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Structure and Substrate Recognition of the *Escherichia coli* Transport Protein NupG from the Nucleoside: H^+ Symporter (NHS) Family

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ABSTRACT: The nucleoside transporter NupG is one of the two principal transport proteins in the inner membrane of *Escherichia coli* that enable the organism to scavenge nucleosides from its external environment. NupG functions in a symport manner driven by the proton motive force and is a member of the Nucleoside:H^+ Symporter (NHS) subfamily of the Major Facilitator Superfamily (MFS) of transporters. NupG has broad substrate specificity, transporting all naturally occurring purine and pyrimidine nucleosides. In studies over many years the *nupG* gene has been cloned and amplified, and the NupG protein has been purified, subjected to biochemical, biophysical and computational analysis, and its X-ray structure determined in the apo state at 3.0 Å resolution. The NupG structure had a typical MFS fold with twelve transmembrane spanning α-helices and distinct N- and C-terminal domains linked by a flexible loop. Preliminary site-directed mutagenesis and molecular docking studies on NupG identified nine putative nucleoside binding pocket residues (R136, T140, F143, Q225, N228, Q261, E264, Y318, F322) and a mutant (D323A) with 20-fold enhanced uridine binding activity. Further biochemical and structural investigations are necessary to better understand the substrate recognition and molecular mechanism of *E. coli* NHS family proteins (NupG, XapB, YegT).

KEYWORDS: Molecular docking, Molecular mechanism, Nucleosides, Site-directed mutagenesis, Substrate recognition, symport, Transport protein.

INTRODUCTION

The nucleoside transporter NupG (418 residues, UniProt: P0AFF4) is one of the two principal transport proteins in *Escherichia coli* that enable the organism to scavenge nucleosides from its external environment (Komatsu, 1973; Mygind and Munch-Petersen, 1975; Munch-Petersen and Mygind, 1976; Leung and Visser, 1977; Munch-Petersen et al., 1979). NupG is found in the inner bacterial membrane where it catalyses the uptake of nucleosides against their concentration gradient, and this is driven by the movement of protons down their concentration gradient due to the proton motive force (Westh Hansen et al., 1987). Nucleosides and protons therefore move in the same direction across the membrane in a symport manner: nucleoside (out) + H^+ (out) → nucleoside (in) + H^+ (in). NupG is a member of the Nucleoside:H^+ Symporter (NHS) subfamily within the Major Facilitator Superfamily (MFS) of transporters (Pao et al., 1998; Xie et al., 2004). NupG has broad substrate specificity because it transports all the naturally occurring purine (adenosine, guanosine, inosine) and pyrimidine (cytidine, deoxycytidine, thymidine, uridine) nucleosides. It can also transport the purine nucleoside xanthosine, but with a very low affinity (Xie et al., 2004, Patching et al., 2005). Consistent with its dependence on the proton motive force, NupG is inhibited by the protonophore uncouplers 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone (CCCP), and by valinomycin.

The other principal nucleoside transporter found in *E. coli* is called NupC (400 residues, UniProt: P0AFF2) (Munch-Petersen and Mygind, 1976; Munch-Petersen et al., 1979; Craig et al., 1994), which is evolutionarily distant from NupG. Like NupG, NupC is a proton-driven symporter, but it is a member of the Concentrative Nucleoside Transporter family with close homologues in humans and other eukaryotes (Gray et al., 2004; Loewen et al., 2004; Johnson et al., 2012; Wright and Lee, 2021; Sun and Patching, 2023). NupG is not closely related to any known eukaryotic nucleoside transporters. NupC shows greater specificity towards pyrimidine nucleosides than NupG because it transports cytidine, deoxycytidine, thymidine, uridine and adenosine. It shows only weak activity with inosine and xanthosine and it does not transport guanosine or deoxyguanosine (Munch-Petersen et al., 1979; Loewen et al., 2004; Patching et al., 2005).

Two other NHS family transporters have been identified in *E. coli*, namely XapB (418 residues, UniProt: P45562) and YegT (425 residues, UniProt: P76417). XapB shares 58.4% sequence identity with NupG and it has been demonstrated as a transporter of xanthosine (Seeger et al., 1995; Nørholm and Dandanell, 2001). YegT shares 30.1% sequence identity with NupG, but its substrate

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specificity has not yet been determined. This article is a concise review and discussion on current knowledge about structure and substrate recognition of NupG and NHS family proteins and what further investigations are necessary to better understand their origin of substrate recognition and molecular mechanism.

