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# Insights into substrate recognition by the *Escherichia coli* Orf135 protein through its solution structure

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ABSTRACT

*Escherichia coli* Orf135 hydrolyzes oxidatively damaged nucleotides such as 2-hydroxy-dATP, 8-oxo-dGTP and 5-hydroxy-CTP, in addition to 5-methyl-dCTP, dCTP and CTP. Nucleotide pool sanitization by Orf135 is important since nucleotides are continually subjected to potential damage by reactive oxygen species produced during respiration. Orf135 is a member of the Nudix family of proteins which hydrolyze nucleoside diphosphate derivatives. Nudix hydrolases are characterized by the presence of a conserved motif, even though they recognize various substrates and possess a variety of substrate binding pockets. We investigated the tertiary structure of Orf135 and its interaction with a 2-hydroxy-dATP analog using NMR. We report on the solution structure of Orf135, which should contribute towards a structural understanding of Orf135 and its interaction with substrates.



## 1. Introduction

Reactive oxygen species generated during respiration can damage a variety of cellular molecules such as nucleotides. These oxidized DNA precursors form aberrant base pairs which can generate mutations. Among these, 8-oxoguanine (8-oxo-G) and its derivatives are well known, and the system in place to avoid mutation by 8-oxo-G has been well studied. 8-Oxo-G mispairs with adenine, and can subsequently cause mutation during replication. In *Escherichia coli,* MutT hydrolyzes 8-oxo-deoxyguanosine triphosphate (8-oxo-dGTP) to its monophosphate to prevent incorporation into the genome [1]. Further, 8-oxo-G within the genome can be excised by MutM, an 8-oxo-G DNA glycosylase, and mispaired adenine can be excised by MutY, an adenine DNA glycosylase [2–5]. Recently, human NUDT5 is also thought to play a role in nucleotide pool sanitization by hydrolyzing 8-oxo-deoxyguanosine diphosphate (8-oxo-dGDP) to 8-oxo-deoxyguanosine monophosphate (8-oxo-dGMP) [6].

In addition to 8-oxo-G, it has been shown that 2-hydroxy-deoxyadenosine triphosphate (2-hydroxy-dATP) is a highly mutagenic precursor since it induces GC-TA transversion during replication [7]. Mammalian MutT homolog 1 (MTH1) sanitizes oxidized DNA precursors including 2-hydroxy-dATP and prevents mutations in organisms. Human MTH1 hydrolyzes 8-oxo-dGTP, 2-hydroxy-dATP, 2-hydroxy-ATP and 8-oxo-dATP to their respective monophosphate forms. *E. coli* Orf135 hydrolyzes 2-hydroxy-dATP, 8-oxo-dGTP and 5-hydroxy-CTP [8–10]. A possible role of Orf135 is to hydrolyze oxidatively damaged nucleotides including 2-hydroxy-dATP, and thereby sanitize the nucleotide pool in cells. In fact, it is known that the frequency of spontaneous and $H_2O_2$-induced mutations is two- to threefold higher in the *orf135*$^-$ strain compared with the wild-type [11]. Additionally, it is known that Orf135 also hydrolyzes 5-methyl-dCTP, dCTP and CTP [12], although the biological significance of this remains unknown.

Orf135, MutT, MTH1 and NuDT5 are members of the Nudix family of proteins which hydrolyze dNTPs, NADH, GDP-mannose, ADP-ribose, diadenosine polyphosphates and diphosphoinositol polyphosphates [13]. Since these substrates consist of a nucleoside diphosphate group linked to some other moiety, the substrates are referred to by the acronym Nudix (Nucleoside DIphosphate linked to X) [13]. Nudix enzymes generally possess a characteristic signature among the family of hydrolases, and are characterized by the presence of a conserved array of 23 amino acids comprising $GX_5EX_7REUXEEXGU$, where U represents a bulky hydrophobic amino acid. From a structural point of view, the motif contains a conserved helix, in which conserved glutamic acid residues

*Abbreviations:* NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum correlation spectroscopy; r.m.s., root mean square.

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**Table 1**[a]

| | SA | $SA_{water\ refined}$ |
|---|---|---|
| Total number of distance constraints | 3504 | |
| Intra residue | 603 | |
| Short range ($\|i-j\|=1$) | 965 | |
| Middle range ($\|i-j\|=2,3,4$) | 574 | |
| Long range ($\|i-j\|>4$) | 1292 | |
| Hydrogen bond constraints | $35\times2$ | |
| *Dihedral constraints* | | |
| $\phi$, $\varphi$ | 93, 93 | |
| *Residual dipole couplings* | | |
| $^1D_{NH}$ | 90 | |
| *R.m.s. deviations from experimental constraints*[b] | | |
| Distance (Å) | $0.013\pm4\times10^{-4}$ | $0.0200\pm5\times10^{-4}$ |
| Angle (°) | 0.23 ± 0.06 | 0.50 ± 0.08 |
| *R.m.s. deviations from idealized covalent geometry* | | |
| Bonds (Å) | $0.0011\pm5\times10^{-6}$ | $0.0040\pm5\times10^{-5}$ |
| Angles (°) | 0.284 ± 0.001 | 0.54 ± 0.01 |
| Impropers (°) | 0.163 ± 0.006 | 1.33 ± 0.08 |
| RDC Q-factor | 11.8 ± 0.5% | 6 ± 0.8% |
| *PROCHECK Ramachandran plot (1–24,31–131)* | | |
| Residues in most favored regions (%) | 89.6 | 93.4 |
| Residues in additional allowed regions (%) | 9.9 | 6.0 |
| Residues in generously allowed regions (%) | 0.3 | 0.6 |
| Residues in disallowed regions (%) | 0.2 | 0.0 |
| *Average atomic r.m.s. deviations from the average structure* | | |
| Back bone (1–24, 31–131) (Å) | 0.26 | 0.41 |
| All heavy (1–24, 31–131) (Å) | 0.72 | 0.88 |

[a] These statistics comprise the ensemble of the 20 structures obtained from 100 starting structures. Structure calculations were performed using CNS version 1.2.
[b] None of these structures exhibited distance violations >0.5 Å, dihedral angle violations >5°.

protrude and chelate metal ions, which plays a crucial role in hydrolysis [13]. Although the hydrolysis center of Nudix enzymes is well conserved, the substrate recognition pockets display structural variation, thus reflecting the variety of substrates which bind the enzymes. Detailed structural information of Orf135 is required in an effort to delineate the nature of the enzyme–substrate interactions involved.

