The Gene \(e.1\) (\(nudE.1\)) of T4 Bacteriophage Designates a New Member of the Nudix Hydrolase Superfamily Active on Flavin Adenine Dinucleotide, Adenosine 5′-Triphospho-5′-adenosine, and ADP-ribose*

The T4 bacteriophage gene \(e.1\) was cloned into an expression vector and expressed in \(Escherichia coli\), and the purified protein was identified as a Nudix hydrolase active on FAD, adenosine 5′-triphospho-5′-adenosine (Ap3A), and ADP-ribose. Typical of members of the Nudix hydrolases, the enzyme has an alkaline pH optimum (pH 8) and requires a divalent cation for activity that can be satisfied by \(\text{Mg}^{2+}\) or \(\text{Mn}^{2+}\). For all substrates, AMP is one of the products, and unlike most of the other enzymes active on Ap3A, the T4 enzyme hydrolyzes higher homologues including Ap4-A, Ap5-A. This is the first member of the Nudix hydrolase gene superfamily identified in bacterial viruses and the only one present in T4. Although the protein was predicted to be orthologous to \(E. coli\) MutT on the basis of a sequence homology search, the properties of the gene and of the purified protein do not support this notion because of the following. (a) The purified enzyme hydrolyzes substrates not acted upon by MutT, and it does not hydrolyze canonical MutT substrates. (b) The \(e.1\) gene does not complement mutT1 in \(vivo\). (c) The deletion of \(e.1\) does not increase the spontaneous mutation frequency of T4 phage. The properties of the enzyme most closely resemble those of Orf186 of \(E. coli\), the product of the \(nudE\) gene, and we propose the mnemonic \(nudE.1\) for the T4 phage orthologue.

The Nudix hydrolase superfamily consists of widely distributed subfamilies of enzymes characterized by a shared structural motif: the Nudix box having a highly conserved consensus sequence,

\[
\text{GXXX}E\text{XXXXXXREUXEEYGU}
\]

SEQUENCE 1

where “X” represents any amino acid, and \(U\) usually represents one of the bulky, hydrophobic amino acids, Ile, Leu, or Val (1). Enzymological and structural studies have shown that this arrangement of amino acids leads to a novel loop-helix-loop motif involved in substrate binding and catalysis (2–8). A recent BLAST(9) search of the data banks for the Nudix signature sequence has revealed over 800 open reading frames from more than 200 species including archaea, eubacteria, and eukarya, and at present, ~50 of the gene products have been identified. These are all enzymes active on nucleoside diphosphate derivatives including (deoxy)nucleoside triphosphates, coenzymes, cell signaling molecules, and nucleotide sugars (1), and we have been systematically identifying and characterizing new members of the superfamily.

An examination of the T4 bacteriophage genome reveals one gene, \(e.1\) (GenBank™ accession numbers NC_000866 and NP_049737) coding for an open reading frame containing a consensus Nudix box signature sequence (Fig. 1). In a recent paper, Kawahata, Arisaka, and Nishikawa (10), using the PSI-BLAST sequence homology search algorithm (11), examined the T4 genome for unidentified open reading frames and predicted that the unknown protein designated by gene \(e.1\) was an orthologue of MutT, the antimiturator (deoxy)nucleoside triphosphatase of \(Escherichia coli\) (12–14). In this paper we describe the cloning of \(e.1\), its expression in \(E. coli\), its purification, and its identification not as an orthologue of MutT but as a new member of the Nudix hydrolases active on FAD, ADP-ribose, and Ap3A.\(^1\) This represents the first member of the Nudix hydrolase superfamily cloned, expressed, and characterized from a bacteriophage. In keeping with the nomenclature for the Nudix hydrolase genes of the \(E. coli\) and human genomes, we propose to assign the mnemonic \(nudE.1\) to this gene in T4 bacteriophage in recognition of its original \(e.1\) designation, and also because the enzyme it signifies most closely resembles NudE in \(E. coli\).