DISCUSSION

The *nupG* gene from *E. coli* K-12 was originally cloned on plasmid pKY2592 (Osaki et al., 1980) and the presence of the gene on the plasmid was demonstrated by restoration of nucleoside transport in a transport-negative mutant (Munch-Petersen and Jensen, 1984). It was later cloned on the multicopy plasmid pBR322 and the *nupG* gene product was tentatively identified in an *E. coli* minicell expression system as a hydrophobic, membrane- bound protein with a relative molecular mass of ~43 kDa (Westh Hansen et al., 1987). The *nupG* gene was then cloned into plasmid pTTQ18 under the control of an IPTG-inducible tac promoter and directly upstream of a coding sequence for a hexahistidine tag. In *E. coli* BLR cells IPTG induction achieved amplified expression of NupG-His_6 to levels of over 20% of total protein in inner membranes (Patching, 2002; Xie et al., 2004). Transport assays in *E. coli* whole cells using radiolabelled nucleosides measured the induced uptake of thymidine, uridine and adenosine by the recombinant NupG-His_6. Transport activity of recombinant NupG-His_6 was also observed in *Xenopus laevis* oocytes, where there was NupG-mediated influx of a range of radiolabelled purine (adenosine, guanosine and inosine) and pyrimidine (cytidine and thymidine) nucleosides (Patching, 2002; Xie et al., 2004).

NupG-His_6 was successfully solubilsed from *E. coli* inner membranes by the mild detergent *n*-dodecyl-β-*D*-maltoside (DDM) and then purified by Ni-NTA chromatography to greater than 95% homogeneity and in yields of >2 mg per litre of culture (Xie et al., 2004). Purified NupG-His_6 was demonstrated to be functional by reconstitution into liposomes and measurement of uptake of [^{3}H]thymidine, which was inhibited by both purine and pyrimidine unlabelled nucleosides (adenosine, deoxyadenosine, guanosine, deoxyguanosine, cytidine, deoxycytidine and uridine). The secondary structure of purified and DDM solubilised NupG-His_6 was demonstrated to be predominantly α-helical by far-UV circular dichroism spectroscopy (Xie et al., 2004).

The ligand specificity of NupG and NupC was assessed by testing 46 natural nucleosides and analogues as inhibitors of [^{14}C]uridine transport by the separate proteins in energised whole *E. coli* cells. It was identified that binding of nucleosides to NupG requires the presence of hydroxyl groups at each of the C-3' and C-5' positions of the ribose moiety, while binding to NupC required only the C-3' hydroxyl substituent. The greater importance of the ribose moiety for binding to NupG was consistent with the evolutionary relationship between NupG and the Oligosaccharide:H^+ Symporter (OHS) subfamily of the MFS (Patching, 2002; Patching et al., 2005).

X-ray crystal structures of wild-type NupG (PDB 7DL9) and a mutant D323A (PDB 7DLA) were obtained at 3.0 Å resolution with both structures in an apo state and in an inward-open conformation (RMSD of 0.3 Å over 384 Cα atoms) (Wang et al., 2021). Consistent with earlier topology prediction (Xie et al., 2004; Patching et al., 2005) and molecular modelling based on the *E. coli* lactose transporter LacY (Vaziri et al., 2013), NupG had a typical MFS fold with twelve transmembrane spanning α-helices (Figure 1). Both the N- and C-terminal ends were at the cytoplasmic side of the membrane and there was a distinct N-domain (TMs 1-6) and C-domain (TMs 7-12) linked by a flexible loop.

