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Crystal structure of the dimer of two essential *Salmonella typhimurium* proteins, YgjD & YeaZ and calorimetric evidence for the formation of a ternary YgjD–YeaZ–YjeE complex

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Abstract: YgjD from COG0533 is amongst a small group of highly conserved proteins present in all three domains of life. Various roles and biochemical functions (including sialoprotease and endonuclease activities) have been ascribed to YgjD and orthologs, the most recent, however, is involvement in the post transcriptional modification of certain tRNAs by formation of N6-threonyl-adenosine (t^6A) at position 37. In bacteria, YgjD is essential and along with YeaZ, YjeE, and YrdC has been shown to be 'necessary and sufficient' for the tRNA modification. To further define interactions and possible roles for some of this set of proteins we have undertaken structural and biochemical studies. We show that formation of the previously reported heterodimer of YgjD–YeaZ involves ordering of the C-terminal region of YeaZ which extends along the surface of YgjD in the crystal structure. ATPγS or AMP is observed in YgjD while no nucleotide is bound on YeaZ. ITC experiments reveal previously unreported binary and ternary complexes which can be nucleotide dependent. The stoichiometry of the YeaZ–YgjD complex is 1:1 with a K_D of 0.3 μ*M*. YgjD and YjeE interact only in the presence of ATP, while YjeE binds to YgjD-YeaZ in the presence of ATP or ADP with a K_D of 6 μ*M*. YgjD doesn't bind the precursors of t^6A, threonine, and bicarbonate. These results show a more complex set of interactions than previously thought, which may have a regulatory role. The understanding gained should help in deriving inhibitors of these essential proteins that might have potential as antibacterial drugs.

Keywords: YgjD; YeaZ; YjeE; essential protein; t^6A; t-RNA; nucleotide binding; ITC; crystallography

Abbreviation Ec, Escherichia coli; ITC, isothermal titration calorimetry; RPF, resuscitation promoting factor; St, Salmonella typhimurium; Tm, Thermotoga maritime; VNC/VBNC, viable but not culturable state; Vp, Vibrio parahaemolyticus.

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Introduction

Kae1/Qri7/YgjD (products of COG0533) belong to a small group of 60 proteins that are present in all three domains of life.[1] Their 'universally conserved' status is likely to reflect involvement in fundamentally important biochemical and cellular processes.

The biological roles of Kae1/Qri7/YgjD proteins have been characterized in terms of overall cellular effects by studies of mutant forms and modulation of their levels of expression. In yeast, Kae1 forms part of the KEOPS (or EKC)[2] complex, which consists of five proteins. KEOPS (EKC) has been implicated in transcription, maintenance of telomeres, and segregation of chromosomes.[3] The COG0533 ortholog found in mitochondria, Qri7, has been reported as essential for maintenance of the mitochondrial genome in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*.[4] In prokaryotes, YgjD has been shown to be an essential gene product and its depletion can lead to significant alterations in cell morphology and ultrastructure.[5]

Several functions have been proposed for the Kae1/Qri7/YgjD ortholog group and their various partner proteins. An early study indicated that an ortholog of the prokaryotic protein YgjD, called Gcp from *Mannheimia haemolytica* (*Mh*Gcp), was an endoglycoprotease specific for heavily *O*-sialoglycosylated substrates such as glycophorin A.[6–8] Paradoxically, for what is now known to be a universally conserved protein only a single *Mannheimia haemolytica* serotype (A1) gave an active enzyme, and it was later suggested that another unidentified secreted protease might display the *O*-sialoglycoprotein endopeptidase activity in these studies.[9] This work has been influential in the annotation of further orthologs and paralogs as well as forming the basis for the MK-M22 class of proteases (MEROPS database).[10] The YgjD ortholog from *Riemerella anatipestifer*, which acts as a cohemolysin (*Ra*CAMP), purified as a recombinant non-secreted protein was reported to have sialoprotease activity.[11]

More recently, completely different biochemical properties were reported for the archaeal COG053 ortholog, PAB1159, namely DNA-binding and apurinic exonuclease activity.[12] However, neither activity was confirmed in a later study using a homolog from a different archaeon.[3] Yet a further property reported for the ortholog from *Escherichia coli* (referred to as Gcp in this case) was the reduction of Amadori-modified proteins, the products of non-enzymatic glycation which are known toxic compounds.[13]

Current evidence points to a role for the COG0533 proteins in a post translational modification of certain tRNAs. This modification involves the formation of N^6-threonyl carbamoyl adenosine (t^6A) at position 37 in the anti-codon stem loop.[14–16] The expression levels of Kae1/Qri7/YgjD correlate with cellular concentrations of t^6A. A subset of tRNAs contain t^6A, which is derived from ATP, bicarbonate, and threonine precursors. This modified base appears to act to maintain accurate decoding by direct structural mechanisms including the prevention of U33-A37 base-pairing interactions.[17] YrdC/Sua5, a further universally conserved protein, is also centrally involved in this tRNA modification and it has been proposed that it catalyses the addition of the carbamoyl threonyl group to appropriate tRNA stem loops.[18,19] The four *E. coli* proteins YgjD, YrdC, YjeE, and YeaZ are reported to be 'necessary and sufficient' for t^6A synthesis *in vitro*.[20] Crystallographic studies of an archaeal ortholog of YrdC, Sua5, identified a ternary complex containing threonine and the non-hydrolysable ATP analog AMPPNP that was proposed to be suitably located for N^6-threonylcarbamoyl adenosine synthesis.[21] A different designation has been made based on studies of an *O*-carbamoyltransferase, TobZ , which contains both YrdC-like and Kae1(YgjD)-like domains, and catalyses analogous reactions to the t^6A modification.[22] It was proposed that Kae1 catalyses the threonylcarbamoyl transfer to yield t^6A_{37} and that YrdC promotes the formation of the threonylcarbamoyladenylate intermediate.

The structure of Kae1 revealed a protein belonging to the ASKHA/HALF superfamily with a bound ATP analog and a nearby zinc ion.[12] The structure of most of the KEOPS complex (Kae1/Bud32/Pcc1/Cgi121, but lacking Gon7) was derived from a series of overlapping structures and showed Kae1 interacting with Bud32, a primordial protein kinase regulated by Cgi121.[3]

Studies of the archaeal MJ1130, a fusion of Kae1 with Bud32 and thus a subset of the KEOPS complex, showed that extensive interactions between Kae1 and Bud32 domains were required for normal function.[23] Both the kinase activity of Bud32 and the ATPase activity of Kae1 appear necessary for the t^6A modification.[14]

Within the prokaryotic domain of life, complexes of YgjD with various other proteins have been identified. One such is the heterodimer of YgjD and its smaller paralog called YeaZ,[5,24] proteins containing 337 and 231 amino acids, respectively in the case of the orthologs from *Salmonella typhimurium*, (*St* YgjD & *St* YeaZ). Close orthologs of YeaZ are restricted to bacteria, where they are not only present in the majority of Gram negative species, but also are found in some Gram positive species. A complex of YeaZ with an ATPase, YjeE, was identified in *E. coli* from bacterial two-hybrid studies and reported to be mutually exclusive with the formation of the YeaZ–YgjD complex.[5] YgjD does not share significant amino acid sequence identity to YjeE, similarly there is no significant sequence identity between the partner proteins of YgjD (YeaZ and

Table I. *Thermodynamic Parameters for Binding of YeaZ and YgjD and YjeE to a preformed YgjD:YeaZ dimer were determined by ITC at 25°C*

