Engineering of hexameric helicases to translocate specific RNA molecules

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Background

The hexameric molecular motors are an established group of ATPases involved in diverse functions: virus replication as packaging motors, nucleic acid replication as helicases and protein degradation within the proteasome. Structural information has provided an insight into their mechanism of action; however, yet to be determined is their dynamic coupling of the conversion of energy into motor function. Particularly of interest is the viral hexameric helicase P4 isolated from cystoviral phage ϕ8. P4 protein assembles into a hexameric ring to form an RNA helicase packaging motor that is structurally related to SF4 and other RecA like ATPases. P4 helicase couples the binding and hydrolysis of ATP to helicase and translocase activity, to unwind long stretches of duplex RNA and concomitantly package RNA into the viral procapsid (figure one)

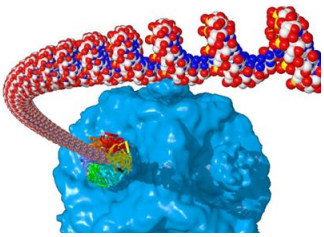

Figure 1: Model of the P4 molecular packaging motor showing the translocation of RNA into the viral capsid

In contrast to other cystoviral P4 helicases isolated from ϕ6, ϕ12 and ϕ13; ϕ8 P4 has a remarkable C-terminal structure which wraps around the outside of the molecule to insert into the central channel where RNA binds. It has been proposed that the C-terminal domain mediates RNA loading and tight coupling of ATPase activity with RNA translocation. This unique feature of ϕ8 P4 makes this helicase particularly interesting and studies to determine the exact motor function and mechanism of RNA translocation is desirable. Moreover, P4 is an effective transmembrane transporter of RNA, however lacks sequence specificity. By engineering specificity into helicases such as ϕ8 P4 they can be used siRNA applications by pumping RNA through membranes and into vesicles for gene silencing and treatment of disease states.

Aims

To engineer phi8P4 hexamer to bind and translocate specific RNA molecules by introducing a series of reactive cysteine residues by site directed mutagenesis; followed by chemical cross-linking to a synthetic DNA oligomer that is complementary to the target sequence. Investigation of several different constructs will be analysed in helicase assays.

Description of work

Cloning, expression and purification of N-terminal His-tagged P4 protein (P4-His)

The gene encoding ϕ8 P4 helicase was subcloned from pET32 into pET28b for incorporation of an N-terminal His tag (pET28b-P4-His). The P4 recombinant plasmid was transformed into XL1 Blue cells for amplification and so a large concentration of isolated plasmid was recovered. Sequencing confirmed the presence of the correct recombinant plasmid with the His tag in frame. Recombinant P4 proteins were expressed in *Escherichia coli* BL21 (DE3) by growth at 37ºC in Luria Bertani medium until OD_{600nm} reached 0.5 to 0.6. Cultures were subsequently chilled on ice and induced with 1mM isopropyl-β-thiogalactopyranoside (IPTG). Induced cells were incubated further for 16 hours at 18 ºC, harvested by centrifugation and lysed by French pressure cell with protease inhibitors. Recombinant P4 proteins were purified to homogeneity using a HiTrap FF nickel column (GE Healthcare, elution with 300-500 mM imidazole) using an AKTA system and concentrations determined by 280nm absorption with a molar extinction coefficient of 11 920 $M^{-1}cm^{-1}$. Detection of P4-His ATPase activity to confirm the recombinant proteins were functional was assayed as described below.

