PROTEIN EXPRESSION AND PURIFICATION 4, 149–159 (1993)

dUTPase from Herpes Simplex Virus Type 1; Purification from Infected Green Monkey Kidney (Vero) Cells and from an Overproducing *Escherichia coli* Strain

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Received August 31, 1992, and in revised form November 27, 1992

Deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase), widespread in nature with a crucial role in the nucleotide metabolism, catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate. The enzyme from herpes simplex virus type 1 (HSV-1 dUTPase) was overproduced in *Escherichia coli* by using the T7 RNA polymerase expression system. The coding region of the HSV-1 dUTPase gene, UL 50, was positioned downstream of the promoter and the ribosome-binding site of the phage T7 gene 10 on the expression vector pET-3a. The resulting recombinant plasmid, pET-3a/UL50, was transformed into *E. coli* BL21(DE3)pLysS cells, conferring expression of HSV-1 dUTPase as 2–3% of the soluble protein inducible by isopropyl thiogalactoside. By chromatography on phosphocellulose and Mono S (Pharmacia LKB) columns a nearly homogeneous preparation of the enzyme with a high specific activity (49 μmol per minute per milligram) was obtained. The recombinant protein was compared with the native dUTPase similarly purified from HSV-1-infected Vero cells (African green monkey kidney fibroblasts). The two proteins showed the same mobility on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the amino-terminal sequences were found to be identical. The molecular mass (39 kDa) and the amino acid composition of the recombinant enzyme are also in accordance with predictions from the DNA sequence. Thus, the overproducing system described here appears suitable for providing HSV-1 dUTPase for detailed studies of molecular properties. © 1993 Academic Press, Inc.

Herpes simplex virus type 1 (HSV-1) encodes three proteins that participate in the metabolism of DNA precursors: ribonucleotide reductase, thymidine kinase, and deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase, EC 3.6.1.23). The enzyme dUTPase catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate (1), providing both (a) a mechanism to prevent incorporation of dUMP into DNA and (b) a pool of dUMP, the precursor of thymidine nucleotides (2). Unsuccessful attempts to construct *Escherichia coli* mutants by insertional inactivation of the structural gene for dUTPase (*dut*) indicate an essential role for the enzyme (3).

Although dUTPase activity has been reported in a variety of organisms, conclusive protein sequence information is so far confined to the enzymes from *E. coli* and HSV-1 where the structural genes have been identified and sequenced. The *E. coli dut* gene, encoding a polypeptide chain for dUTPase of 152 amino acid residues (4), has been cloned in expression plasmids providing enzyme for detailed studies (5). The three-dimensional structure, recently determined by X-ray crystallography to a resolution of 1.9 Å (6), shows a trimeric arrangement of identical subunits.

Identification of the structural gene for HSV-1 dUTPase, UL50, was made by Preston and Fisher (7). The complete sequence determination of the unique long region in the HSV-1 (strain 17) genome (8), including UL50, gave a deduced molecular mass for the enzyme of 39,125 Da, 371 amino acid residues, and an isoelectric point of 8.0. These figures are in good agreement with a biochemical investigation by Caradonna and Adamkiewicz (9) who separated and partially characterized the viral and cellular dUTPase activities from HSV-1 (strain F)-infected HeLA S3 cells. These authors also showed that the viral enzyme is a monomer. By DNA sequence comparisons, putative genes for dUTPases have also been identified in other members of the herpesvirus family (10,11).

McGeoch (12) discovered that the so-called pseudoproteases, encoded by certain poxviruses and retrovi-

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1046-5928/93 $5.00

ruses and of unknown function, have five conserved sequence motifs in common with the dUTPases from *E. coli* and herpes viruses. He suggested that the pseudoproteases could be dUTPases. This prediction was confirmed when retroviral dUTPase activity was identified in several nonprimate lentiviruses (13).

In our laboratory we have been investigating the dUTPase from *E. coli* and earlier work on cloning and overproduction (5) has greatly facilitated those studies. Here, we extend our investigations to dUTPase encoded by HSV-1. The enzyme has been partially purified from virus-infected Vero cells and we have made a genetic construct for overproduction of the enzyme in *E. coli*. A method is described for purification of the recombinant enzyme to near homogeneity.

MATERIALS AND METHODS

Chemicals. The detergent polyoxyethylene 10 tridecyl ether (i-$C_{12}E_{10}$) was obtained from Sigma. Triton X-100 was from Riedel-de Haen AG. Analytical chemicals were from Sigma or Boehringer. Recombinant *E. coli* dUTPase was prepared as described previously (5).

Buffers. Extraction buffer: 20 mM Hepes, pH 7.3, 50 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin 2 μg ml^{-1}, pepstatin A 2 μg ml^{-1}. The extraction buffer was used with or without 0.1% detergent (polyoxyethylene 10 tridecyl ether).

Buffer A: 20 mM Hepes, pH 7.3, 50 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, leupeptin 2 μg ml^{-1}, pepstatin A 2 μg ml^{-1}, and 0.01% polyoxyethylene 10 tridecyl ether.

Buffer B: 50 mM MES, pH 6.5, 100 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, leupeptin 2 μg ml^{-1}, pepstatin A 2 μg ml^{-1}, and 0.01% polyoxyethylene 10 tridecyl ether.

Bacterial strains, plasmids, and viruses. Plasmid pIIE1, a pAT153 derivative carrying a 4-kilobase-pair (kbp) *Eco*RI fragment of the HSV-1 chromosome, containing the viral dUTPase gene (UL50) (7), was kindly provided by Dr. V. Preston (Department of Virology, Glasgow, UK).

E. coli DH5a F' (Promega) was used for initial cloning and in the experiments on mutagenesis. *E. coli* BL21(DE3) pLysS, carrying a chromosomal copy of the T7 RNA polymerase gene under control of the *lac* UV5 promoter, was generously provided by Dr. F. W. Studier (Brookhaven National Laboratory, Upton, NY). The plasmid pLysS confers resistance to chloramphenicol and harbors the T7 lysozyme gene. Plasmid pET-3a (14), in a modified version containing a *Hin*dIII site downstream of the transcriptional termination signal (15), was kindly provided by Dr. P. Elias (Department of Medical Biochemistry, Göteborg, Sweden).