EXPERIMENTAL PROCEDURES

Materials

The expression plasmid pET-24a(+) (Km') and \(E. coli\) HMS174 (DE3) were from Novagen (Madison, WI). Phages Li/a (a lysozyme amber mutant, referred to as \(\text{am}^c\) in the text) and \(\text{hi}^{NB} 3004\) were from the collection of Larry Gold, University of Colorado, Boulder. The bacteria CR63 (\(\text{supD}\)), CR63a (\(\text{supD}\), \(\lambda\) lysogen), NapIV (\(\text{supD}\)), and NapIV (\(\text{supD}\)) were from the bacterial strain collection of Larry Gold (15). T4 strains used as templates for cloning the \(e.1\) gene were gifts of Linda Reha-Krantz, University of Alberta, Canada, and Lindsay W. Black, University of Maryland. The T4 DNA used as a template for PCR was extracted by following the procedure described by Sambrook and Russell (16). The plasmid pTrc99A (Amp') and Sephadex G-100 were from Amersham Biosciences, and the restriction enzymes, PCR kits, calf intestinal alkaline phosphatase, and inorganic pyrophosphatase were from Stratagene (La Jolla, CA). The plasmid pCR®2.1-Topo® is a product of Invitrogen. The homogeneous \(E. coli\) MutT (deoxy)nucleoside

\(^1\) The abbreviations used are: Ap3A, diadenosine triphosphate, adenosine 5′-triphospho-5′-adenosine; Ap4A, diadenosine polynucleotides; Km', kanamycin resistance; Amp', ampicillin resistance.
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Methods

Cloning of Gene e.1 into pET24a(+) and pTrc99A—Gene e.1 was amplified from strain T4 DNA using the polymerase chain reaction. Ndel and BamHI sites were incorporated at the start and the end of the gene, respectively. The amplified gene was purified, digested with Ndel and BamHI, and ligated into pET-24a(+) cloning vector under the control of the T7 lac promoter. The resultant plasmid, pETe.1, was transformed into E. coli DH5α for storage and into HMS174 (DE3) for protein expression.

Gene e.1, containing NcoI and BamHI sites, was amplified from the above pETe.1 and ligated into the respective sites of pTrc99A to form pTrc.1 under the control of the trc promoter.

Construction of a nudE.1 Deletion Mutant (nudE.1Δ1 Phage)—The plasmid pCR2.1nudE.1 contains a constructed Swa1 deletion that removes the amino-terminal half of the nudE.1 gene. This mutant allele is referred to as nudE.1Δ1, e.m. phage (10⁷/ml) was used to infect exponentially growing NapIV supD (2 × 10⁷/ml) with or without plasmid pCR2.1nudE.1. After 90 min, chloroform was added to induce lysis, and the diluted progeny were plated on permissive and non-permissive NapIV. e.m.-infected cells containing the pCR2.1nudE.1 plasmid produced a 100-fold greater frequency of e. phage than the control infection (data not shown). The recombination event that selected the e. allele may replace the nudE.1 gene of the phage with the nudE.1Δ1 allele of the plasmid. 24 e. phage plaques were screened for the nudE.1Δ1 allele by PCR on DNA extracted by the method of Joewick and Miller (18). One of the 24 plaques yielded phage with the deletion in e.1.

Expression and Enzyme Purification—One colony of the expression strain pETe.1/HMS174(DE3) was inoculated into 40 ml of LB medium containing 50 μg/ml carbenicillin, incubated at 37 °C overnight, and inoculated into 2 liters of the same medium. When the culture reached an A₆₀₀ of 0.3, it was transferred to an incubator at 22 °C and grown to an A₆₀₀ of 100. At this point, isopropanol was added to a final concentration of 0.5%, and the cells were grown for an additional 12 h, harvested, and washed in buffered saline. The washed cells were suspended in 2.5 volumes of 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.1 mM diithiothreitol (buffer A), disrupted in a French press, and centrifuged to remove cell debris, and the supernatant was adjusted to about 10 mg of protein/ml with buffer A (Fraction I). Streptomycin was added to a final concentration of 1.5%, and the precipitated nucleoproteins were removed by centrifugation. The supernatant (Fraction II) was brought to 50% saturation with solid ammonium sulfate, and the precipitate was collected and dissolved in a minimal volume of buffer A (Fraction III). Fraction III was chromatographed on a Sephacryl G-100 gel filtration column in buffer A plus 200 mM sodium chloride. The fractions containing the e.1 protein were pooled and stored at −80 °C (Fraction IV).

Enzyme Assays—The enzyme assay measures the conversion of a phosphatase-resistant substrate to a phosphatase-sensitive product. A standard reaction mixture (50 μl) containing 50 μl Tris-Cl, pH 8.0, 5 mM Mg²⁺, 2 mM substrate, 4 units of alkaline phosphatase, and 0.1–2 milliunits of enzyme was incubated at 37 °C for 15 min. The inorganic orthophosphate liberated was assayed by the colorimetric procedure of Ames and Dubin (19). When (deoxy)nucleoside triphosphates are tested as substrates, the assay must be modified since alkaline intestinal phosphatase is active on triphosphates, and also there is a slow hydrolysis of triphosphates to inorganic orthophosphate during the course of color development. In this case, the alkaline phosphatase is replaced by 0.5 unit of inorganic pyrophosphatase, and the reaction is terminated by the addition of Norit, which adsorbs the residual nucleoside triphosphates. An aliquot of the Norit supernatant is then used for the determination of inorganic orthophosphate as above. In both assays, a unit of enzyme catalyzes the hydrolysis of 1 μmol of substrate/min.