Protein in Injector	Protein in Cell	Protein in Cell	ΔH_{obs} (kcal/mol)	ΔS (cal K/mol)	K_{Dapp} (μM)	n	c
YeaZ (430 μM)	YgjD (43 μM)	–	–5.2 (±0.1)	12.4 (±0.2)	0.29 μM (±0.1)	0.9 (±0.1)	127 (±8)
YjeE (460 μM)	YgjD (37 μM)	YeaZ (42 μM)	1.6 (±0.1)	29.1 (± 0.7)	5.8 μM (±1.1)	1.0 (±0.2)	6.4 (±1.1)

The binding was measured in 50 mM KPO_4, pH7.2, 0.1M NaCl, 1 mM DTT, by ITC. The concentrations of the proteins used are shown in the table. The data were fitted to a single site binding model.
The c values fall within the range 1–1000 that allows the isotherms to be accurately deconvoluted with reasonable confidence to derive the K values (Wiseman et al. 1989).
Shown are the values for n, the stoichiometry of binding; $K_{D(app)}$, the apparent equilibrium dissociation constant; ΔH_{obs}, the observed entropy change for single site binding; and ΔS^0, the standard entropy change for single site binding. For each value the standard deviations for three experiments are shown in parentheses.

YjeE) and those forming the KEOPS/EKC complex with Kae1, for example, Bud32/Pcc1/Cgi121/Gon7. Pull-down assays showed the involvement of YrdC in forming complexes involving either YgjD or YeaZ, while they did not bind to YjeE.[20] Another proposed function for YeaZ is as a highly specific protease that cleaves the C-terminal region of YgjD in *E. coli*.[5]

Crystal structural analysis of the prokaryotic proteins, YgjD, YeaZ, and YjeE have, to date, only contained single protein species of either YeaZ or YjeE. The structure of YjeE from *Haemophilus influenzae* showed no homology to other proteins apart from a Walker motif, the latter being indicative of nucleotide binding.[25] An ATP-ase activity for *Hi*YjeE was observed, although another report revealed tight binding of ADP but only slow hydrolysis of ATP once contaminating ATPases were removed.[26]

Crystal structures for YeaZ from *Escherichia coli (Ec),*[27] *Salmonella typhimurium (St),*[28] *Thermotoga maritime (Tm),*[29] and *Vibrio parahaemolyticus(Vp)*[30] have been determined. YeaZ has a 2-lobed structure which belongs to the ASKHA superfamily. The bacterial orthologs are very similar in structure while these have some differences compared to *Tm*YeaZ, where the C-terminus forms an α-helix (α-6) in place of a region containing no regular secondary structure in the former. We noted that the relative orientation of the 2 lobes in *St*YeaZ, as seen in the crystal structure, were incompatible with nucleotide binding, consistent with the results from calorimetric measurement in solution. In order for YeaZ to bind nucleotides it seemed that relative domain movements and/or binding to a partner protein might be necessary.[28] It has been proposed that an alternative dimer interface AB/form 2, observed in the *Tm*YeaZ crystal structure, if utilised in the YgjD–YeaZ complex, might allow nucleotide binding to extend from YeaZ across the heterodimer boundary.[29,30] It was further suggested that the different conformational states of these two dimeric complexes could form the basis for a switching mechanism between partner proteins.

*St*YeaZ has been reported to act as a resuscitation promoting factor (RPF) in *Salmonellae*,[31] having a role in the initiation of escape from the 'viable but not culturable state' (abbreviated as VNC or VBNC). The mechanism of this effect is not yet known although it does imply that YeaZ must also be capable of being secreted. Whether YgjD is involved in escape from VNC is also unknown. We reported that there was no resemblance between the structures of Gram positive[32] and Gram negative RPFs.[28]

Our studies are aimed at the characterization of the structure and biochemical properties of essential proteins of unknown function in *Salmonella typhimurium* that might represent novel drug targets, including YeaZ,[28] YegS,[33] and YcbL.[34] New drugs are urgently required as the continual rise of high level resistance to current anti-bacterials presents increasing threats to human and animal health. We have now extended our earlier structural and functional work on YeaZ to include studies of its interactions with partner proteins and small molecule ligands. Thus we report crystal structure determination of the heterodimeric complex of YeaZ and YgjD bound to different nucleotides. Additionally, we describe extensive calorimetric studies to assess interactions between three proteins essential for t^6A synthesis in bacteria, YeaZ, YgjD, and YjeE together with the effects of binding of ATP/ADP and putative precursors for the tRNA modification such as bicarbonate and threonine.

Results

YgjD and YeaZ form a binary complex that is unaffected by ADP or ATP

The interaction between YgjD and YeaZ was investigated by isothermal titration calorimetry (ITC) and the results are summarized in Table I and Figure 1(A). Examination of Table I and Figure 1(A) shows that YgjD and YeaZ bind with a 1:1 stoichiometry and K_D of 0.29 μM in an exothermic reaction.

Both proteins were also screened by ITC for their ability to bind nucleotides. YeaZ showed no evidence of binding ADP or ATP, whereas YgjD showed exothermic binding of ADP and ATP. The binding of