TABLE 1

Extraction of dUTPase from *E. coli* Cells Overproducing HSV-1 dUTPase

Extraction method	Growth conditions (°C)	Total protein (mg)	Total enzyme activity (μmol min^{-1})
Without detergent	24	1.9	0.72
With detergent	24	2.2	5.2
Without detergent	37	2.0	1.0
With detergent	37	2.2	4.5

Note. E. coli BL21(DE3)pLysS pET-3a/UL50 cells were grown and induced at 24 and 37°C. Aliquots of 10 ml of the bacterial culture (250 ml) were centrifuged (Sorvall SS-34 rotor, 4000*g*, 10 min, 4°C), and the cells resuspended in 2 ml of extraction buffer with or without detergent (polyoxyethylene 10 tridecyl ether). After freezing and thawing (three rounds) the extraction mixtures were centrifuged (Sorvall SS 34-rotor, 17,000*g*, 1 h, 4°C). Aliquots of the supernatants were assayed for enzyme activity after dilution in the respective buffer used in the extraction.

The expression vector pMG524 (16), harboring the λp_L promoter followed by a multiple cloning site, was used in conjunction with the *E. coli* strain N4830 (Pharmacia LKB), encoding the temperature-sensitive phage repressor cI857.

RΔ305 (17), a thymidine kinase-deficient mutant of HSV-1 (strain F), was used to infect Vero cells (African green monkey kidney fibroblasts).

Assay of dUTPase activity. Assay for dUTPase was carried out with [^{3}H]dUTP (Amersham, UK) according to Tye *et al.* (18). Nonradioactive dUTP (Sigma) was purified on a Mono Q (Pharmacia LKB) column. Protein solutions to be assayed were diluted up to 1000-fold in Buffer A except in the experiment on extraction of dUTPase from the bacterial cells (Table 1) where dilutions were made in the respective extraction buffer. The polyethyleneimine–cellulose sheets (Polygram, Machery-Nagel, Germany) for thin-layer chromatography were prewashed in 1.0 M formic acid.

Protein determination. Protein concentrations were determined according to the microprotein assay by Bradford (19) using bovine serum albumin as the standard.

Extraction of native HSV-1 dUTPase from infected Vero cells. Growth and infection of Vero cells, harvesting, disruption, and subsequent separation of the homogenate into nuclear and cytosolic fractions have been described previously (20).

Cloning procedure. DNA manipulations were carried out by standard methods (21). Restriction enzymes and DNA ligase from Boehringer or New England Biolabs

(*Nde*I) were used following recommendations by the manufacturers.

Mutagenesis was performed according to Amersham (kit RPN 1523) with a mutagenesis primer 5'-CTAACAGG^{C}A^{T}ATGAGTCAGTGGGG-3' synthesized and purified at BM Enheten, University of Lund. Mutant M13 phages were selected by hybridization with the mutagenesis primer, 5'-end labeled with [γ^{32}P]ATP (Amersham, UK). The base changes introduced were confirmed by DNA sequencing using the universal M13 primer and a sequencing kit from U.S.B.

Expression of recombinant HSV-1 dUTPase in E. coli. Growth and induction of *E. coli* were performed essentially according to the recommendations by Studier *et al.* (14). A single colony of *E. coli* BL21(DE3) pLysS harboring the recombinant plasmid pET-3a/UL50 was grown overnight in 2× YT medium (21) containing 150 μg ml^{-1} ampicillin and 25 μg ml^{-1} chloramphenicol. On a preparative scale, an aliquot (4 ml) of the overnight culture was used to inoculate 250 ml 2× YT in 40 mM potassium phosphate buffer, pH 7.5, also containing antibiotics as above. When the culture, grown at 24 or 37°C, reached an optical density of 0.7 at 600 nm, isopropyl thiogalactoside (IPTG) was added to a final concentration of 0.5 mM and the incubation was allowed to proceed for additional 4 h at the respective temperature. Cells were harvested by centrifugation (Sorvall GSA rotor, 4000*g*, 10 min, 4°C) and resuspended in extraction buffer.

Protein purification. Phosphocellulose (Whatman P-11) was pretreated according to the manufacturer. The cation-exchange column Mono S HR 5/5 and the gel-filtration column Superose 12 HR 10/30 were obtained from Pharmacia LKB. The FPLC system (Pharmacia) was run at room temperature. Otherwise, purification of the enzyme was carried out at 4°C. Dialysis bags (Spectra/por 3) immersed in Na-carboxymethyl cellulose (Acquacide I, Behring Diagnostics) were used for concentrating protein solutions.

Gel electrophoresis and protein blotting. One-dimensional SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (22) was run on a Protean equipment from Bio-Rad. The gel contained 10% acrylamide in the resolving gel and 4% in the spacer gel. Blotting to polyvinylidene difluoride (PVDF) membrane (Millipore) was performed according to Wittbjer *et al.* (23) using a transfer time of 90 min.

Two-dimensional (2-D) separation according to O'Farrell (24) was carried out using a GE-2/4 LS equipment (Pharmacia). The proteins were first separated by isoelectric focusing on a gel rod (2-D Pharmalyte 3-10, Pharmacia) followed by size separation on a gradient SDS–PAGE (12–20%). To establish the gradient, a gel rod was sliced into pieces of 1 cm on which pH values were measured.

Molecular-weight markers (LMW calibration kit, Pharmacia) were run in parallel. Proteins were visualized by staining with 0.1% Coomassie brilliant blue R-250.

Amino acid analysis. Analysis was carried out on a performic acid-oxidized sample of recombinant HSV-1 dUTPase hydrolyzed in 6 M HCl for 20 h at 110°C. A LKB Plus amino acid analyzer Model 4151 with ninhydrin detection was used.