RESULTS AND DISCUSSION

Gene Cloning, Expression, and Protein Purification—The cloned gene was sequenced, and it was congruent with that reported for e.1 (nucleotides 67037–67490 of the T4 genome, GenBank™ accession number NC_000866). The gene expressed well at 37 °C, but almost all of the extracted protein was insoluble and precipitated during centrifugation. At 22 °C, the expression rate was reduced, but the yield was comparable to that at the higher temperature, and under these conditions the protein was found almost entirely in the soluble fraction suitable for purification. After chromatography on a Sephacryl G-100 sizing column, the protein appeared highly purified when analyzed by electrophoresis on a denaturing gel, but as shown in Fig. 2, a satellite band is visible slightly below the bulk of the protein. Analyses of the amino-terminal regions of both bands indicated that the less abundant, faster moving species was five amino acids shorter than the major band, most likely resulting from an internal start on a methionine codon located in this position. No attempts were made to remove this slightly truncated species from the bulk of the protein.

Identification of Enzyme Activity—Since the e.1 gene was predicted to be an ortholog of MutT (10), the first candidate substrates we tried were (deoxy)nucleoside triphosphates, known to be rapidly hydrolyzed by E. coli MutT (13). However, no significant hydrolysis was observed. Since all enzymes containing the Nudix box signature sequence so far discovered hydrolyze several different nucleotide diphosphate derivatives, we surveyed a large selection and found that FAD, Ap₃A, and ATP were hydrolyzed in the presence of ADP-ribose were favored substrates. These data are shown in Table I. For comparison, the specificity of MutT is also shown. It is obvious that the two enzymes have strikingly different specificities with no overlap. In each case, the preferred substrates are not substrates of the other. On the other hand, the T4 enzyme resembles Orf186 of E. coli (20) in its...
spectrum of favored substrates, the most notable difference being the higher activity on FAD. This is also shown in Table I where the enzymes can be directly compared. Because of their similarity, we proposed the mnemonic nudE I to designate the T4 gene to signal its relationship to the orthologous gene coding for Orf186 named nudE in the E. coli Genetic Stock Center, Yale University. A summary of the kinetic properties of the T4 enzyme is shown in Table II. Although FAD has the highest \( V_{\text{max}} \), it also has the highest \( K_m \). In terms of catalytic efficiency, \( K_{\text{cat}}/K_m \), ApA and ADP-ribose have 2–3 times higher activity, respectively.

**Products of the Reaction**—A common mechanism of virtually all members of the Nudix hydrolase superfamily studied so far is a nucleophilic attack by water on a pyrophosphate linkage in the substrate. An analysis of the products of hydrolysis of FAD, ADP-ribose, and a series of diadenosine polyphosphates indicates that the T4 enzyme also uses this mechanism. As identified by high performance liquid chromatography, all three substrates liberate AMP as one of the products, and no inorganic orthophosphate is formed during the course of the reaction. The stoichiometry may therefore be described by the following equations.

FAD + H\(_2\)O \(\rightarrow\) AMP + FMN (Eq. 1)

ADP-ribose + H\(_2\)O \(\rightarrow\) AMP + ribose-5-P (Eq. 2)

ApA + H\(_2\)O \(\rightarrow\) AMP + Apn-1 (Eq. 3)

Several aspects of the hydrolysis of the diadenosine polyphosphate series of compounds are surprising. First, although ApA is the best substrate of the group, ApA is hydrolyzed at about 70% of the rate, and there is practically no diminution in rate for the next two higher homologues. This is uncharacteristic for enzymes hydrolyzing diadenosine polyphosphates, Nudix hydrolases or otherwise, since those enzymes active on ApA are generally not active on the higher homologues. An example may be seen in Table I where Orf186 is highly active on ApA but has little or no activity on ApA. Second, no inorganic orthophosphate is formed during the course of hydrolysis indicating that the immediate products formed (ADP, ATP, adenosine tetraphosphate, etc.) are not further degraded. Many of the ApA pyrophosphatases further degrade their initial products. Third, one of the hydrolytic products is always AMP independent of the length of the polyphosphate linker. This means that the nucleophilic attack must always occur on the \( \alpha \) or \( \beta \) phosphorus, which is unique among this class of enzymes.