Design, expression and purification of structure based P4 mutants

Structure based mutants were designed to remove exposed cysteine residues and introduce novel cysteine residues into the external perimeter, for precise dye and DNA oligomer labelling and examination of RNA interactions with the protein using FRET. Point mutations were introduced into the P4 gene using template plasmid pET28b-P4-His. The amino acid substitutions C128A and C128S were introduced by site-directed mutagenesis (QuikChange, Stratagene) and the mutant plasmids were sequenced to confirm the mutations. Recombinant P4 proteins were expressed in *Escherichia coli* BL21 (DE3) and purified using a HiTrap FF nickel column as described above. Purified mutant P4 proteins were analysed for solubility and activity, as described below, before introduction of novel cysteine residues near the C-terminus. Residue 128 is close to the ATPase active site of the protein, imposing possible failure of activity if involved in enzyme catalysis. After confirmation that the cysteine-less mutant P4 protein had activity, I continued with the four point mutations; A285C, A287C, A290C, A304C which were separately introduced into the C128A mutant plasmid by site-directed mutagenesis, sequencing confirmed the mutations to be successful. Recombinant P4 proteins were expressed in *Escherichia coli* BL21 (DE3) and purified before analysis of solubility and activity.

Detection of P4 activity

ATPase activity of recombinant P4 proteins was measured using the EnzChek® Phosphate Assay kit. The concentration of the recombinant and wildtype P4 proteins was standardised for accurate comparison. The activity was measured with and without poly(A) RNA to confirm RNA induced ATPase activity.

Results

Expression and purification of P4-His was successful shown by SDS-PAGE analysis in figure two. P4-His was assayed for ATPase activity; figure three shows the activity of the wildtype P4 protein compared to P4-His at a standardised concentration of 1 mgmL^{-1}. As expected both the wildtype and N terminal Histag P4 proteins have RNA induced ATPase activity, exhibiting no activity (blue and green lines) when RNA is not present. Although P4-His has less activity than the wildtype protein, structure based P4 mutants were created using P4-His since the His tag significantly aids purification. SDS-PAGE comparison of the wildtype and P4-His proteins in showed that P4-His has two bands whereas the wildtype only has one band. This suggests truncation of P4-His, possibly at the N-terminus, which may have affected the activity measurements shown.

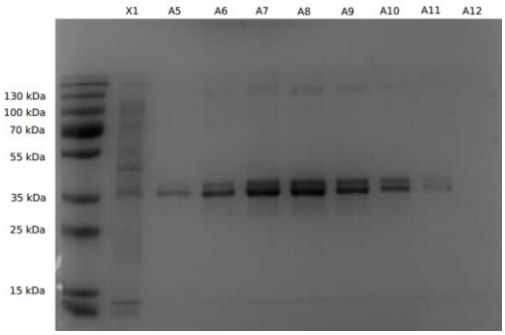

Figure 2: SDS-PAGE analysis of the fractions obtained by HiTrap purification of P4-His. Well 1: NEB protein ladder. Well 2: wash fraction. Wells 3 to 10: purified fractions A5 to A12. Bands of highest intensity and purity in wells 5, 6 and 7 therefore fractions A8, A9 and A10 pooled together and P4-His concentrated

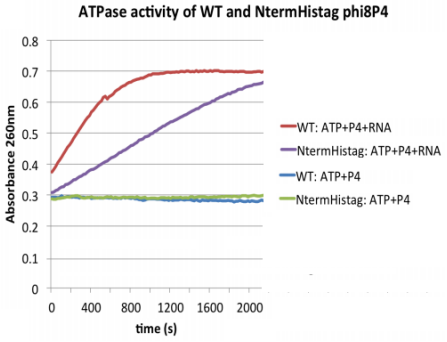

Figure 3: Activity of wildtype P4 protein compared to P4-His. Both wildtype P4 and N terminal Histag P4 have RNA induced ATPase activity. P4-His has 20% less activity of the WT protein

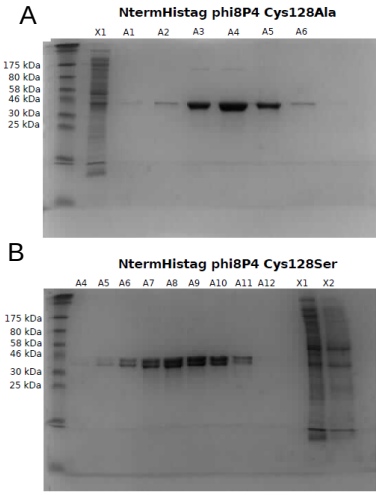

Figure 4: SDS-PAGE analysis of the fractions obtained by HiTrap purification of Cysteineless P4 mutants. (A) Well 1: NEB protein ladder. Well 2: wash fraction. Wells 3 to 8: purified fractions A1 to A6. (B) Well 1: NEB protein ladder. Wells 2 to 10: purified fractions A4 to A12. Wells 12 and 13: wash fractions. In each case the fractions of highest intensity pooled together and mutant P4 concentrated