Edman degradation. Samples of HSV-1 dUTPase, blotted onto PVDF membranes, were subjected to Edman degradation using an automatic gas phase sequencer.

RESULTS

Purification of HSV-1 dUTPase from infected Vero cells. Caradonna and Adamkiewicz (9) described phosphocellulose chromatography for the separation of HSV-1 and host cell dUTPases. The phosphocellulose chromatography used by Elias *et al.* (20) was found to perform in a similar manner and was utilized to estimate the content of HSV-1 dUTPase in extracts from virus-infected Vero cells. The cytosolic extract was found to contain appreciable amounts of the viral enzyme, significantly higher (about 7×) than a 1.7 M NaCl extract of the nuclear pellet, and was chosen as a starting material for purification of the native HSV-1 dUTPase.

Ammonium sulfate was added to the cytosolic extract under stirring at 4°C to a 70% saturation and left overnight. The precipitate was collected by centrifugation (Sorvall GS-3 rotor, 11,000*g*, 30 min, 4°C) and stored at −80°C. About 10 g of the ammonium sulfate precipitate (corresponding to cytosolic extract from approximately 20 g of Vero cells) was dissolved in a small volume of Extraction buffer containing 0.1% detergent (polyoxyethylene 10 tridecyl ether) and extensively dialyzed against Buffer A. In the extract, which included pepstatin A, leupeptin, and PMSF, the enzyme retained its activity several days in the cold room. If these inhibitors were omitted, a rapid loss of dUTPase activity could be observed. The dialysate was centrifuged at 90,000*g* in a Beckman Ti 60 rotor for 2 h at 4°C. About 80% of enzyme activity was recovered in the supernatant which was used for purification of the HSV-1 dUTPase as summarized in Table 2.

Of the clear supernatant, 20 ml (containing 220 mg protein) was loaded on a phosphocellulose column (1.6 × 10 cm) and chromatographed as described in the legend to Fig. 1A. Two peaks of enzyme activity were located in the chromatogram by the dUTPase assay. The first peak, containing the host cell dUTPase (see Discussion), appeared at 0.27 M NaCl. The HSV-1 dUT-

TABLE 2

Preparations of Native and Recombinant HSV-1 dUTPase

Purification step	Total protein (mg)	Total enzyme activity (μmol min^{-1})	Specific enzyme activity (μmol min^{-1} mg^{-1})	Purification (fold)	Yield (%)
		A			
Lysate supernatant[a]	220	3.4	0.015	1	100
Phosphocellulose	5.5	3.1	0.56	37	91
Mono S	1.8	2.0	1.1	73	59
		B			
Lysate supernatant	118	201	1.7	1	100
Phosphocellulose	4.4	132	30	18	66
Mono S	2.4	117	49	29	58

Note. (A) HSV-1 dUTPase from a cytoplasmic extract of about 20 g virus-infected Vero cells. (B) Recombinant enzyme from 500 ml of *E. coli* strain BL21(DE3)pLysS pET-3a/UL50 culture induced at 24°C.

[a] The host dUTPase from the Vero cells present in the lysate supernatant becomes separated from the viral enzyme in the phosphocellulose step. Its contribution to the total activity in the lysate supernatant, estimated to 22% from the chromatogram in Fig. 1A, has been subtracted.

Pase, eluted as the second peak at 0.40 M NaCl, was used for further purification.

The HSV-1 dUTPase from the phosphocellulose column was rechromatographed on a cation-exchange column. The peak fractions (Fig. 1A) were pooled (18 ml), concentrated in Aquacide, diluted 10-fold in Buffer B without NaCl, and loaded on a Mono S column attached to FPLC equipment. The experimental conditions for the chromatography are described in the legend to Fig. 2A. HSV-1 dUTPase was eluted at 0.39 M NaCl. Fractions containing the enzyme were pooled, concentrated with Aquacide, and subjected to SDS–PAGE (Fig. 3, lane 10) and protein blotting for Edman degradation.

Use of detergent improved the purification of the native dUTPase and the most dramatic effect was observed in the initial dialysis of the ammonium sulfate precipitate. When the dialysis was performed in the absence of detergent, the recovery of activity in the supernatant obtained in the subsequent ultracentrifugation was 30–50%. The presence of detergent increased the recovery to about 80%. On phosphocellulose chromatography, the addition of detergent increased the activity of peak 2 containing HSV-1 dUTPase (at 0.40 M NaCl) whereas the activity of the host (Vero) cell dUTPase in peak 1 (at 0.27 M NaCl) appeared unaffected.

Expression vector pET-3a/UL50. Digestion of plasmid pIIE1 with *Bal*I gave a 1710-bp fragment, carrying the dUTPase gene, UL50. The isolated fragment, containing the coding base pairs (1113 bp) and flanking regions, was ligated into the λp_L vector pMG524, predigested with *Sma*I, and treated with alkaline phosphatase. The recombined DNA was used to transform *E. coli* N4830. Positive clones harboring the fragment in the desired orientation with respect to the λp_L promoter were identified by restriction enzyme analysis. No overexpression of dUTPase could be detected, however, when this construct was tested by heat induction at 42°C.

Another expression system, the pET vector (14), containing start signals for transcription and translation from bacteriophage T7 gene 10, was used for further attempts. In order to position the start codon of UL50 relative to the ribosome-binding site of the vector, the 5′ flanking sequences of UL50 had to be deleted. This was achieved by site-directed mutagenesis. The 1710-bp HSV-1 fragment was moved from pMG524 to M13mp19 using the *Eco*RI and *Hin*dIII sites in these vectors. Single-stranded DNA was prepared and mutagenesis was carried out with a mutagenic primer designed for the purpose of introducing a *Nde*I restriction site (CATATG) at the start codon (ATG) (see Table 3). Since the mutation frequency was unexpectedly low (10%), screening was performed by hybridization. The replicative form of the selected M13 clone was prepared and hydrolyzed by *Nde*I and *Hin*dIII to generate a 1.5-kb fragment which was inserted in pET-3a. The resulting construct (Fig. 4), called pET-3a/UL50, was transformed into *E. coli* BL21(DE3) pLysS cells.