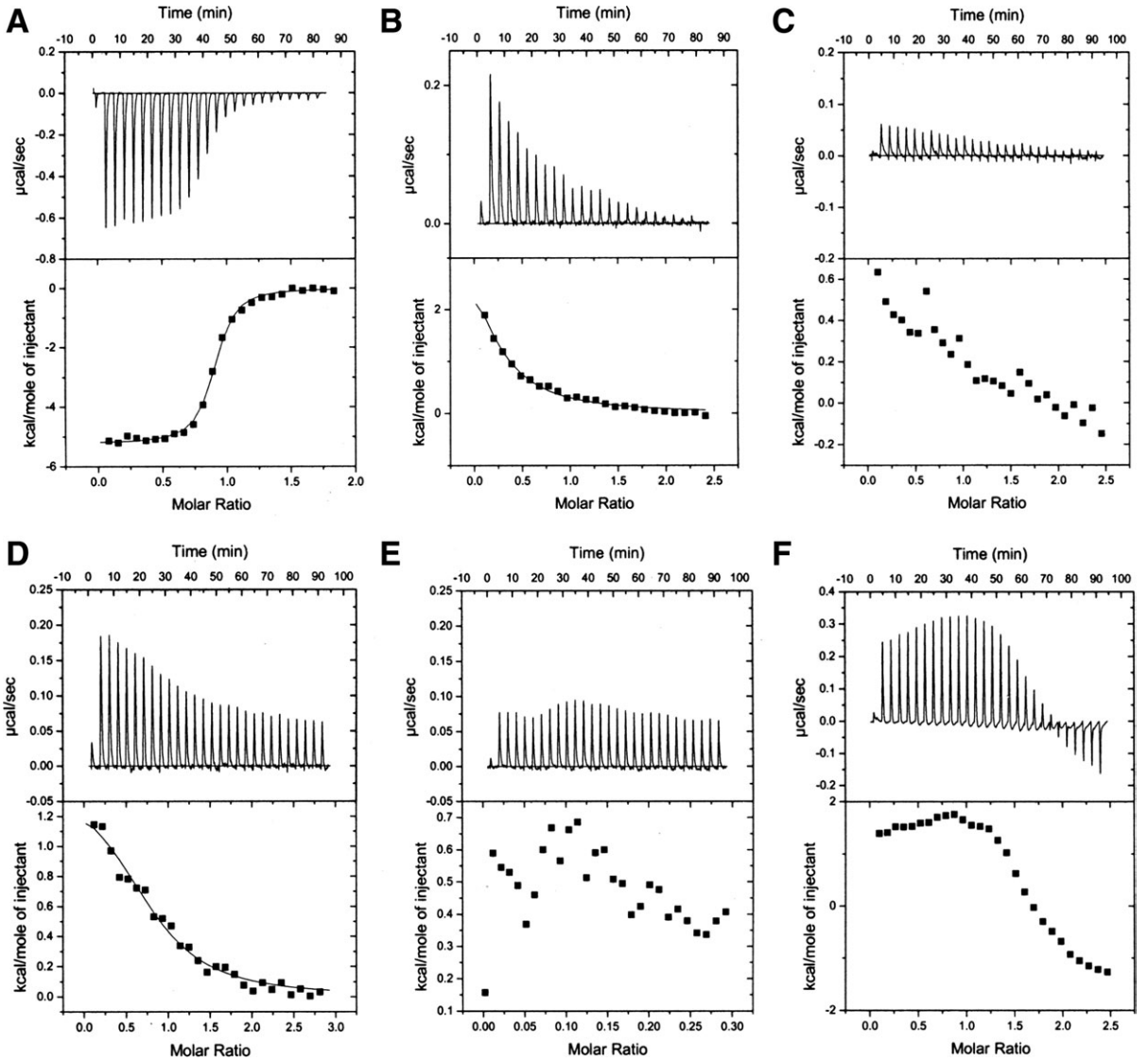

Figure 1. ITC analysis of the interactions between YjeE, YgjD, and YeaZ. All the ITC experiments were carried out in 50 m*M* KPO4, pH7.2, 0.1*M* NaCl, 1 m*M* DTT. (A) 430 µ*M* YeaZ titrated into 43 µ*M* YgjD. (B) 482 µ*M* YjeE titrated into 37 µ*M* YgjD in the presence of 1 m*M* ATP. (C) 459 µ*M* YjeE titrated into a mixture of 39 µ*M* YgjD and 39 µ*M* YeaZ. (D) 459 µ*M* YjeE titrated onto a mixture of 42 µ*M* YgjD and 37 µ*M* YeaZ in the presence of 1 m*M* ADP. (E) 459 µ*M* YjeE titrated into buffer in the presence of 1 m*M* ADP. (F) 459 µ*M* YjeE titrated into a mixture of 39 µ*M* YgjD and 39 µ*M* YeaZ in the presence of 1 m*M* ATP. In each titration, the upper panel shows the heat exchange upon injection (1 × 2 µL and 24 ×10 µL) of the protein from the injector and the lower panel shows the integrated heat pulses, normalized per mole of injectant.

ATP was complex and could not be analyzed quantitatively (data not shown), whereas the binding of ADP was adequately described by a single site binding model (data not shown). The *c* values for the interaction were below 1, therefore the data could not be deconvoluted to derive accurate K_D estimates.[35] When the titration of YeaZ onto YgjD was repeated in the presence of 1 m*M* ADP or ATP, the K_D for the binding of YgjD to YeaZ was unaffected.

Nucleotides modulate the interactions of YjeE with YgjD and a YgjD–YeaZ dimer

YjeE was screened by ITC for its ability to bind nucleotides. The binding of ATP gave a complex pattern, whereas the binding of ADP was adequately described by a single site binding model (data not shown). The c values for this interaction were below 1, therefore the data could not be deconvoluted to derive K_D estimates.[35]

The binding of YjeE to YgjD or YeaZ was assessed alone and in the presence of ADP or ATP. No heat exchanges greater than baseline heats of dilution were observed when YjeE was titrated into YeaZ, implying no binding (data not shown). When YjeE was titrated into YgjD, endothermic binding was observed but only in the presence of ATP [Fig. 1(B)]. This experiment was repeated on three separate occasions and in each case the data were adequately described by a single site binding model.

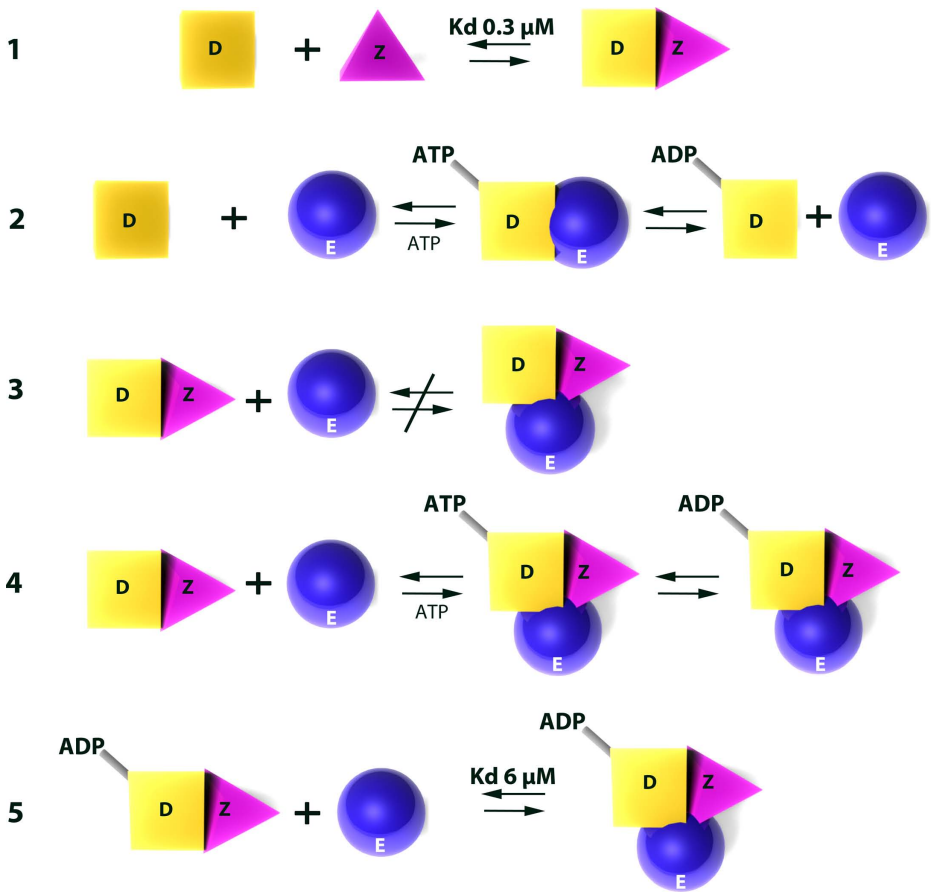

Figure 2. Interactions between YgjD (D) , YeaZ (Z) , YjeE (E) and nucleotides as deduced from ITC. 1 = Z and D interact with a 1:1 stoichiometry and a K_d of approximately 0.3 µ*M*. 2 = D and E interact only in the presence of ATP. 3 = E does not interact with a Z–D dimer in the absence of nucleotides. 4 = E interacts with a Z–D dimer in the presence of ATP or ADP. 5 = E interacts with a Z–D dimer with a 1:1 stoichiometry and a K_d of approximately 6 µ*M* in the presence of ADP. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

However the '*c*' values for this interaction were below 1, therefore the K_D values were not derived.