**Other Properties of the Enzyme**—The T4 enzyme has an absolute requirement for a divalent cation, and under optimal concentrations Mn\(^{2+}\) is about 60% as effective as Mg\(^{2+}\). No other cations tested including Zn\(^{2+}\), Ca\(^{2+}\), or Co\(^{2+}\) are effectual. In keeping with almost all of the Nudix hydrolases, the enzyme has an alkaline pH optimum of about 8.0. Although the purified enzyme has a calculated molecular mass of 17,653 Da and migrates on an electrophoretic denaturing gel with the expected mobility (see Fig. 2), it eluted from a calibrated gel filtration column at the position characteristic of a 30-kDa protein. One interpretation of this anomaly is that the protein is a homodimer with an asymmetric Stokes radius, causing it to appear smaller than the 35 kDa we would expect of a dimer. Another possibility is that the protein is retarded by nonspecific interactions with the column matrix. We favor the former interpretation because this same column has given reliable measurements, under the same conditions, for several other proteins. It is interesting that E. coli Orf186, the enzyme most closely resembling the T4 enzyme in respect to substrate spec-
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**TABLE III**
Non-complementation of mutT<sup>+</sup> by T4 e.1

| SB3/pTrc99A | SB3/pTrc99A mutT | SB3/pTrc99A e.1 |
|-------------|-----------------|-----------------|
| 4364 ± 1599 | <10             | 3948 ± 1611     |

**TABLE IV**
Burst sizes of nudE.1<sup>+</sup> and nudE.1ΔI

| Phage       | Experiment 1 | Experiment 2 |
|-------------|--------------|--------------|
| nudE.1<sup>+</sup> | 290          | 100          |
| nudE.1ΔI    | 270          | 140          |

* Burst size is number of phage produced/infected bacterium.

**Effect of nudE.1 Deletion on T4 Replication**—To assess the importance of gene nudE.1 to phage growth, a deletion mutant, nudE.1ΔI, was constructed as described under “Methods,” and its growth was compared with wild type phage. Table IV shows the results of two separate experiments in which the number of phage/bacterium (burst size) for the wild type and the mutant are listed. No significant differences are evident between the parental and mutant phage. Thus nudE.1 does not appear to be required for normal phage growth and replication under these conditions. According to Kutter et al. (22), approximately half of the 300 or so genes in T4 phage have unknown functions and are in regions that are likely to be deletable under standard laboratory conditions. It stands to reason that genes important in the physiology and life cycle of T4 inhabiting the colon, its normal ecological niche, may have diminished value when infecting its host, E. coli, growing under optimized conditions of nutrition, atmosphere, climate, and population density. This is especially true of the NudE.1 enzyme since its enzymatic activity parallels Orf186 of its host. As pointed out previously (1), the Nudix hydrolases are surveillance enzymes that are involved in removing potentially toxic metabolites such as ADP-ribose, preventing the accumulation of normal metabolic intermediates, and redirecting them to other areas of metabolism or removing stress-induced signaling molecules such as diadenosine polyphosphates. In its normal habitat of low oxygen tension, overcrowding, and limited nutrition, NudE.1 might play a supportive role in phage growth by carrying out these functions.

Finally it should be noted that this is another example of proteomics gone astray. Since the first member of the Nudix hydrolase superfamily was MutT (1) there has been a tendency to assume that open reading frames containing the Nudix box signature sequence are proteins functionally related to the MutT enzyme, despite the fact that MutT itself is only one member of a large and still growing list of enzymes with unrelated functions. The Nudix box motif is the binding and catalytic site for hydrolysis of nucleoside diphosphate derivatives, but the specificity for individual substrates lies outside this region. We have been partially successful in uncovering landmarks distal to the Nudix box that designate family members with related enzymatic activities (23), and we are continuing our search for these signposts so that reliable predictions can be made relating sequence homology to function.

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The deletion of nudE.1 does not increase the forward mutation frequency to acridine resistance (W.L. Xu, P. Gauss, J.Y. Shen, C. A. Dunn, and M. J. Bessman, unpublished observations).

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**Fig. 3. Products of the hydrolysis of Ap₃A, Ap₄A, and Ap₅A.** Standard reaction mixtures (omitting alkaline phosphatase) were incubated at 37 °C, applied to a calibrated reverse phase C₄ column, and analyzed by high performance liquid chromatography. The bottom panel shows the profile for nucleotide standards.
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