponding to a 17-kDa polypeptide (Fig. 1), which, when treated according to the outlined purification procedure, results in an essentially pure protein (>95% as visualized on the gel). Attention is called to a faint band in the high molecular weight region of the gel. This most likely represents an aggregated form of the protein, not a contaminant. It is consistently seen in varying amounts in different preparations of the enzyme. It is not present in extracts of cells transformed with the vector lacking the insert (lane 2) and a sequence analysis of the N-terminal 10 amino acids of the final preparation indicates that the enzyme is at least 98% homogeneous with respect to these residues (data not shown). It is noteworthy that the bulk of the Orf17 protein is released from the cells merely by freezing them and extracting with a dilute buffer. This results in a crude extract much more highly enriched in Orf17 than procedures involving more complete disruption of the cells, such as sonication. One other member of the MutT family of proteins, the GDP-mannose hydrolase (11), is also readily extractable from previously frozen cells. Perhaps these two expressed enzymes share a common cellular compartment such as the periplasmic space, which may be breeched in the freeze-thaw cycle, or they may share some other feature not readily apparent. At any rate, this mild extraction procedure facilitates the subsequent purification, because the bulk of cellular proteins is left behind in the cell pellet.

**Properties of the Enzyme**

**Specificity**—As shown in Table I, the enzyme hydrolyzes all eight of the canonical (deoxy)nucleoside triphosphates with a marked preference for dATP. The next most favored substrate is approximately 6-fold lower in catalytic efficiency (kcat/Km). The enzyme recognizes not only the nucleotide base, but the sugar, as well, since in each case, the ribo nucleotide is less active than its corresponding deoxy equivalent. Especially noteworthy is the low activity of dGTP relative to dATP, amounting to an order of magnitude difference in catalytic efficiency. This is in marked contrast to the MutT nucleoside triphosphatase, which prefers dGTP, hydrolyzing it at a 30-fold higher rate (7) and with a catalytic efficiency 2 orders of magnitude higher than with dATP (13).

**pH Optimum**—The enzyme is maximally active at pH 8.6, diminishing to 50% activity at pH 7.6 or 9.9 (data not shown). This distinctly alkaline optimum is similar to the pH versus rate profile of three other members of the E. coli MutT family i.e. MutT (7), NADH pyrophosphatase (10), and GDP-mannose hydrolase (11).

**Divalent Cation Requirement**—As with all other members of
the MutT family, a divalent cation is required for activity, and 
Mg\(^{2+}\) is most effective. The optimal concentration is 5 mM for 
free Mg\(^{2+}\), and substitution of Mn\(^{2+}\), Ca\(^{2+}\), or Zn\(^{2+}\) at 1 mM or 
5 mM reduces the rate 10-fold or more. Mg\(^{2+}\) did not activate 
the enzyme until its concentration exceeded that of the sub-
strate, suggesting that two metal ions are involved in the 
catalysis, as has been demonstrated for the MutT enzyme (15).
The salts, NaCl or KCl, reduced the enzymatic activity by 50% 
at concentrations of 200 mM.

**Products and Mechanism of the Reaction**—The nature of the 
products formed during the enzymatic hydrolysis of dATP were 
determined in standard colorimetric assay mixtures scaled up 
20-fold. Two reactions were run in parallel, except for the 
omission of yeast inorganic pyrophosphatase in one of them. 
Aliquots were removed over a 30-min period and analyzed for 
nucleotides by HPLC and inorganic orthophosphate as de-
scribed under “Methods.” In the absence of inorganic pyrophos-
phatase, no inorganic orthophosphate was detected throughout 
the incubation. Fig. 2 reports the results of the analysis of the 
incubation mixture containing yeast inorganic pyrophos-
phatase. The disappearance of dATP is reflected in the appear-
ance of dAMP and PP\(_i\) (calculated as 2 P\(_i\)) in approximately 
equivalent amounts. No dADP was detected during the course 
of the incubation and no P\(_i\) was detected in the absence of 
inorganic pyrophosphatase. Thus, the reaction describing the 
hydrolysis may be written as follows.

\[
dATP + H_2O \rightarrow dAMP + PP_i
\]

**REACTION 1**
The nature of the products are the same as those reported for 
two other related enzymes, the MutT and MutX nucleoside 
triphosphatases.