YjeE was further screened for its ability to bind to a preformed YgjD–YeaZ heterodimer in the presence and absence of ADP or ATP. No strong evidence for binding was observed in the absence of nucleotides [Fig. 1(C)]. The small heat exchanges seen in [Fig. 1(C)] are at a level below the ITC instrument's sensitivity for recording accurate measurements. Consequently, these heat exchanges cannot be reliably interpreted as evidence of binding. In contrast, significant endothermic reactions were seen in the presence of ADP [Fig. 1(D)] or ATP [Fig. 1(F)]. The interaction in the presence of ADP showed a residual heat of dilution [see Fig. 1(D)] that was accounted for by the relatively high heat of dilution seen in the buffer baseline control [Fig. 1(E)]. The interaction in the presence of ATP was complex and was not adequately described by a simple binding model, therefore the data could not be analyzed quantitatively [Fig. 1(F)]. However, the interaction between YjeE and the YgjD-YeaZ heterodimer, in the presence of ADP, was adequately described by a single site binding model that indicating a 1:1 stoichiometry and a K_D of 5.8 µ*M* [Table 1, Fig. 1(D)]. The interactions between YjeE, YgjD, & YeaZ and their proposed modulation by nucleotides are summarized in Figure 2.

Screening YgjD and YeaZ for the ability to bind threonine and bicarbonate

ITC was used to screen YgjD (43.2 µ*M* in the cell) or YeaZ (40 µ*M* in the cell) for their ability to bind threonine or bicarbonate. Each ligand (3 m*M* in the injector) was tested individually in the presence and absence of ATP (3 m*M* in the injector and the cell) and together in the presence and absence of ATP. In all cases no heat exchanges above the background heat of dilution could be detected implying no binding.

Testing for YeaZ-mediated proteolysis of YgjD

Digestion experiments (see Methods) using 2:1 and 3:1 molar ratios of YgjD–YeaZ were carried out at 25 and 6°C in the presence and absence of Zn^{2+}, ATP and Zn^{2+}/ATP. No evidence of any digestion of YgjD

Table II. *Data Collection and Refinement Statistics for StYeaZ-YgjD*

Crystal	ATPγS SeMet Remote ANMOI[a]	ATPγS SeMet Peak ANMOI[a]	Native ADP (AMP)
Beamline	ESRF BM14	ESRF BM14	Diamond I04
Wavelength (Å)	0.9755	0.9785	0.9801
Space Group	*P*1	*P*1	*C*2
Unit cell parameters (Å)	$a=61.2$, $b=68.2$, $c=87.7$ $\alpha=107.7$, $\beta=92.6$, $\gamma=116.4$	$a=61.2$, $b=68.1$, $c=87.6$ $\alpha=107.7$, $\beta=92.6$, $\gamma=116.4$	$a=121.1$, $b=61.6$, $c=83.8$ $\beta=101.1$
Resolution range (Δ)	50–1.65(1.71–1.65)	50–1.90(1.97–1.90)	50–2.30(2.38–2.30)
Redundancy	6 (3.8)	4 (3.8)	4.0 (4.1)
Completeness (%)	96.4 (87.3)	90.3 (51.9)	96.7 (100.0)
R_{merge}[B] (%)	6.7 (47.2)	5.2 (14.8)	11.6 (64.7)
I/σI	31.2 (2.5)	35.0 (9.8)	25.1 (5.2)
R_{work}[c] (%)/R_{free}[c] (%)	15.9/18.6	–	18.2/23.2
r.m.s.d bond lengths (Å)	0.007	–	0.009
r.m.s.d. bond angles (Å)	1.1	–	1.0
Mean B-factors; all/main chains/ side-chains and waters (Å^2)	21.7/17.8/25.2	–	29.7/25.8/33.7

Figures in brackets are for outer shell data: [a]ANMOI: anomalous, no merge, original index; [b]$R_{merge}=\sum|I_{obs}-I|/\sum I$; [c]$R=\sum_{hkl}|F_o(hkl)-F_c(hkl)|/\sum_{hkl}|F_o(hkl)|$. PDB codes 3ZEU & 3ZET.

was detectable by SDS PAGE under any of the conditions tested.

Crystallization, data collection, and structure determination of the YgjD–YeaZ complex

Extensive trials yielded no useable crystals of YgjD as a single protein species or as part of the ternary complex YgjD–YeaZ–YjeE. Dynamic light scattering showed YeaZ to be dimeric in solution and YgjD as monomeric. Mixing the two showed a clear upshift in the hydrodynamic radius consistent with the formation of a heterodimer (data not shown), which crystallized under several conditions.

The structure of the YgjD–YeaZ complex containing SeMet labeled YeaZ was solved by MAD methods. Initial phases were obtained by solving the YeaZ seleno-methionine substructure using data truncated at 2.0 Å resolution. Phases were then extended to 1.65 Å resolution, resulting in the building of 96% of the residues in RESOLVE. Further refinement and manual rebuilding gave the final statistics listed in Table II; representative electron density in the region of the nucleotide binding site on YgjD is shown in Figure 3. Data to 2.3 Å resolution were collected from crystals of YgjD–YeaZ grown in the presence of ADP. Refinement of the structure revealed that the ADP had degraded to AMP [Fig. 3(B)].

Structure of the YgjD-YeaZ heterodimer: subunit contacts and the role of YeaZ C-terminus

The crystal structure of YgjD from the heterodimer with YeaZ shows the typical ASKHA superfamily

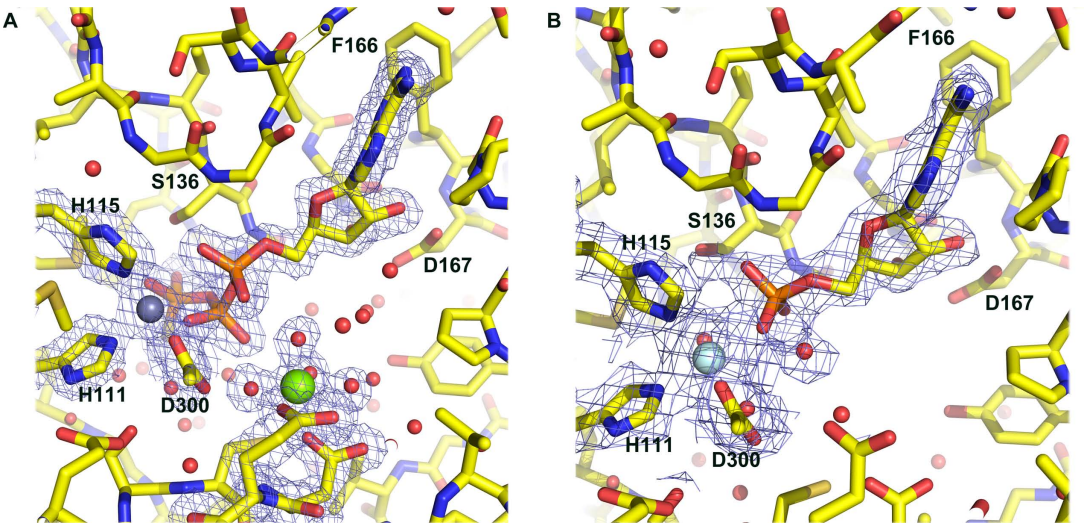

Figure 3. Electron density for the nucleotide binding site on YgjD. Protein and nucleotide skeletal models are shown in standard atom colors with water molecules colored red. (A) ATPγS, with zinc ion (medium blue) and magnesium ion (green). (B) AMP, with cadmium ion light blue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