The new strain, *E. coli* BL21(DE3) pLysS pET-3a/UL50, was tested for overexpression at 37°C by IPTG induction. At the time of induction, cells were titered on selective plates in order to detect plasmid instability and a relatively high frequency of the cells (14), 2–3%, were found to have lost sensitivity to IPTG.

After 2 h of induction, the cells were harvested and a crude extract was obtained by detergent lysis according to Studier *et al.* (14). The specific activity of the extract was 2.0 μmol min^{-1} mg^{-1} which compares favorably with an extract from *E. coli* BL21(DE3) pLysS cells (Fig. 3, lane 2), showing a specific activity of 0.02 μmol min^{-1}

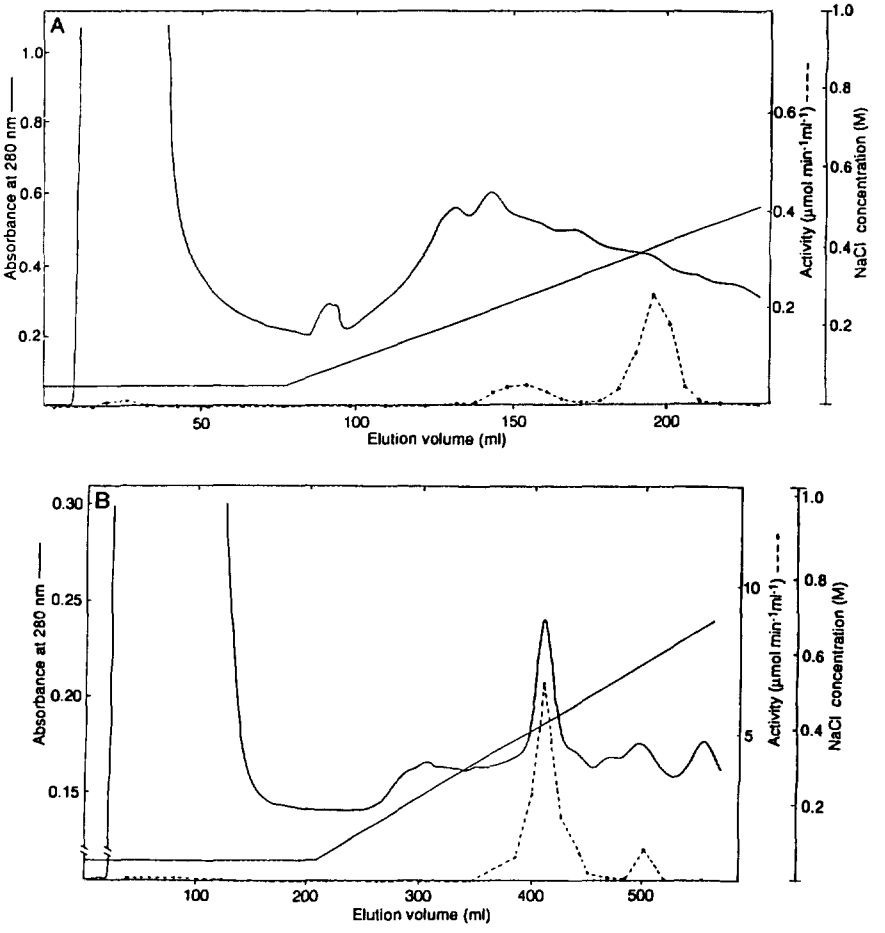

FIG. 1. (A) Phosphocellulose chromatography of HSV-1 dUTPase from virus-infected Vero cells. The column (1.6 × 10 cm), equilibrated in Buffer A, was loaded with 20 ml (containing 220 mg protein) of the supernatant from the centrifuged dialysate of the cytosolic fraction from infected Vero cells. The flow rate was 0.3 ml min^{-1} and fractions of 6 ml were collected. After a washing of the column with 3 vol of Buffer A, elution was performed using a linear NaCl gradient from 0.05 to 1.0 M in 300 ml of Buffer A. (B) Phosphocellulose chromatography of recombinant HSV-1 dUTPase. The column (2.6 × 10 cm) was equilibrated with Buffer A and the centrifuged supernatant (98 ml containing 118 mg protein) of a lysate from 500 ml of bacterial culture was applied. The flow rate was 0.8 ml min^{-1} and fractions of 8.5 ml were collected. After a washing of the column with 3 vol of Buffer A, elution was performed using a linear NaCl gradient from 0.05 to 1.0 M in 500 ml of Buffer A.

mg^{-1}, interpreted as a background originating from the host (*E. coli*) dUTPase.

The crude extracts were examined by SDS–PAGE and 2-D separation. Upon induction, a novel band of 39 kDa appeared on SDS–PAGE (Fig. 3, lanes 2 and 3). On a 2-D separation, the inducible band showed an isoelectric point of about 8.0 (not shown).

Growth and induction at 24°C (room temperature) compared to that at 37°C was found to give slightly higher yields of enzyme activity (see Table 1) and these conditions were accordingly chosen for large-scale preparations.

Extraction of HSV-1 dUTPase from E. coli cells. Lysis of *E. coli* BL21(DE3) pLysS cells is greatly facilitated by the expression of T7 lysozyme encoded by the plasmid pLysS (14). A crude extract is obtained by freezing and thawing which disrupts the bacterial inner membrane and exposes the peptidoglucan layer toward degradation. In addition, T7 lysozyme specifically inhibits transcription by T7 RNA polymerase resulting in a tight regulation of gene expression in the uninduced state.

