Cloning and Expression of the Uracil–DNA Glycosylase Inhibitor (UGI) From Bacteriophage PBS-1 and Crystallization of a Uracil–DNA Glycosylase–UGI Complex

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ABSTRACT  The uracil-DNA glycosylase inhibitory protein (UGI) from the bacteriophage PBS-1 has been cloned and overexpressed. The nucleotide sequence is identical to that for the previously described PBS-2 inhibitor. The recombinant PBS-1 UGI inhibits the uracil-DNA glycosylase from herpes simplex virus type-1 (HSV-1 UDGase), and a complex between the HSV-1 UDGase and PBS-1 UGI has been crystallized. The crystals have unit cell dimensions a = 143.21 Å, c = 40.78 Å and are in a polar hexagonal space group. There is a single complex in the asymmetric unit with a solvent content of 62% by volume and the crystals diffract to 2.5 Å on a synchrotron radiation source. © 1995 Wiley-Liss, Inc.

Key words: DNA repair, PCR, Bacillus subtilis, herpes simplex virus, protein–protein interaction

INTRODUCTION

Uracil–DNA glycosylase (UDGase) is a ubiquitous base-excision DNA repair enzyme responsible for the removal of the normal RNA base uracil, from DNA, by hydrolysis of the N-glycosidic bond linking the base to the deoxyribose sugar.1 Uracil arises in DNA either by misincorporation of dUTP during DNA synthesis, or by deamination of cytosine in existing DNA and is extremely damaging both to the template function and regulated expression of the genome. UDGases have been identified in a very wide range of organisms including bacteria, archaea, plants, and mammals, and DNA viruses such as herpes and pox, and show a high degree of conservation of their amino acid sequence across large evolutionary distances.2

The Bacillus subtilis bacteriophages PBS-1 and PBS-2 are apparently biologically unique in using uracil as a component base in their DNA, in place of thymine.3 This presents these bacteriophages with a serious problem, as their DNA will be totally fragmented as a result of the UDGase activity of the host bacterium. In fact, PBS-1 and PBS-2 prevent the destruction of their uracil–DNA by producing a small protein that rapidly and irreversibly inactivates the host UDGase.4,5 Suprisingly, this uracil-DNA glycosylase inhibitor (UGI) is not specific for the UDGase from B. subtilis alone but will inactivate homologous UDGases from other sources, including the human and herpes simplex enzymes.6,7 The UGI acts by formation of a non-covalent complex with UDGase, which is essentially irreversible and the two proteins can only be separated under denaturing conditions.8 The estimated binding constant for the PBS-2 UGI and the E. coli UDGase is 1.3 μM.9

Recently we have determined the crystal structure of the UDGase from herpes simplex virus type-1 (HSV-1) at 1.75 Å resolution.10 In the three-dimensional structure, it is possible to identify an elongated channel in which extended DNA substrates bind, and a highly conserved pocket that binds uracil with high specificity and provides the active site of the enzyme. The universality of UGI activity against all UDGases suggests that the inhibitor interacts with this highly conserved part of the UDGase structure; however, the situation may be more complicated, since inhibition by UGI is not prevented by DNA or uracil, and kinetic studies suggest a multi-step process of inactivation that may involve substantial conformational changes in the enzyme, inhibitor, or both.8,9

We have now cloned and expressed the UGI from bacteriophage PBS-1 in multimilligram quantities in E. coli. Here we report the successful crystallization of a complex between PBS-1 uracil–DNA glycosylase inhibitor and the uracil–DNA glycosylase from HSV-1, which will ultimately provide a detailed understanding of the unusual and highly specific protein–protein interaction between UDGases and UGI.

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MATERIALS AND METHODS
Cloning and Expression of PBS-1 UGI

Lysozyme from a PBS-1 infection of *B. subtilis* was a gift from Dr. Jeff Errington (University of Oxford). The PBS-1 UGI gene of unknown sequence was amplified by a polymerase chain reaction (PCR) reaction using oligonucleotide primers designed on the basis of the known PBS-2 UGI coding sequence, and incorporating restriction sites for subsequent cloning. The PCR reaction gave a band of approximately 250 base pairs on an agarose gel. This band was extracted and cleaned, and cut with *NdeI* and *HindIII* and then ligated to plasmid pRSETB (Invitrogen Corp.), which had been cut with *NdeI* and *HindIII*. The sequence of the amplified fragment was identical to that previously reported for the PBS-2 UGI gene. The cloned PBS-1 UGI coding sequence was expressed under the control of a T7 promoter induced by addition of 1 mM isopropyl thiogalactoside (IPTG) in *E. coli* strain BL21 (DE3). Maximal expression was obtained 3 hours after induction. Bacteria expressing UGI were lysed by sonication and centrifuged at 20,000 rpm for 90 minutes. The supernatant was heated to 100°C for 15 minutes to denature *E. coli* proteins and centrifuged again at 20,000 rpm for 30 minutes. This supernatant was loaded onto a DE52 column and eluted with a gradient of 0 → 0.4 M NaCl. High molecular weight material was removed by ultrafiltration on an Amicon YM30(K) membrane. The resultant material was approximately 70% pure and gave a yield of 15 mg/L of bacterial culture.