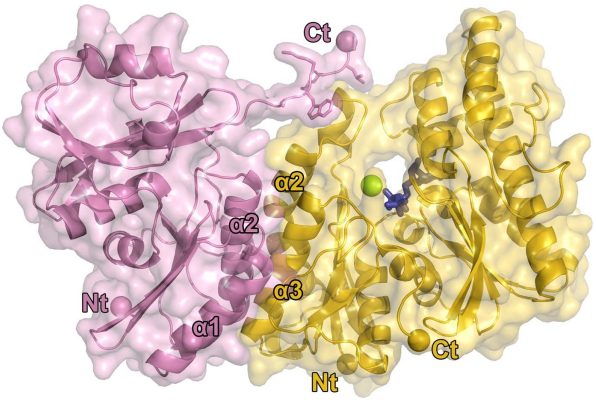

Figure 4. Crystal structure of the complex of YeaZ and YgjD: YeaZ is colored purple & YjgD is in gold. The ATPγS is shown in dark blue, while magnesium is colored green. Some components of the subunit interface of the two proteins are labeled including α-helices and the extended C-terminus of YeaZ. Other N- & C-termini are also marked. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

fold, with the protein organized into 2 lobes. Each lobe consists of one long helix, a β-sheet and a largely helical sub-domain [Fig. 4]. Overall the secondary structure is similar to that of Kae1 with some differences in loop regions. Overlapping the α-carbons of YgjD and Kae1 (PDB: 2IVN) shows an rms deviation of 1.9 Å for 321 equivalent residues with 37% sequence identity. The secondary structure of YgjD is related to that of YeaZ apart from the extended C-terminus of the latter as well as differences in loop regions which accommodate additional residues in YgjD [Fig. 6].[28]

Comparison of the structure of YgjD–YeaZ complexes in either the presence of ATPγS or with AMP showed small conformational changes in side chains of YgjD that were confined to the vicinity of the β-phosphate or γ-S-phosphoryl group [Fig. 7]. Similarly, *St*YeaZ, whether present in the heterodimeric binary complex with *St* YgjD or as a single protein[28] adopts an overall similar conformation but with one notable exception. Although the C-terminal ~13 amino acids are disordered in the crystal structure of *St*YeaZ,[28] 11 of these residues become fully ordered in the complex with YgjD by formation of an extended arm that has many contacts with YgjD, which are described in more detail below.

The configuration of the YgjD–YeaZ heterodimer mimics one of the two putative YeaZ homodimers suggested from the crystal packing of earlier YeaZ structures.[27–30] Although this so-called A_2/form 1 dimer has a smaller surface contact area than does the AB/form 2 dimer, it is the most commonly observed in the previously reported YeaZ crystal structures.[29,30]

The subunit contacts for YgjD–YeaZ are formed from a number of interactions, which are mainly hydrophobic in nature, although some hydrogen bonds are also present [Fig. 5(D)]. The most prominent feature is the packing of 2 pairs of α-helices located in the N-terminal regions of each protein (α-2 & α-3 from YgjD and α-1 & α-2 from YeaZ), forming a 4-helix bundle (see Figs. 4 and 5). A set of conserved hydrophobic residues noted at the interface region of the A_2 dimer of *Vp*YeaZ[30] (Leu39, Leu46, Ile73, Ile77, Leu81 in *St*YeaZ) are largely contained within the 2 interface helices [Fig. 5(C)]. However, the closest contact distances with YgjD are generally more than 4.0 Å apart except in a few cases, for example, residue Leu39, where CD1 is 3.6 Å from the CG of Arg 96 and 3.7 Å from the main-chain O of Leu321 in YgjD. A closer contact is between the CD1 of Leu61 in YeaZ to Tyr100 CD2 (YgjD) (3.6 Å).

The second set of inter-protein contacts involve the extended C-terminus of YeaZ that runs along the surface of YgjD [Figs. 4 and 5(D)], are varied in nature and include both hydrophobic and hydrogen-bonding interactions, for example, Val222 CG1 & CG2 (YeaZ) with Tyr 39 CE2 & OH (YgjD); Trp224 CD1 (YeaZ) with Val43 CG1 (YgjD); Ser220 OD1 (YeaZ) to Arg49 NH1 (YgjD) and Arg219 CO main chain (YeaZ) to Arg49 NH2 (YgjD) [Fig. 4]. By contrast, this C-terminal region is disordered in the *St*YeaZ structure[28] and proteolytically removed in *Vp*YeaZ.[30]

The third set of contacts involves a region near the C-terminus of YgjD. However, as it is about ten residues from the C-terminus, the latter thus does not extend over the surface of the partner protein in the manner observed for the C-terminus of YeaZ binding to YgjD. Interactions include Thr324 OG1 with Gln36 NE2 of YeaZ, the latter forming a bifurcated interaction to Val321 C=O main chain in YgjD [Fig. 5(B)]. The C-terminal region of an ortholog of YgjD from *Staphylococcus aureus* (*Sa*Gcp) has been shown from mutational studies to be important for heterodimer formation with YeaZ.[36] Mutations in the orthologous *Sa* Gcp at Tyr317-Phe322 (Phe313-Thr318 in *St* YgjD) abolish dimer formation, while mutating Asp324-Asn329 (Asp320-Thr324 in *St* YgjD) or Ser332-Tyr336 (Pro327-Leu331 in *St* YgjD) reduce binding to YeaZ. Direct intersubunit contacts are present in one case (Val323 main-chain NH (YgjD) to Thr 35 (YeaZ) and Val 323 C=O main-chain (YgjD) to Thr35 OG1(YeaZ)), while the other two regions, including the most disruptive site (Phe313 to Thr318 in *St* YgjD), are not close to the subunit interface [Fig. 5(B)]. In the latter cases mutations might interfere with dimerization indirectly via disrupting the four-helix bundle.

Comparison of the structure of YgjD with other proteins

A list of the top 10 structures most closely related to YgjD in the PDB from DALI searches is shown in Table III, some duplicate entries were omitted. The