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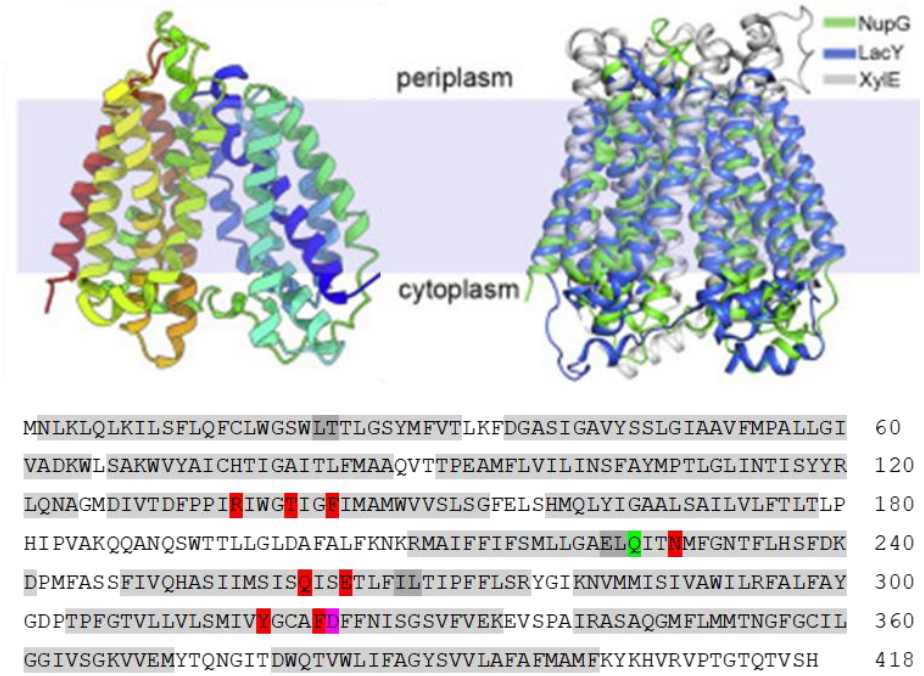

Figure 1. Structure of *E. coli* nucleoside transporter NupG. Top left. X-ray crystal structure of NupG shown in ribbon representation and rainbow coloured from the N-terminal end (blue) to the C-terminal end (red). **Top right.** Structure of NupG (green) superimposed with the X-ray crystal structures of the *E. coli* lactose transporter LacY (blue) (PDB 1PV6) (Abramson et al., 2003) and the *E. coli* xylose transporter XylE (grey) (PDB: 4GBY) (Sun et al., 2012). **Bottom.** Amino acid sequence of NupG highlighted to show: TM helices based on the crystal structure (grey), breaks in TM helices (dark grey), position of mutant D323A (pink), residues that when mutated to Ala abolished uridine binding (red), residue that when mutated to Ala did not affect uridine binding (green). The structures at the top of the figure were adapted from Wang et al. (2021).

In a preliminary investigation of substrate recognition by NupG following the crystal structure, the same study used ITC to measure the binding of uridine to purified NupG (in MES buffer, pH 6.0, with 0.02% DDM), with the wild-type protein producing a binding affinity (Kd value) of 199.67 ± 15.01 μM (Wang et al., 2021). Several single-site specific alanine mutants of NupG were constructed informed by superimposition of the NupG structure with those of LacY (Abramson et al., 2003) and the *E. coli* xylose transporter XylE (Sun et al., 2012), and by molecular docking of NupG with uridine (Figure 1). Mutation D323A in the middle of TM10 enhanced uridine binding by 20-fold (Kd = 9.67 ± 2.87 μM). Mutations R136A, T140A and F143A in TM5, N228A in TM7, Q261A and E264A in TM8, and Y318A and F322A in TM10 all apparently abolished uridine binding. Mutation Q225A in TM7 did not significantly affect uridine binding (Kd = 227.67 ± 88.34 μM) (Wang et al., 2021).

In a molecular docking model of the nucleoside binding site in NupG (Wang et al., 2021), the hydrophilic residues R136, T140 and E264 formed hydrogen bonds with the ribose moiety of uridine (Figure 2). These residues are identically conserved in the three members of the NHS family found in *E. coli*: NupG, XapB, YegT (Table 1). This may be expected as the ribose moiety is the same in all the naturally occurring nucleosides. The hydrophilic residues Q225, N228, Q261 and Y318 formed hydrogen bonds with the base moiety of uridine (Figure 2). It is surprising that these residues are identically conserved with XapB (Table 1), so some other residues must be involved in defining the different nucleoside specificities of NupG and XapB. The NupG residues Q225, N228 and Y318 are not identically conserved in YegT, as they correspond to YegT residues A226, Y229 and H310, respectively (Table 1). The hydrophobic residues F143 and F322 were also in the binding pocket of NupG (Figure 2) and appeared to be essential for nucleoside binding (Wang et al., 2021). These residues are identically conserved in XapB, but correspond to YegT residues W138 and Y314, respectively (Table 1). The substrate specificity of YegT is yet to be determined experimentally.