It had been demonstrated previously that the mechanism 
of hydrolysis of dGTP by MutT involves a nucleophilic attack on 
the β-phosphorus of the triphosphate, rather than on the more 
generally favored α-phosphorus, which would yield the same 
products (16). It was therefore of interest to determine whether 
the Orf17 enzyme, having a different specificity but producing 
similar products, also utilizes the same mechanism of attack. 
Accordingly, the hydrolysis of dATP by Orf17 was conducted in 
25% H\(_2\)O\(^{18}\) and the products were analyzed by \(^{31}\)P NMR spec-
troscopy. The proton decoupled 243-MHz \(^{31}\)P NMR spectrum of 
the reaction mixture is shown in Fig. 3. The course of the 
reaction, as measured by NMR spectroscopy, independently 
confirms the direct conversion of dATP to dAMP and PP\(_i\). Peaks 
corresponding to the substrate dATP and the products dAMP

![Fig. 2. Products of the reaction. A standard colorimetric assay (see “Methods”) was scaled up 20-fold and was monitored by HPLC to quantify dATP (●) and dAMP (○). No dADP was detected during the course of the reaction. PP\(_i\) (○) was converted to P\(_i\) by the inorganic pyrophosphatase included in the reaction mixture and was calculated as one-half of the P\(_i\) measured in the Fiske-Subbarow assay (31). No P\(_i\) was formed in a parallel reaction in the absence of inorganic pyrophosphatase.](image)

![Fig. 3. \(^{31}\)P NMR spectrum of the Orf17 dATPase reaction in H\(_2\)O\(^{18}\). The sample contained 6 mM dATP, 50 mM Tris, pH 9, 11 mM MgCl\(_2\), 25% H\(_2\)O\(^{18}\), 20% D\(_2\)O, 2 mM EDTA, and 1.2 units of Orf17 dATPase in a final volume of 650 μl. It was passed through a Chelex 100 column prior to the addition of MgCl\(_2\), EDTA, and enzyme. Orf17 dATPase was dialyzed against 50 mM Tris, pH 7.5, 1 mM EDTA, 0.1 mM DTT, and 5 g/liter Chelex 100. The spectrum was acquired and processed as described under “Methods.” The peaks for dAMP and PP\(_i\) are expanded and shown to the right of each respective peak in the spectrum. The resonance corresponding to pyrophosphate consists of two peaks, one downfield peak for pyrophosphate containing only \(^{18}\)O and one upfield for pyrophosphate labeled by \(^{18}\)O. The resonance for dAMP appears as a single peak, indicating no \(^{18}\)O labeling.](image)

and PP\(_i\) are observable, whereas no peaks corresponding to P\(_α\) 
(−12.4 ppm) or P\(_β\) (−8.3 ppm) of dADP are observable and the 
peak corresponding to P\(_i\) (0 ppm) is negligible. The spectrum for 
the reaction consists of a doublet centered at −13.0 ppm cor-
responding to the α-phosphorus of dATP, a triplet centered at 
−21.4 ppm corresponding to the β-phosphorus of dATP, and 
a doublet centered at −8.0 ppm corresponding to the γ-phos-
phorus of dATP, as well as resonances corresponding to dAMP (1.3 
ppm) and inorganic pyrophosphate (−7.8 ppm). The resonance 
at 1.3 ppm corresponding to the phosphorus of dAMP shows a 
single peak, indicating no \(^{18}\)O labeling of this product. Two 
resonances corresponding to inorganic pyrophosphate are ob-
erved, one resonance at −7.764 ppm corresponding to pyro-
phosphate containing only \(^{18}\)O and the other at −7.776 ppm 
corresponding to pyrophosphate labeled by \(^{18}\)O with a chemical 
shift difference of 0.012 ppm (2.85 Hz). \(^{18}\)O directly bonded to 
phosphorus causes an upfield chemical shift of the phosphorus 
resonance relative to the chemical shift of the resonance for
unlabeled phosphorus (17, 18). In pyrophosphate, the two phosphorus atoms are strongly coupled such that the resonances for unlabeled and $^{18}$O-labeled phosphorus collapse into an apparent singlet 0.012 ppm upfield of the resonance for unlabeled pyrophosphate (19). The intensity ratio of the resonances for unlabeled and $^{18}$O-labeled phosphorus is 3:1 consistent with 25% H$_2^{18}$O. Again, one peak was detected for dAMP and two peaks were detected for inorganic pyrophosphate with an intensity ratio of approximately 1:2 in respect to unlabeled PPi and $[^{18}$O]PPi (data not shown). Thus, Orf17 dATPase is similar to the MutT dGTPase in catalyzing a nucleophilic attack by H$_2$O (or initially by the enzyme, followed by H$_2$O) on the $\beta$-phosphorus of dATP or dGTP, respectively.