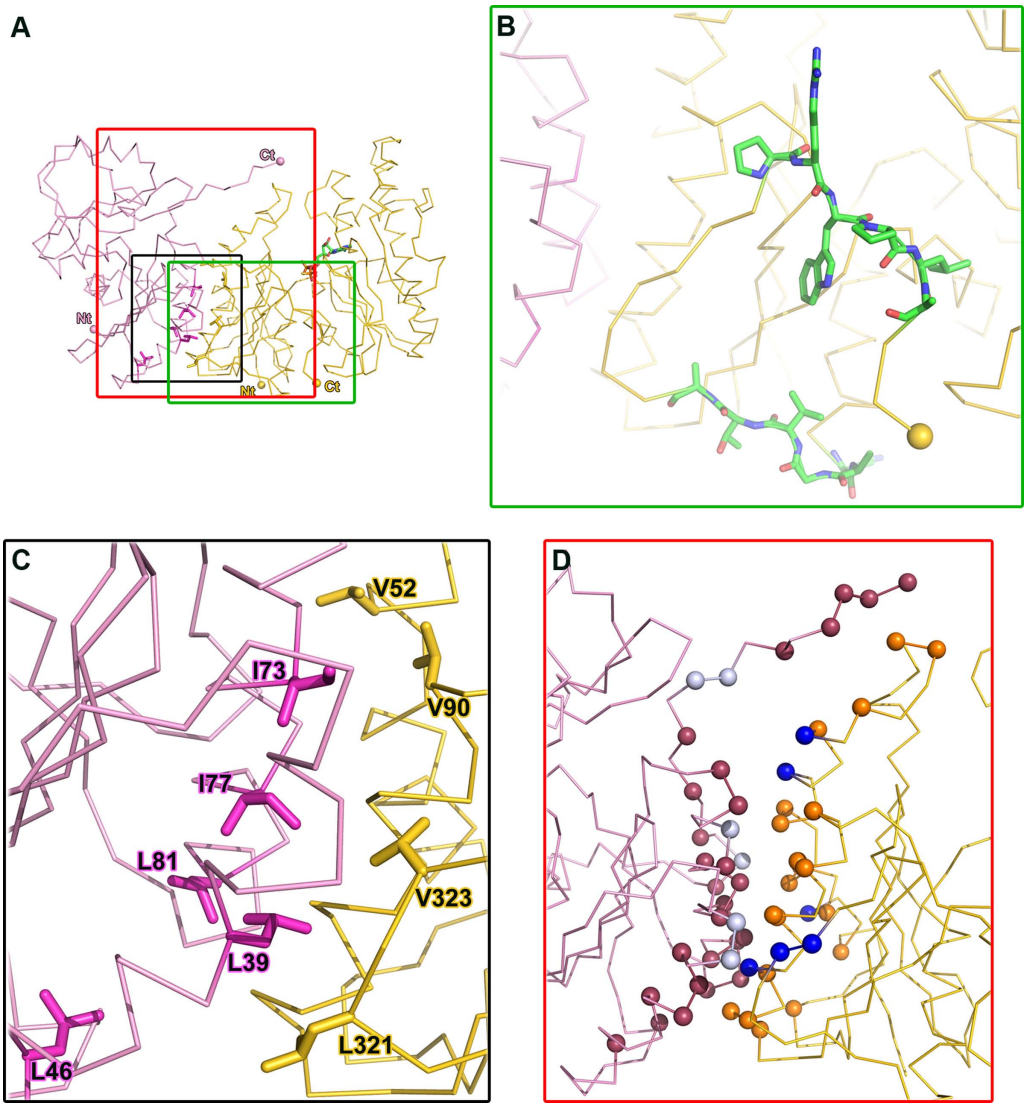

Figure 5. General and expanded views of YgjD–YeaZ contacts. (A) Overall view of the Ygjd–YeaZ heterodimer with expanded regions marked. (B) mutational sites equivalent to those that affect dimer stability in the *S. aureus* ortholog (residues 313–318 & 327–331) yet are distal to the contact regions for YgjD–YeaZ. (C) The conserved hydrophobic residues near to the interface in a YeaZ dimer (purple) and adjacent residues in YgjD in the heterodimer (yellow) are shown. (D) Intermolecular contacts as determined by PISA (Orange/purple hydrophobic interactions, blue/light blue ionic/hydrogen bonds). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

most similar structures are the archaeal orthologs, Kae1 and further down the list are various paralogs including YeaZ and TobZ.

Overlapping YeaZ and YgjD revealed that the smaller number of residues in the former is due to shorter loop regions that interconnect the secondary

Table III. *DALI Hits from St YgjD Search-some Duplicate Entries have been Omitted*

PDB Code	Protein	*Z* Factor	Rmsd (Å)	Length Aligned (Residues)	Length Sequence 2 (Residues)	% Sequence Identity
2ivn	Pa Kae1	42.4	1.9	321	325	37
3eno	Ta Kae1	40.9	1.9	321	329	32
2vwb	Mj Kae1–Bud32	34.7	2.0	308	507	32
3vf2	TobZ	22.6	3.2	270	566	22
2gel	YeaZ	18.6	3.2	184	218	17
1hux	2hgCoA activator	18.5	3.7	253	259	16
1x9j	Butyrate kinase	16.2	4.1	248	373	13
3tsq	HypF	15.6	2.6	154	647	23
3qbw	ANaMuramic kinase	15.5	4.7	274	356	12
3dze	Glycerol kinase	15.3	4.3	268	495	16

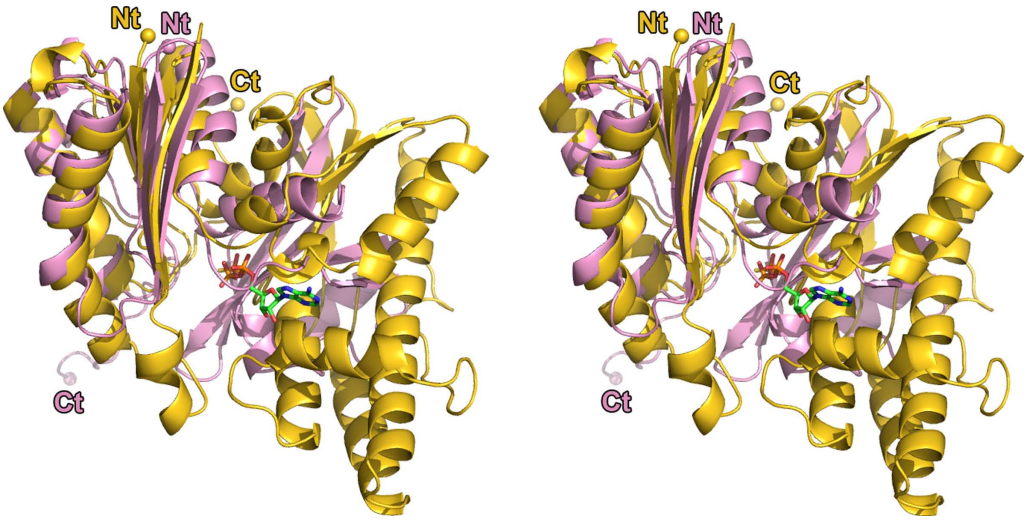

Figure 6. Stereo view of the Overlap of YeaZ and YgjD. YeaZ (colored pink) and YgjD (colored gold) both have modified HSP70-actin like folds. YeaZ has domain rotation which prevents nucleotide binding. YgjD has normal relative domain arrangement allowing the binding of ATPγS (shown in standard atom colors). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

structure as inferred from sequence alignments[28] and comparison with the Kae1 structure.[12] The overlap of YeaZ and YgjD shows a 1.6 Å rms deviation of α-carbons for 141 equivalent residues. There is a difference in the relative domain positions between YeaZ and YgjD [Fig. 6], in the case of YgjD the conformation is compatible with nucleotide binding, indeed ATPγS or AMP are bound in the two crystal structures presented here. In contrast, *St* YeaZ has lobes A and B configured in different relative positions than in most homologs,[28] which together with short connecting loops might contribute to the inability to bind nucleotides.[28,30] The relative domain positions of YeaZ do not change on formation of the complex with YgjD (Fig. 6). YeaZ largely retains the same conformation (apart from the C-terminal region) when present in homodimer or heterodimer, as reflected in an rmsd of 0.6 Å for 215 equivalent α-carbons.

Nucleotide and metal ion binding sites on YgjD

The nucleotide binding site of the YgjD crystal structures shows either ATPγS or AMP bound, the latter apparently having degraded from ADP initially used. The interactions of the 2 nucleotides with the protein are essentially the same for the purine rings with a small shift in position for the ribose hydroxyls interacting with Asp167 and Gly163 (Fig. 7). Larger differences are observed in the region of the β-phosphate/γ-thiophosphate groups where His139 and Ser136 are displaced (Fig. 7).