Crystallization of UDGase-UGI complex

HSV-1 uracil-DNA glycosylase was expressed and purified as described. Purified UDGase was added in molar excess to UGI prepared as above, and the mixture dialyzed into a buffer of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF). This was loaded onto a Q-Sepharose Fast-Flow column (Pharmacia, Gaithersburg, MD) and eluted with a NaCl gradient from 0 → 1.0 M. The 37 kDa UDGase–UGI complex eluted in the first peak. Purified UDGase–UGI complex was concentrated to 34 mg/ml by ultrafiltration using an Amicon YM10 ultrafiltration membrane and a Centricon 3 (Amicon, Beverly, MA) ultrafiltration spin-column in a buffer consisting of 10 mM Tris-HCl, pH 8.0, 0.1 mM PMSF, and 0.02% sodium azide. Crystallization screens were performed using conditions close to the original UDGase crystallization conditions, using a sparse matrix screen based on that of Jancarik and Kim, and were implemented as microbatch experiments under paraffin oil.

Data Collection and Processing

A crystal of smallest dimension 60 µm was mounted in a glass capillary and used to collect 18 × 1° oscillation images on a 30 cm MAR Image Plate Detector using 0.92 Å radiation on station 9.5 at the SRS Daresbury Laboratory, Warrington, UK. Unit cell parameters were determined using REFI[A] and images were integrated using MOSFLM (A.G.W. Leslie, MRC, Cambridge, U.K.) and reduced using the ROTAVATA/AGROVATA and TRUNCATE programs of the CCP4 Software Suite.

RESULTS AND DISCUSSION

Although the nucleotide sequence encoding the PBS-1 UGI was unknown at the outset, PCR primers based on the presumably homologous PBS-2 UGI gene were successful in amplifying a product of the expected size from a PBS-1 DNA template. The PCR was efficient and specific despite the nature of the template, which contained uracil in place of thymine, and has a very high proportion of A-U base pairs. Subsequent sequencing of the amplified DNA showed that the PBS-1 and PBS-2 UGI genes are in fact identical at the nucleotide level.

Addition of purified recombinant PBS-1 UGI to HSV-1 UDGase resulted in complete loss of UDGase activity, demonstrating that the recombinant UGI is native and fully active. PBS-1 UGI and HSV-1 UDGase formed a tight complex when mixed in vitro. The molecular weight of the complex was not sufficiently different from that of UDGase itself to allow separation on that basis; however, the charge properties of the complex between the basic UDGase and the acidic UGI were significantly different from the free UDGase and readily allowed purification of the complex by ion-exchange chromatography.

Thin hexagonal rod-like crystals used in this initial analysis were obtained under conditions of 17.5 mg/ml UDGase–UGI complex, 15% PEG 8000. Subsequently, larger crystals have been obtained using protein at 43 mg/ml, 10.6% PEG 8000, 70 mM (NH₄)₂SO₄, 35 mM citrate/phosphate buffer, pH 6.2. On a synchrotron radiation source, such a crystal diffracted to 2.5 Å resolution, suggesting that a medium resolution structure determination of the complex will ultimately be possible. Autoindexing of the oscillation images gave a trigonal unit cell with dimensions a = 143.21 Å, c = 40.78 Å, which was subsequently used to integrate the images. The images were integrated and reduced assuming the different Laue symmetries compatible with a trigonal lattice. Of these, only P3 and P6 gave acceptable values for merging symmetry-related reflections, suggesting the presence of a hexagonal space group.

Calculation of Vₐ with a complex molecular weight of approximately 37 kDa gives values of 3.26 for Z = 6, and 1.63 for Z = 12. The value of 3.26 corresponds to a solvent content of approximately 62% by volume, which is within the range observed for protein crystals and consistent with the drop-off in intensity observed beyond 2.5 Å, indicating a polar hexagonal...
space group with a single complex in the asymmetric unit. Crystal morphology makes observation of the 001 reflections very difficult, and these have not been measured in this preliminary study, so we cannot distinguish at present between space groups P6₁, P6₂, P6₃, P6₄, or P6₅. Refinement of the crystallization conditions has now yielded crystals of sufficient size for data collection using a laboratory source, and structure determination of these is now in progress.

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REFERENCES