Kinetics—Standard procedures were used to determine the general kinetic parameters of the dATPase. Initial estimates of the $K_m$ and $V_{max}$ were obtained from double-reciprocal plots of substrate versus initial velocity (21) and then refined using a nonlinear least squares analysis weighted to substrate concentration (22)(data not shown). The values are reported in Table II.

An interesting aspect of the reaction emerged early in the project, when we noticed that the rate of dATP hydrolysis decreased markedly during the course of the incubation. Further investigation implicated product inhibition as the cause, since inclusion of inorganic pyrophosphatase in the reaction mixture restored initial rates. This is shown in Fig. 4A, where the time course of dAMP formation is compared in the presence and absence of inorganic pyrophosphatase. That PPi, and not dAMP is the product responsible for the inhibition is pointed up by the relative $K_i$ values determined from the plots in Fig. 4 (B and C). Note that the concentrations of dAMP and PPi differ by 100-fold in the two graphs, and the $K_i$ values calculated from these data are approximately 4 mM for dAMP and 20 mM for PPi. MutT nucleoside triphosphatase, which shares many of the properties of the Orf17 enzyme, has a 50-fold higher $K_i$ for PPi, and relatively little product inhibition.2 We have searched for possible covalent enzyme-substrate intermediates involving dAM$_32$P or 32PPi, without success, and neither of these labeled products exchanges with dATP within the limits of our measurements during the course of the reaction. Failure to detect a covalent deoxyadenylate-enzyme intermediate by exchange of 32PPi into dATP is consistent with a nucleophilic attack at the $\beta$-phosphorus rather than the $\alpha$-phosphorus, as found by $^{18}$O incorporation into pyrophosphate. Failure to detect a covalent pyrophosphoryl-enzyme intermediate by exchange of dAM$_32$P into dATP indicates either that the intermediate does not exist, or that it forms irreversibly.

TABLE II

| Parameter | $V_{max}$ | $K_{cat}$ | $K_i$ (dAMP) | $K_i$ (PPi) |
|-----------|-----------|----------|--------------|-------------|
|           | $18.0 \pm 0.6 \mu$mol min$^{-1}$mg$^{-1}$ | $5.2 \pm 0.16 \text{ s}^{-1}$ | $0.79 \pm 0.08 \text{ mM}$ | $4.2 \pm 0.2 \text{ mM}$ | $19 \pm 3 \mu$M |

In this regard, it is interesting to speculate about the Orf17 dATPase activity within the E. coli cell. Josse (23, 24), in his elegant studies on E. coli inorganic pyrophosphatase, has shown that the enzyme is constitutive and accounts for approximately 0.2% of the cellular protein. It hydrolyzes PPi at the rate of about 1.5 mmol min$^{-1}$ mg$^{-1}$, and it has a $K_m$ of 5 $\mu$M. Thus one might expect that the intracellular PPi concentration would be kept well below the 20 $\mu$M $K_i$ for the Orf17 dATPase, and that this enzyme would be active in the cytoplasm. However, Kukko and Heinonen (25) and Kukko-Kalske et al. (26) have shown that the intracellular concentration of PPi in E. coli

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2 S. K. Bhatnagar and M. J. Bessman, unpublished observations.
is 0.5 mM and is independent of very large fluctuations in the amount of inorganic pyrophosphatase in the cells. At 0.5 mM PPi, the dATPase would be expected to be almost completely inactive. Further investigations will be necessary to elucidate the mechanism of inhibition by PPi, and perhaps its significance will emerge when the physiological role of the enzyme (data not shown), the results in Table III clearly demonstrate that Orf17 does not complement mutT gene.

The conservation of cytoplasmic PPi concentrations may not be germane to its normal residence, the consideration of cytoplasmic PPi concentrations may not be germane to its in situ activity. Further investigations will be necessary to elucidate the mechanism of inhibition by PPi, and perhaps its significance will emerge when the physiological role of the dATPase is understood.