An experiment was carried out to find out conditions for extraction of induced *E. coli* BL21(DE3)pLysS pET-3a/UL50 cells (Table 1). Cells grown at 37 and 24°C, respectively, were disrupted by freezing and thawing (three rounds) and the release of enzyme was examined in the presence and absence of detergent (polyoxyethylene 10 tridecyl ether). The best results were obtained by freezing and thawing in the presence of 0.1% detergent using cells grown at 24°C. A higher concentration of detergent, 0.2%, did not increase the recovery of dUTPase activity. Freezing and thawing in the absence of detergent resulted in a similar release of protein but a

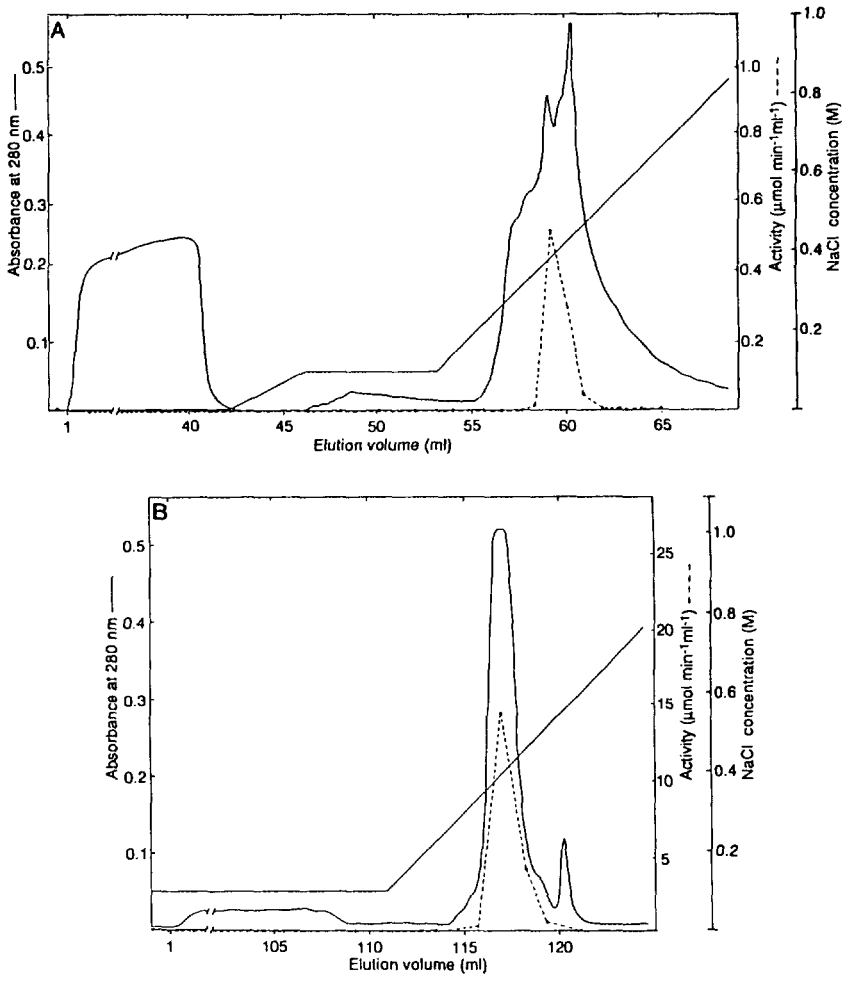

FIG. 2. (A) Cation-exchange chromatography of HSV-1 dUTPase from virus-infected Vero cells. The column (Mono S HR 5/5), attached to a FPLC equipment was equilibrated with Buffer B. Three top fractions from the main peak of enzyme activity in the phosphocellulose chromatography in Fig. 1(A) were pooled (18 ml) and concentrated to 4 ml using Aquacide. The protein solution was diluted 10-fold in Buffer B without NaCl and loaded on the column at a flow rate of 1 ml min^{-1}. Elution was performed using a two-step gradient. First step: from 0 to 0.1 M NaCl in 4 ml of Buffer B, followed by 6 ml 0.1 M NaCl in Buffer B. Second step: from 0.1 to 1.0 M NaCl in 18 ml of Buffer B. (B) Cation-exchange chromatography of recombinant HSV-1 dUTPase. The column (Mono S HR 5/5), attached to a FPLC equipment was equilibrated with Buffer B. Top fractions from the main peak of activity in the phosphocellulose chromatography in Fig. 1(B) were pooled and diluted 10× with Buffer B containing 50 mM NaCl instead of 100 mM NaCl. Of the protein solution obtained, 107 ml (containing 1.0 mg of protein) was loaded on the column. The flow rate was 1 ml min^{-1} and elution was performed with a gradient from 0.1 to 1.0 M NaCl in 18 ml of buffer B.

significantly lower activity. The results show the importance of detergent for the extraction of HSV-1 dUTPase. In a separate experiment (not shown), we found Triton X-100 to be equally effective for the extraction of dUTPase as polyoxyethylene 10 tridecyl ether.

When a crude extract, initially obtained without detergent, was diluted in Buffer A (which contains 0.01% detergent) before assay, a twofold stimulation of activity was observed. Therefore, the differences in activity in Table 1 would reflect an effect of detergent on the extraction process as well as on the enzyme extracted. A maximal "activation" of partly purified detergent-depleted enzyme was obtained at a concentration of 0.01% polyoxyethylene 10 tridecyl ether or Triton X-100, detergents with similar critical micelle concentrations (0.023 and 0.022% (25). The former detergent was preferred since it has no uv absorbance and gives a negligible disturbance in protein determination by Bradford. In this context, we determined whether the activity of purified *E. coli* dUTPase was affected by presence of detergent. No effect was observed.