For the AMP complex, Cd^{2+} used as a crystallization additive, replaces a zinc ion. Additionally, Mg^{2+} positioned more distally to the nucleotide site to which it is indirectly linked by water molecules in the ATPγS complex is absent in the AMP complex (Fig. 3).

Discussion

The crystallographic & calorimetric studies of three of the proteins involved in prokaryotic t^6A synthesis: YgjD, YeaZ, & YjeE described here, has allowed more detailed understanding of their interactions with nucleotides and involvement in formation of protein:protein complexes. A more extensive network of oligomerization states in solution has been revealed, including the formation of a ternary complex. We also throw some light on potential functional properties of the three proteins, which have not as yet been fully biochemically characterized.

ITC studies of YeaZ, YgjD, and YjeE showed that binding or lack of binding of ligands could be easily distinguished and in some cases allowing K_D determination & quantification of the formation of the YgjD–YeaZ complex previously observed from qualitative interaction network studies[24] and bacterial two-hybrid screening.[5] We also showed the YgjD–YeaZ complex to be equimolar and relatively stable as indicated by a K_D value of <300 nM, while the strength of heterodimer formation is unaffected by the presence of ADP or ATP.

When comparing the ITC results with the bacterial two-hybrid data[5] there was no evidence for YeaZ–YjeE complex formation under any conditions tested. While YgjD–YjeE was observed, its occurrence was nucleotide dependent and pull-down experiments with His-tagged proteins did not detect this interaction.[20] However, this latter result is consistent with our ITC results as ATP (required for YgjD–YjeE complex formation), wasn't present in the pull-down protocol. In the current study it may be of relevance to note that all the proteins used lacked any additional heterologous amino acids.

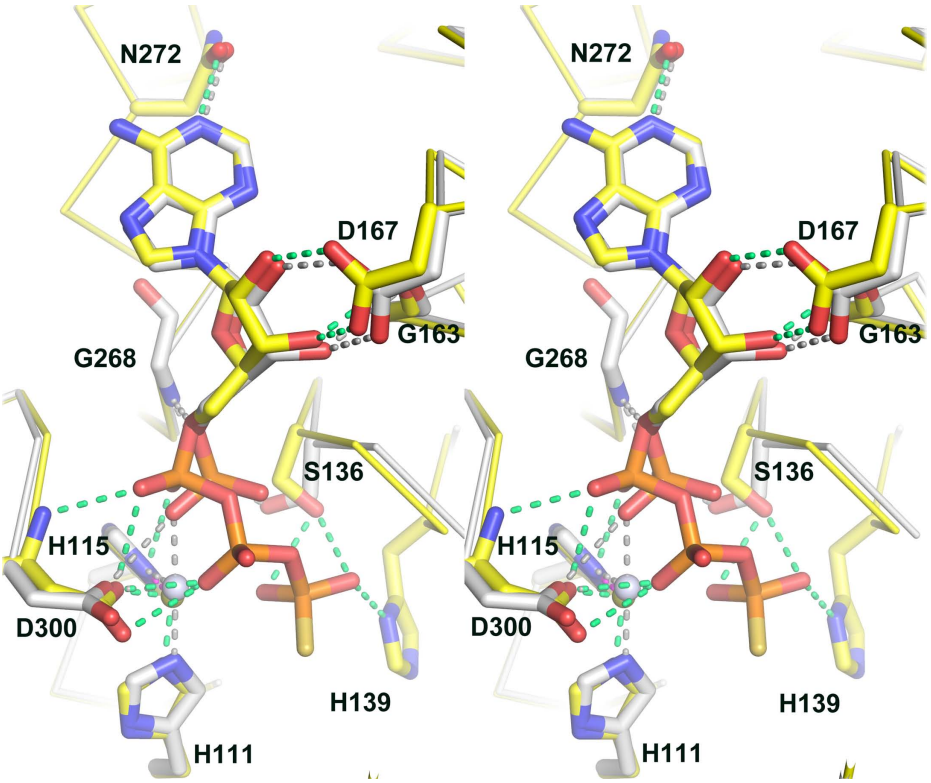

Figure 7. Stereo view showing the interactions of ATPγS and AMP with YgjD. Standard atom colors are used for ATPγS with hydrogen bonds shown as green broken lines. For AMP the carbon atoms are shown in grey, as are hydrogen bonds. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Surprisingly, instead of the mutually exclusive formation of YgjD–YeaZ & YeaZ–YjeE complexes reported previously, we found evidence for a nucleotide-dependent ternary YgjD–YeaZ–YjeE complex when YjeE was titrated onto a preformed YgjD–YeaZ dimer. Highly purified YjeE has been shown to possess only a weak ATPase activity, yet it binds the product ADP tightly.[26] Commensurate with this observation, the ternary complex formation between YjeE and a pre-formed YgjD–YeaZ heterodimer is adequately described by a single site binding model in the presence of ADP but not in the presence of ATP [see Fig. 1(D,F)]. These properties are characteristic of a signal or energy transduction system where such complexes may exist only transiently that will not necessarily be observable *in vivo* as in the case of a two-hybrid screen.

The crystal structure of the YgjD–YeaZ complex showed the A_2/form 1 dimer configuration as previously observed in the YeaZ homodimer. Differences in the conformation of YeaZ in the heterodimer occur in the C-terminal region, which undergoes a dramatic change compared to the YeaZ homodimer. The disordered C-terminal residues become fully ordered, forming interactions in an extended conformation located along the surface of YgjD. The observed YgjD overall fold was similar to that of the earlier Kae1 orthologs with some alterations in loop regions. The AB/form 2 dimer of YeaZ has some conserved hydrophobic residues situated on the outer surface,[29,30] thus potentially placed for a further protein interaction to form a trimer. One possibility is that the proposed switching between the A_2 and AB dimers could be facilitated by the interactions with YjeE as we showed that YjeE could form a ternary complex of YgjD–YeaZ–YjeE. Such a complex would potentially be capable of binding ATP across the YgjD–YeaZ boundary[30] and switching between the two states could form part of a signaling system. In the structure of the fusion protein of Bud32 with Kae1, the majority of the interactions between the different domains were confined to the second domain of Kae1[23] and could present another potential interface for YgjD to form a ternary complex. The crystallography also showed that AMP could be bound to YgjD. It is not clear whether AMP would bind under physiological conditions as in the current case the zinc ion in YgjD had been replaced by cadmium.

YgjD has recently been suggested to be a ligase for threonine with bicarbonate and it proved possible to model these latter two ligands into the putative active site of Kae1.[15] Alternatively, binding of tRNA in the correct orientation for Sua5(YrdC) to modify position 37 of the tRNA has been proposed.[14] The finding in this current study that YgjD is not able

to bind two of the precursors of t^6A synthesis (threonine/bicarbonate), argues against its potential role as a ligase. However, the observation that YrdC binds YgjD and YeaZ[20] may mean that substrate binding to YgjD can only occur in higher order complexes with YrdC, potentially allowing substrate channeling between the different enzyme active sites.

The earlier reported activity of the YgjD ortholog, Gcp, as a sialoprotease might have been complicated by a contaminant in the material purified from a native source.[9] A wide ranging series of tests for sialoproteolysis that we conducted with *St* YgjD and *St* YeaZ–*St* YgjD using glycophorin-A as a substrate, with commercially supplied *Mh*Gcp as a positive control showed no cleavage under any conditions (data not shown). Nevertheless, a recombinant YgjD ortholog has been reported to be an active sialoprotease (*Ra*CAMP), thus the question of the proteolytic properties and resulting further possible physiological roles of COG053 orthologs remains open. However, the ability to catalyze such apparently diverse reactions as sialoprotease and t^6A modification by YgjD seems somewhat surprising.