In this study, we have determined the solution structure of Orf135 by NMR techniques. Based on inspection of the determined structure and monitoring the NMR signals when adding substrate, we have identified a substrate binding pocket. We also discuss the molecular recognition mechanism of Orf135 and detail a structure comparison with other Nudix enzymes. Molecular recognition of the 2-hydroxy-A base is a particularly important issue. To date, structural investigations of MTH1 have provided the sole information pertaining to the molecular recognition of 2-hydroxy-A [14].

## 2. Materials and methods

### 2.1. Sample preparation and NMR experiments

Orf135 was expressed and purified as previously described [15]. Briefly, the protein was expressed as a GST-fusion protein in *E. coli* BL21 Star (DE3) (Invitrogen), and subsequently purified by GSH column chromatography. Following the removal of GST by HRV3C proteinase, Orf135 was finally purified by gel-filtration chromatography. Purified Orf135 was prepared in $KH_2PHO_4$-$K_2HPO_4$ (pH 6.8) 93% $H_2O$/7% $^2H_2O$ buffer containing 50 mM KCl for the NMR experiments. NMR experiments were performed on a Bruker DMX500, a Bruker AVANCE 500 with cryogenic probe, a Bruker AVANCE 600 with cryogenic probe, or a Bruker DRX800 with triple axis gradient probe at 303 K. All spectra were processed using NMRPipe [16], and analyzed by Sparky [17]. The $^1H$, $^{13}C$ and $^{15}N$ assignments were obtained from standard multidimensional NMR methods [15].

### 2.2. Structure determination

Inter-proton distances were derived from 2D NOESY, 3D $^{15}N$ edited NOESY–HSQC, and 3D $^{13}C$ edited NOESY–HSQC. Additionally, dihedral $\phi$ and $\psi$ angles derived from TALOS were also used [18]. Residual $^1D_{NH}$ couplings were obtained by comparison with $^{15}N$–$^1H$ couplings obtained from isotropic and anisotropic samples. The anisotropic sample was prepared by adding 15 mg/ml Pf1 phage. The couplings were measured using 3D HNCO-TROSY experiments performed in an interleaved manner [19]. Structural restraint collection was performed using CYANA version 3.00 with the CANDID protocol [20]. An ensemble of 100 Orf135 structures were calculated using CNS version 1.2 based on the obtained structural restraints including residual $^1D_{NH}$ couplings using a standard simulated annealing protocol [21]. Finally, structures were refined with a water refinement protocol using CNS version 1.2 [22].

The final 20 lowest energy ensemble structures were checked by PROCHECK-NMR [23], and graphics were created using MOLMOL [24] and PyMOL (DeLano Scientific, San Carlos, CA). The lowest energy structure among the ensemble was used as a representative structure in order to perform structural comparisons, and to generate ribbon and molecular surface models.

### 2.3. Substrate binding analyses

Signal perturbations of $^1H$ and $^{15}N$ amide resonances of $^{15}N$ uniformly labeled Orf135 were monitored upon addition of an equimolar amount of non-hydrolyzed ribonucleotide analog of 2-hydroxy-dATP, α, β-methylene 2-hydroxy-adenosine triphosphate (2-hydroxy-AMPCPP) to 0.2 mM Orf135 sample in 20 mM HEPES buffer (pH 7.5) containing 20 mM KCl, 1 mM DTT, 1 mM $MgCl_2$ and 5% $^2H_2O$. The $^1H$–$^{15}N$ HSQC experiments were performed at 303 K. Reduced signal intensities were analyzed using Sparky [17]. Changes in signal intensity were evaluated by calculating the ratio of the intensity difference caused by perturbation and reference spectra, (Iref − Iper)/Iref, where Iref and Iper represent signal intensities in the reference and perturbed spectrum, respectively.

## 3. Results and discussion

### 3.1. Structure determination and description of overall structure

The elution volume of Orf135 in the gel filtration chromatography corresponded to the molecular weight of the monomer, while the line shapes of the NMR spectrum were relatively narrow. These data indicated that Orf135 exists as a monomer in solution. Almost all of the $^1H$, $^{13}C$ and $^{15}N$ NMR signals were assigned using standard multi-dimensional NMR techniques [15]. More than three thousand distance restraints derived from NOEs were collected, and 90 N–H residual dipole couplings were also used (Table 1). Fig. 1A depicts the backbone of the final 20 structures derived from NMR data showing that the atomic coordinates throughout the protein molecule have been well defined, with the exception of the C-terminal residues and the long loop located between strand β2 and helix α1 (Fig. 1A and B). It should be noted that steady state {$^1H$}–$^{15}N$ heteronuclear NOE indicated that the former part of this loop is flexible in solution (residues 25–30). As shown in Fig. 1C, the values are relatively low, which indicate flexibility, in contrast to the secondary structure parts associated with higher values, which indicate rigidity. The average r.m.s. deviation calculated