Purification of recombinant enzyme. The procedure for purification of recombinant HSV-1 dUTPase is summarized in Table 2. Two 250-ml cultures of *E. coli*, grown and induced at 24°C, were kept on ice for 10 min and harvested by centrifugation (Sorvall GSA rotor, 4000*g*, 10 min, 4°C). The bacterial pellet was resuspended in 100 ml of extraction buffer containing 0.1%

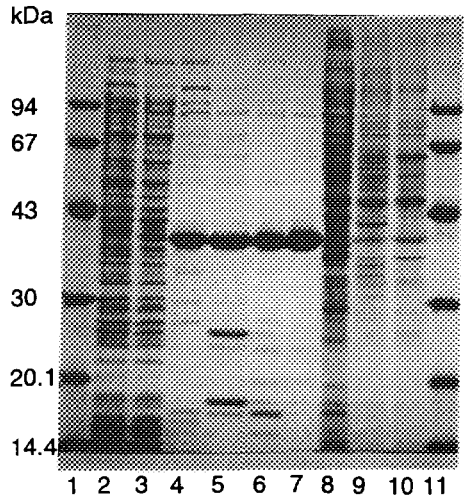

FIG. 3. SDS-PAGE of recombinant and native HSV-1 dUTPase. Recombinant HSV-1 dUTPase: Lane 2, 80 μg crude extract of BL21(DE3) pLysS cells; Lane 3, 80 μg crude extract of IPTG-induced BL21(DE3) pLysS pET-3a/UL50 cells; Lane 4, 20 μg protein of the peak fraction from the phosphocellulose chromatography; Lane 5, 20 μg protein from a Superose 12 chromatography of a phosphocellulose fraction; Lane 6, 20 μg of precipitate obtained from the Mono S chromatography; Lane 7, 20 μg of a washed precipitate from the Mono S chromatography. HSV-1 dUTPase from virus-infected Vero cells: Lane 8, 80 μg of the supernatant from the ultracentrifuged Vero cell dialysate; Lane 9, 25 μg of protein from the HSV-1 peak in the phosphocellulose chromatography of the Vero cell extract; Lane 10, 25 μg protein of the peak fraction from the Mono S chromatography. Molecular weight markers: Lanes 1 and 11.

detergent (polyoxyethylene 10 tridecyl ether). The cells were lyzed by three rounds of freezing and thawing, the lysate was centrifuged (Sorvall SS-34 rotor, 17,000*g*, 1 h, 4°C), and the supernatant assayed for enzyme activity (see Table 2).

The supernatant was directly loaded on a phosphocellulose column (2.6 × 10 cm), equilibrated in Buffer A, and chromatographed as described in the legend to Fig. 1B. The major part (>90%) of the protein material loaded on the column appeared in the flowthrough together with about 3% of the total enzymic activity applied. The nonretarded activity probably originates from *E. coli* dUTPase which is an acidic protein (4). The dUTPase activity, eluted at 0.40 M NaCl, coincided with the main protein peak in the chromatogram (Fig. 1B). A smaller peak of activity, not investigated further, occurs at 0.58 M NaCl. The material in the main peak was of high purity as judged from a Coomassie-stained SDS-PAGE (Fig. 3, lane 4) and the specific activity of the pooled fractions was 30 μmol min^{-1} mg^{-1}.

The HSV-1 dUTPase obtained in the phosphocellulose chromatography in Fig. 1B was rechromatographed on a cation-exchange column as described in the legend to Fig. 2B. Fractions containing dUTPase were pooled and diluted 10-fold in Buffer B containing 50 mM NaCl instead of 100 mM NaCl. Protein (1.0 mg in 107 ml) was loaded on a Mono S HR 5/5 column previously equilibrated with Buffer B and elution was performed as described in the legend to Fig. 2B. The eluted enzyme (at 0.39 M NaCl), with a specific activity of 49 μmol min^{-1} mg^{-1}, precipitated after chromatography. As seen in lane 7 on the SDS-PAGE (Fig. 3), dUTPase was enriched in the precipitate. Analysis of the amino acid composition of this precipitate also demonstrated high purity (Table 4). The precipitation was apparently reversible since the enzyme activity (determined after 500- to 1000-fold dilution in Buffer A) was retained. Using phosphocellulose and Mono S chromatography, about 2.5 mg of nearly homogeneous protein (Fig. 3, lane 6) was obtained from 500 ml of bacterial culture (Table 2).

Addition of $MgCl_2$ (10 mM) followed by incubation at 4°C for 2 h resulted in solution of the precipitate and the precipitation could also be circumvented by the presence of 10 mM $MgCl_2$ during the Mono S chromatography. The specific activity of the enzyme (routinely assayed in the presence of 10 mM $MgCl_2$) was not affected by these additions.

Protein obtained from the phosphocellulose step was analyzed by gel filtration using two serially connected Superose 12 columns at high ionic strength, 0.4 M NaCl in Buffer A. Only a modest purification and a low recov-

TABLE 3

Amino-Terminal Sequences of Recombinant and Native HSV-1 dUTPase and Encoding DNA Sequences

DNA sequences	
Recombinant enzyme	CAT-ATG-AGT-CAG-TGG-GGA-TCC-GGG-GCG-ATC-CTT-GTC-CAG-
Native enzyme	AAG-ATG-AGT-CAG-TGG-GGA-TCC-GGG-GCG-ATC-CTT-GTC-CAG-
Protein sequences	
Recombinant enzyme	Ser-Gln-Trp-Gly-Ser-Gly-Ala-Ile-Leu-Val-Gln-
Native enzyme	Xxx-Gln-Xxx-Gly-Ser-Gly-Ala-Ile-Leu-Val-Xxx-
Encoded sequence	Met-Ser-Gln-Trp-Gly-Ser-Gly-Ala-Ile-Leu-Val-Gln-

Note. The DNA sequence for the native enzyme is from McGeoch *et al.* (8) which was mutagenized in the present study to include a *NdeI* restriction endonuclease site (CATATG) for the recombinant enzyme. Protein sequences were determined by Edman degradation. Xxx denotes uninterpretable results. The amino-terminal sequence encoded from the DNA structures has been included for comparison.