YeaZ has been proposed to catalyze the proteolytic cleavage of YgjD.[5] Despite extensive screening under different conditions of temperature, added metal ions and nucleotides, we could find no evidence for proteolysis of YgjD by YeaZ. Also, the structural studies showed that the C-terminus of YgjD was intact in the crystals with no sign of proteolytic cleavage having occurred, even though YgjD–YeaZ were subjected to long incubation times at high protein concentrations during the course of crystallization experiments. The difference in species (*S. typhimurium* against *E. coli*) used in the two experiments seems unlikely to explain the variation in proteolysis as these bacteria are closely related. Indeed, for the more divergent Gram positive *S. aureus* system no digestion of the YgjD ortholog (*Sa*Gcp) by YeaZ was observed.[36] In assessing other differences from the Handford et al. experiment, a few factors may be relevant. Firstly, their YgjD construct contained additional residues before the C-terminal 6His-tag, which potentially could cause some local unfolding that may favor proteolysis. It can be noted that, from the structural studies reported here, the C-terminal region of YgjD is functionally important in A_2 heterodimer formation with YeaZ. Secondly, we isolated YgjD and YeaZ to the high levels of purity required for crystallography by using a different procedure (i.e. hydrophobic and ion-exchange column steps) to that reported in earlier studies. In our experience, such protocols appear to be particularly effective in the removal of any protease contaminants from our *E. coli* expression system. From a structural biology perspective, it has been pointed out that YeaZ lacks resemblance to any known protease active site. This fact together with the absence of a putative zinc-binding motif has been taken as evidence against the likelihood of YeaZ being a protease.[29] The question of the biochemical role of YeaZ thus still remains unanswered. As there is still no evidence for nucleotide binding to YeaZ, it may have an essentially structural role in the formation of higher order protein complexes, perhaps as part of a signaling system. The link, if any, between t^6A synthesis and the YeaZ-induced recovery from VNC has still to be established.

This study has shown that the range of protein:-protein and protein:nucleotide interactions in the YgjD, YeaZ, YjeE system is more extensive than previously thought. Clearly a greater understanding of the role of such complexes in regulating t^6A biosynthesis is needed and also how they, or their components, may interact with YrdC; the fourth essential protein in the system. While YgjD/Kae1 and YrdC/Sua5 form common links between the prokaryotic and eukaryotic/archaeal t^6A biosynthesis systems, there are significantly differing associated proteins such as YeaZ/YjeE or Bud32/Pcc1/Cgi121/Gon7. More detailed characterization of the differing systems may shed light on any common biochemical functions.

Methods and Materials

Cloning and expression of St YgjD & St YjeE

The coding sequences of the *St ygjD* and *yjeE* genes (optimised for *E. coli* expression) were synthesised by the Blue Heron and GeneArt companies respectively and subcloned into the plasmid pET3A using 5′ and 3′ Nde1 and BamH1 sites. The resulting plasmids were designated pMUT149 (*ygjD*) and pMUT301 (*yjeE*).

YgjD, YjeE, & YeaZ purification

YgjD was purified in 50 m*M* potassium phosphate pH7.2, 1 m*M* DTT from 25 g *E. coli* BL21AI cells transformed with pMUT149 and induced at 15°C by arabinose by sequential chromatography on phenyl sepharose and Q-sepharose columns yielding 500 mg of YgjD at greater than 95% purity.

YjeE was purified in 50 m*M* potassium phosphate pH7.2, 1 m*M* DTT from 45 g *E. coli* BL21AI cells transformed with pMUT301 and induced at 30°C by arabinose by sequential chromatography on Q-sepharose and, following precipitation by ammonium sulfate in the range 25-35% saturation, sephacryl S300 columns yielding 850 mg of YgjD at <95% purity. YeaZ was purified as described previously.[28]

Isothermal titration calorimetry

The rationale underpinning the use of ITC to characterize protein–protein and protein–ligand interactions was as described previously.[37]

The heat due to the binding reaction was obtained as the difference between the heat of reaction and the corresponding heat of dilution. Studies of the interaction of certain combinations of YeaZ, YgjD, and YjeE with or without ATP or ADP were undertaken. Additionally the potential binding of threonine and bicarbonate to YgjD in the presence or absence of ATP was assessed.

Crystallization and structure determination

Samples for crystallization were prepared as follows: a 1:1 mix of SeMetYeaZ (8 mg/mL) and native YgjD (8 mg/mL) were independently buffer exchanged into 0.1*M* Tris pH8.0, 0.2*M* ammonium sulphate, 0.2*M* NaCl, 10 m*M* $MgCl_2$, and 1 m*M* TCEP prior to mixing (maximum protein concentrations during buffer exchange YgjD 2 mg/mL, YeaZ ~40 mg/mL). Approximately 8000 fold buffer exchange was used to minimize phosphate levels from the purification buffer – 1 m*M* ATPγS was then added to the mixture and buffer exchanged (1 round, 20fold), then concentrated to 16 mg/mL of total protein.

Crystals of *St* YgjD-YeaZ were obtained under several conditions. Optimization gave the best crystals of SeMet labeled YeaZ native YgjD in complex with ATPγS under the following conditions: 16% PEG3350, 0.1*M* Tris pH8.0, 0.2*M* ammonium sulphate. The complex of unlabeled YgjD–YeaZ grown in the presence of ADP were obtained using: 20 %v/v glycerol, 16 %w/v PEG8000, 0.080 M sodium cacodylate pH 6.5, 0.160*M* magnesium acetate with 20 m*M* cadmium chloride dihydrate as an additive. Crystals were frozen prior to X-ray data collection in liquid nitrogen. Data were collected at two synchrotron sources: the ESRF using the BM14 MAD beamline and I04 at Diamond, RAL, UK. Data were processed with HKL2000 or Xia2 and statistics are shown in Table II. The structure was solved by MAD methods with RESOLVE[38] using data from crystals containing selenomethionine-labeled YeaZ and native YgjD grown as a complex with ATPγS. Further complexes were solved using X-ray data from unlabeled proteins grown in the presence of ADP, although this had apparently degraded as AMP was observed. Additional model refinement was in CNS, PHENIX or Autobuster, iterated with several further rounds of rebuilding using 'O' or Coot. Final refinement statistics are listed in Table II. For similarity searches DALI was used (http:// ekhidna.biocenter.helsinki.fi/dali_server/), while more detailed overlaps were carried out with SHP.[39] Subunit contacts were assessed with PISA (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html).

Proteolysis experiments

Proteolysis experiments to test the proposed cleavage of YgjD by YeaZ were carried out in 50 mM PIPES pH7.2, 1 m*M* DTT in a final volume of 60 μL. YgjD (20 μ*M*) was incubated with YeaZ (40 and 60 μ*M*) in the presence of Zn^{2+} (830 μ*M*) or ATP (8.3 m*M*) or Zn^{2+} & ATP or in the absence of both ligands. Incubations were carried out at 6°C for 20 hours at 37°C for 3 hours and the proteins were analyzed by SDS PAGE using a 12% separating gel.

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Competing Interest Statement: ARH & DKS have no current conflict of interest but previously acted as consultants and were co-founders of Arrow Therapeutics (along with IC). Such financial interests ended by 2011.

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