**What Is the Role of Orf17 dATPase?**—Although the orf17 gene is located close to ruvC, it probably is not involved in ruvC function (4). Our original interest in the Orf17 protein derived from a small region of amino acid homology common to it and MutT, as shown in Fig. 5A. This same region is highly homologous to MutX of *S. pneumoniae* (1), to a MutT homologue of *P. vulgaris* (8), and to a human 8-oxo-dGTPase (9). All of these latter four proteins are involved in preventing mutations of the AT → CG variety, and all of the genes coding for these proteins complement mutT strains. In addition, they all hydrolyze nucleoside triphosphates to PPi and nucleoside monophosphates. For these reasons, it was of interest to determine whether or not Orf17, which catalyzes similar reactions, is also involved in preventing mutations. Accordingly, a plasmid containing orf17 was used to transform a mutT strain of *E. coli*. Although the transformed strain produced large quantities of the enzyme (data not shown), the results in Table III clearly demonstrate that orf17 does not complement mutT. Thus, it is most likely not the putative suppressor of mutT referred to by Ray (27) or Desiraju (28). Furthermore, a strain of *E. coli*, lacking the orf17 gene, has no significant enhancement in mutation frequency when compared to its wild type parent (Table III). Thus Orf17 does not share the antimutator properties of MutT. A broader spectrum of tests will have to be performed in order to see if orf17 is involved in other mutational pathways.

On the other hand, it is quite possible that Orf17 dATPase does not play a role in antimutagenesis. We have included in Fig. 5B three additional proteins with the highly homologous mutT signature sequence, yet having no nucleoside triphosphatase activity and no apparent role in preventing mutations. Orf257 and Orf1.9 are newly discovered enzymes, which hydrolyze NADH (10) and GDP-mannose (11), respectively, and the

**TABLE III**

**Mutation frequencies of bacterial strains**

| Bacterial strain | Plasmid | Mutations/million cells |
|------------------|---------|------------------------|
|                  |         | 10^4                     |
|                  |         | 10^5                   |
|                  |         | 10^6                   |
|                  |         | 10^7                   |

*SB3 (mutT<sup>-</sup>)*  | pTrc99A    | 1200 ± 560              | 900 ± 520                |
|                        | pTrcMutT  | 7 ± 1                   | 4 ± 2                    |
|                        | pTrcOrf17 | 2000 ± 1200             | 2100 ± 1500             |
| AB1157 (orf17<sup>-</sup>) | HRS1024 (orf17<sup>-</sup>) | 7 ± 11 | <1 |

*SB3 (mutT<sup>-</sup>)* is a strain lacking a functional MutT protein, AB1157 (orf17<sup>-</sup>) is a wild type parent strain of HRS1024 (orf17<sup>-</sup>), and HRS1024 (orf17<sup>-</sup>) is a strain lacking a portion of the orf17 gene.

*Numbers represent the average (± standard deviation) mutation frequency of five separate cultures. NaN and strep<sup>p</sup> are colonies resistant to nalidixic acid or streptomycin, respectively.

**—,** not determined because both AB1157 and HRS1024 already contain a streptomycin resistance factor.
motif of an
vealsthissignature sequencetobelocatedintheloop1-helix1
mentation of diverse metabolic reactions involving nucleoside
signature sequence represents a nucleoside diphosphate bind-
nucleoside diphosphate linkage. It seems likely that the MutT
their recognition and hydrolysis of metabolites containing a
observations.
their substrate preferences and detailed reactions. It will be of
zymes3 all contain the MutT signature sequence, but differ markedly in
phosphatases.3 The feature common to all these enzymes is
We are presently characterizing two new enzymes bearing the
MutT signature sequence, which also are not nucleoside
nosophatase has recently been cloned from human tissue (29).
Finally, the results presented here introduce a note of cau-
tion in ascribing MutT antimutator function to newly discov-
ered proteins containing the MutT signature sequence. The
Orf17 dATPase described here, as well as the NADH pyrophos-
all contain the MutT signature sequence, but differ markedly in
without the enzyme, along with a clone overexpressing it, pro-
sequently for comparative experiments designed to elucidate the metabolic role of the enzyme.

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