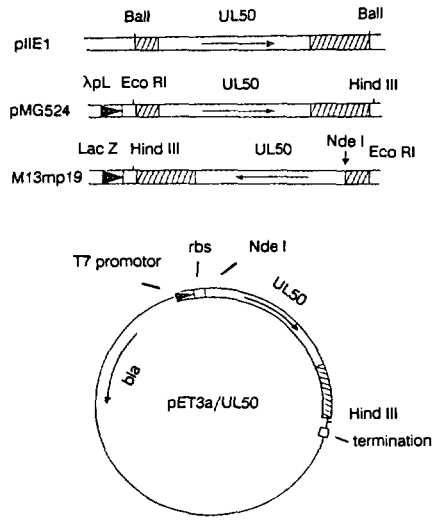

FIG. 4. Outline of construction of the expression plasmid pET-3a/UL50 containing the HSV-1 dUTPase gene, UL50. UL50 was obtained from pIIE1 by *BalI* digestion and inserted in the multiple cloning site of pMG524 behind the λp_L promoter. Using the *Eco*RI and *Hind*III sites, the gene was moved to M13mp19 to enable introduction of a *NdeI* site by mutagenesis. The new *NdeI* site and the *Hind* III site were used to position UL50 in pET-3a behind the promoter and ribosome-binding site of T7 gene 10. Noncoding parts of the *BalI* fragment are indicated by hatched areas.

ery were obtained although the protein profile of the chromatogram indicated a high purity of the preparation. The dUTPase prepared in this way was analyzed by SDS–PAGE (Fig. 3, lane 5). In addition to the dUTPase at 39 kDa, prominent bands of lower molecular mass can be observed, indicating a susceptibility of the dUTPase toward proteolysis.

Amino acid composition. Determination of the amino acid composition of the recombinant HSV-1 dUTPase was performed on a precipitate from the Mono S step washed with Buffer B without NaCl, and finally Millipore water. The result is shown in Table 4.

Amino-terminal amino acid sequence. The results of the Edman degradation are presented in Table 3. During degradation of the dUTPase prepared from Vero cells, no residue could be identified in the first and third cycles. Degradation of recombinant dUTPase revealed the sequence for the first 11 cycles. The result is identical to residues 2–12 in the sequence predicted from the nucleotide sequence of the structural gene.

DISCUSSION

A dUTPase from herpes simplex virus was first reported by Wohlrab and Francke (26) and Caradonna and Cheng (27). These authors observed a rise in dUTPase activity upon viral infection which they could show to originate from a new dUTPase. Caradonna and Adamkiewicz (9) carried out a biochemical study of the dUTPase activity and succeeded in separating the viral dUTPase from the host cell enzyme. They partially characterized semipurified preparations of the enzyme.

This report also deals with the purification and properties of the dUTPase encoded by HSV-1. A limited study was performed on the native HSV-1 dUTPase from virus-infected mammalian (Vero) cells. A recombinant vector, pET-3a/UL50, was constructed for the overexpression of the HSV-1 dUTPase in *E. coli* and a procedure developed for the purification of the recombinant enzyme to a high degree of purity. The recombinant enzyme appears to be identical or closely similar to the native enzyme derived from virus-infected Vero cells as supported by the following evidence: (a) Their mobilities on SDS–PAGE are indistinguishable. (b) The chromatographic behaviors on phosphocellulose and Mono S columns are identical. (c) Enzyme activities of the two proteins are stimulated by detergent whereas neither *E. coli* dUTPase nor green monkey kidney (Vero) dUTPase show this property. (d) The amino-terminal sequences are identical over the range investigated (Table 3).

The studies of the HSV-1 dUTPase from virus-infected mammalian cells were carried out using a cytosolic extract of Vero cells infected with HSV-1. Wohlrab *et al.* (28) found that the cytosolic extract from HSV-1-

TABLE 4

Amino Acid Composition of Recombinant HSV-1 dUTPase

Residue	Predicted	Observed
Ala	44	43.4
Cys	3	3.4
Asn + Asp	27	27.2
Gln + Glu	22	23.1
Phe	12	12.0
Gly	40	40.0
His	8	6.7
(Ile)	12	(10.7)
Lys	5	5.1
Leu	36	35.6
Met	6	5.2
Pro	37	37.2
Arg	28	27.8
Ser	17	17.6
Thr	24	23.5
(Val)	38	(34.1)
Trp	6	nd
Tyr	6	6.2

Note. The values (number of residues per enzyme molecule) refer to a sample of performic acid-oxidized dUTPase hydrolyzed in 6 M HCl, 20 h, 110°C. The slow release of Ile and Val has not been corrected for (figures in parentheses) and Trp was not determined. The composition predicted from the DNA sequence (8) has been included. The deviation of Met content can be accounted for by the absence, probably by post-translational removal, of the Met encoded as amino-terminal residue as demonstrated by the Edman degradation.

infected cells contains less HSV-1 dUTPase than the nuclear pellet although the difference was not very large. The dUTPase from HSV-2, on the other hand, was almost exclusively in the cytosolic fraction. The cytosolic extract used in our studies (20) was prepared by a slightly different procedure than used by Wohlrab *et al.* (28) and was found to contain appreciable amounts of the HSV-1 enzyme. As a first step of purification we ran a phosphocellulose chromatography under conditions resembling those of Caradonna and Adamkiewicz (9). A second step, cation-exchange chromatography, gave the enzyme in a purity of 2–3% and after a SDS–PAGE it could be recovered in a sufficiently homogeneous form for amino-terminal sequencing.

The structural gene for dUTPase in the genome of HSV-1 has previously been mapped. Wohlrab *et al.* (28) performed a genetic analysis utilizing as phenotypic character the difference between HSV-1 and HSV-2 in distribution of dUTPase activity between the nuclear and cytosolic fractions. The region of the HSV-1 chromosome identified by Wohlrab *et al.* as harboring the dUTPase gene was independently analyzed in great detail by Hall *et al.* (29) with respect to mRNAs transcribed. It was also cloned by Preston and Fisher (7) into the vector pAT153 and placed downstream of a control region from the virus regulating the expression of immediate-early proteins. The plasmid derived, pIIE1, was found capable of inducing transient expression of dUTPase in BHK cells which could be prevented by insertional inactivation (30). These findings provided conclusive identification and mapping of the dUTPase gene in the HSV-1 chromosome.