Successful expression and purification of the cysteine-less P4 mutants was confirmed by SDS-PAGE (figure 4). Detection of mutant P4 ATPase activity demonstrated that both mutants surprisingly have more activity compared to the wildtype protein, possibly due to loss of activity of the wildtype protein during storage or degradation. Substitution of cysteine residues to produce a functional protein is difficult as they usually occupy important roles in stabilisation and catalysis, resulting in an insoluble or inactive protein when modified. However, in this circumstance it appears that both cysteine-less mutants are fully functional. Confirmation that both cysteine-less P4 mutants exhibited ATPase activity proceeded with introduction of novel cysteine residues into the C-terminal domain of the C128A mutant. Each P4 mutant created was assayed for ATPase activity as shown in figure five. Each of the different variants with a new cysteine residue exhibit RNA induced ATPase activity therefore each of the mutants can be labelled with dyes. P4 mutants which are shown to have activity after labelling with dyes will be labelled with a DNA oligomer and sequence specificity will be engineered into P4 to translocate complementary RNA molecules

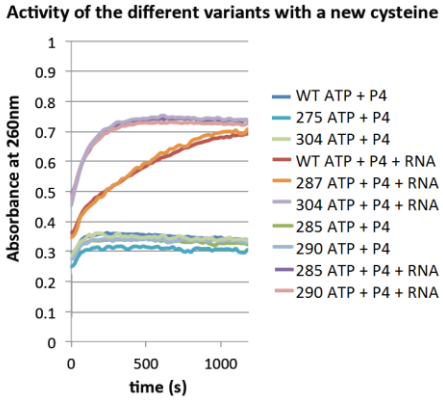

Figure 5: Activity of the four P4 mutants with novel cysteine residues introduced at positions A285C, A287C, A290C, A304C compared to wildtype

Future Directions

This project has demonstrated that P4 helicase mutants with novel cysteine residues can be created with stability, solubility and functionality. Future experiments involve modifying the P4 mutants by labelling the hexamer subunits with fluorophores, such as Alexa-488 and Alexa-594, and screening the labelled proteins for activity. Specificity can then be engineered into P4 by labelling with a DNA oligomer using the novel cysteine residues and studying how this affects translocation through helicase activity and RNA binding assays. Moreover, active fluorophore labelled mutants can be identified and their interactions with labelled RNA studied and characterised using FRET. Single molecule fluorescence can be used to examine interactions between P4 helicase and RNA by labelling them both with a fluorescent dye, and immobilising the RNA to a surface (figure 6). Translocation of the RNA through the helicase is visualised by changes in FRET measurements between the two dyes.

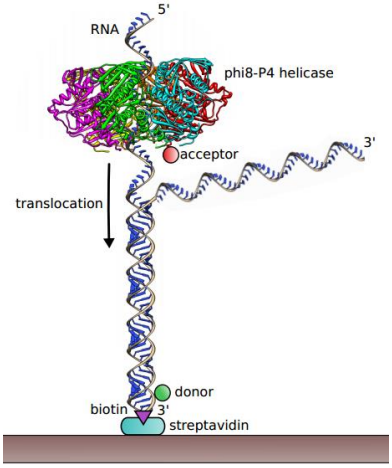

Figure 6: Diagram showing positions of dye labelling and RNA translocation through P4 helicase in fluorescence microscopy studies

Departures from the Original Proposal

Due to time constraints, chemical modification of the hexamers with DNA oligos and assaying P4 for engineered specificity was not achieved in these 8 weeks. However, I have consolidated and refined my knowledge of molecular cloning, expression, purification, activity assays and data analysis; and I am much more competent in the lab