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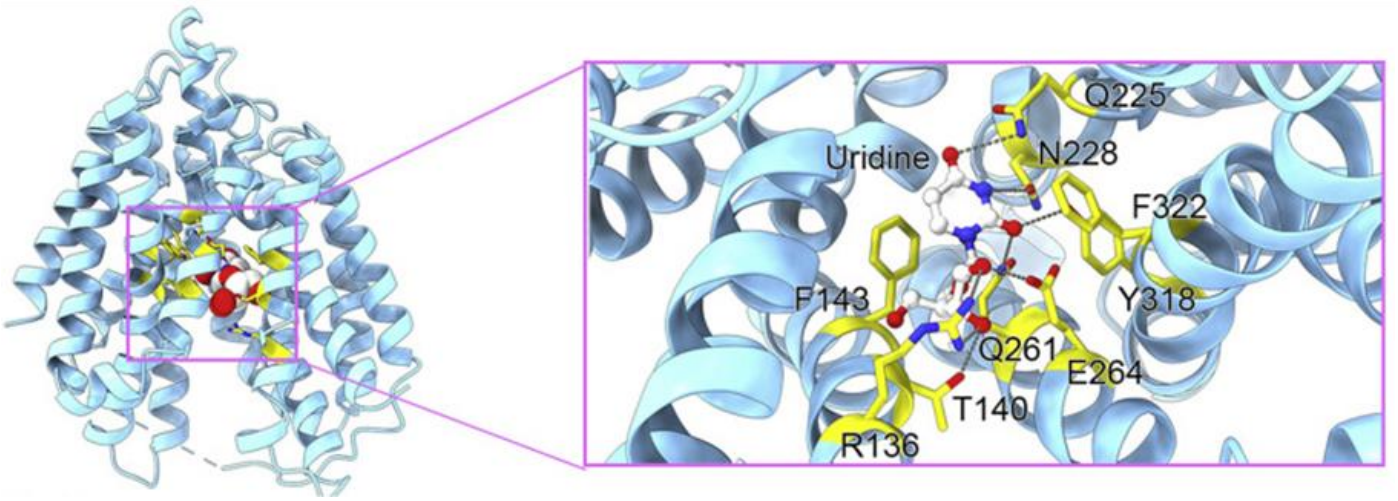

Figure 2. Putative substrate-binding site of NupG based on the docking model of the NupGD323A–uridine complex. Left. The overall structure is represented as ribbons, and uridine is represented as red spheres. **Right.** Detailed binding interaction between residues in the putative substrate binding site (yellow sticks) and uridine (white sticks). This figure was reproduced from Wang et al. (2021).

The location of the NupG D323A mutant is not close to the binding pocket of NupG, but it does have an important role in nucleoside binding/transport and it is identically conserved in both XapB and YegT (Table 1). In preliminary investigations, it was shown that the D323A mutant maintained its enhanced uridine-binding affinity in different buffers with a range of pH values (5, 6 and 8), whilst wild-type NupG had decreased binding affinity at pH 8 (Wang et al., 2021). It was suggested that this property is similar to that of LacY mutant E325A, where residues E325 and K319 were identified as protonation sites, and that D323 may be the proton-escaping site of NupG during proton-coupling (Wang et al., 2021).

CONCLUSION

More comprehensive biochemical and structural investigations are necessary to further understand substrate recognition and to elucidate molecular mechanism in NHS family proteins. This should help to answer the following questions: (i) What residues are responsible for the differences in substrate specificity between NupG and XapB? (ii) What is the substrate specificity of YegT? (iii) What are the structural differences between NHS family proteins? - obtain crystal structures of XapB and YegT. (iv) What effect does the D323A mutation have on XapB and YegT substrate binding and what effect does it have on transport activity in NHS proteins? (v) How does D323 couple substrate binding and protonation in NupG?

Table 1. Conservation of nucleoside binding site residues in *E. coli* NHS family proteins. Residues identified in the putative nucleoside binding site of the NupG structure are compared with the corresponding residues in XapB and YegT. NupG residues are coloured to show the position of mutant D323A (pink), residues that when mutated to Ala abolished uridine binding (red), and the residue that when mutated to Ala did not affect uridine binding (green). XapB and YegT residues identically conserved with NupG are in bold.

	Residue		
TM helix	NupG	XapB	YegT
TM5	R136	**R136**	**R131**
	T140	**T140**	**T135**
	F143	**F143**	W138
TM7	Q225	**Q225**	A226

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	N228	**N228**	Y229
TM8	Q261	**Q261**	**Q252**
	E264	**E264**	**E255**
TM10	Y318	**Y318**	H310
	F322	**F322**	Y314
	D323	**D323**	**D315**

CONFLICT OF INTEREST

The author has no conflict of interest to declare.

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