In this investigation, we utilized the vector of Preston and Fisher (7), pIIE1, as a source of the HSV-1 dUTPase gene (UL50) in constructs for overproduction of the HSV-1 enzyme. In our initial attempt, UL50 was placed downstream of the λp_L promoter. This promoter has previously been successfully utilized to overproduce dUTPase from *E. coli* (5). However, the HSV-1 dUTPase was not expressed in a similar construct. The absence of a strong and correctly positioned Shine–Dalgarno sequence may explain the result.

The pET expression system (14), providing a ribosome-binding site as well as a strong promoter (both from bacteriophage T7 gene 10), was tried next. UL50 was moved from the λp_L vector to M13mp19 and a *Nde*I restriction site (see Table 3), created by site-directed mutagenesis, enabled a desired positioning of UL50 in pET-3a. The recombinant plasmid, called pET-3a/UL50, was found to direct an inducible overproduction of HSV-1 dUTPase in *E. coli* BL21(DE3) pLysS cells. This result, together with successful cloning carried out in other laboratories (15,31,32), demonstrates the usefulness of the system of Studier *et al.* (14) for the overexpression of HSV-1 proteins.

Release of the dUTPase from the bacterial cells presented some problems. Yeda pressing, routinely used for the overproduced *E. coli* dUTPase (5), was not very successful (data not shown). Considerable amounts of protein were released but the yield of enzyme activity was poor. Instead, we tried the lysis procedure suggested by Studier *et al.* (14). Presence of detergent proved to increase substantially the amount of dUTPase activity extracted (Table 1). A similar effect of detergent has previously been observed for a dTTPase-dUTPase induced in *Bacillus subtilis* after infection with bacteriophage ϕe (33). After centrifugation of the extract, the supernatant could be directly loaded onto a phosphocellulose column. Chromatography gave excellent recovery and purification. Rechromatography on a Mono S column resulted in a nearly homogeneous HSV-1 dUTPase preparation as judged from SDS–PAGE (Fig. 3, lane 7) and amino acid analysis (Table 4).

The purified enzyme was found to easily form a precipitate. No appreciable loss of activity could, however, be noted, suggesting that the precipitation is reversible. The addition of $MgCl_2$ (10 mM, as in the assay mixture) was found to prevent or retard the formation of precipitate, indicating the presence of metal binding site(s) on the enzyme influencing the solubility of the protein. dUTPases from various sources like *E. coli* (34), *Drosophila* (35), and human lymphoid cells (36) have all been found to be dependent on divalent metal ions, e.g., Mg^{2+}, for activity. Addition of EDTA or other chelating agents inhibits activity and HSV-1 dUTPase is no exception (9). Whether the metal ion acts by binding to the protein, the substrate, or both requires further investigation.

The amino acid composition determined for the recombinant HSV-1 dUTPase is in excellent agreement with that predicted from the nucleotide sequence of the structural gene, UL50 (Table 4). The amino-terminal sequence derived by Edman degradation is also in agreement with the DNA sequence (Table 3). However, a serine residue is found in the first cycle in the degradation of the recombinant protein and the Met encoded as the amino-terminal residue appears to be missing in the recombinant protein as well as in the enzyme from infected Vero cells. Removal of an amino-terminal Met, when followed by a Ser, would be expected according to general rules for post-translational *in vivo* processing of amino-termini (37).

The isoelectric point determined here (about 8.0) agrees with that previously obtained by Caradonna and Adamkiewicz (9) on partially purified preparations of HSV-1 dUTPase and with a theoretical value (8.0) from the amino acid sequence deduced from the nucleotide sequence of the structural gene (8). The molecular mass determined for HSV-1 dUTPase by SDS–PAGE (39 kDa) is also in agreement with the DNA sequence. Hall *et al.* (29) demonstrated a 1.5-kb mRNA transcribed

from a region of the HSV-1 chromosome, later shown to encode the viral dUTPase, and *in vitro* translation of this transcript gave a polypeptide with a molecular mass of 39 kDa. Preston and Fisher (7) presented evidence for the 1.5-kb mRNA being the transcript encoding HSV-1 dUTPase and were later on able to show that HSV-1 dUTPase activity is associated with a polypeptide 39 kDa in size (30). Caradonna and Adamkiewicz (9) found a slightly lower value of 35 kDa using SDS–PAGE and gel filtration.

Only a few reports about specific activities for different dUTPases are available. The high value found here, 49 μmol min^{-1} mg^{-1}, for the HSV-1 enzyme is consistent with the high degree of homogeneity of the enzyme preparation obtained. It is also high in comparison with dUTPases from other sources. The human (from HeLa cells) and the rat spleen enzymes have been purified to near homogeneity and values of 16 and 17.6 μmol min^{-1} mg^{-1} have been reported (9,38). The value reported (5) for the *E. coli* enzyme is 55 μmol min^{-1} mg^{-1}. Expressed as activity per polypeptide chain, the figure for the bacterial enzyme is significantly lower than that for the HSV-1 dUTPase.

dUTPase has an important role in nucleotide metabolism but the enzymological properties remain to a large extent uncharacterized. Herpesvirus produces a dUTPase of its own, indicating a function for the enzyme in the life cycle of the virus, and the viral enzyme has also been suggested as a target for the development of specific antiviral compounds (39,40). The overproducing system developed here makes it possible to obtain HSV-1 dUTPase in sufficient amounts for detailed studies of structure and function, which should be helpful in understanding the biological role of the enzyme, in particular for the herpes viral systems.

ACKNOWLEDGMENTS

We thank Drs P. Elias, V. Preston, and F. W. Studier, for providing *E. coli* strains and plasmids. We are grateful to Drs David Eaker and Åke Engström at Uppsala University for help with the analysis of the amino acid composition and the Edman degradation. This work was supported by grants from Swedish Cancer Society, Swedish Natural Science Research Council, Magn. Bergvalls Stiftelse, and Kungliga Fysiografiska Sällskapet in Lund.

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