STUDIES ON RIBONUCLEASE E OF *ESCHERICHIA COLI* AND ITS ASSOCIATION WITH THE ENZYME POLYNUCLEOTIDE PHOSPHORYLASE

by

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ABSTRACT

Messenger RNAs (mRNA) in *Escherichia coli* are highly labile molecules due to the combined action of a number of exo- and endoribonucleases that orchestrate their degradation. Two of these enzymes, ribonuclease E (RNase E) and polynucleotide phosphorylase (PNPase) have been implicated as key components of a purported mRNA degradation complex, otherwise known as the "degradosome" (Py *et al.*, *Nature* **381**, 169-172 (1996)). The purpose of these studies was to identify the site of interaction of PNPase with RNase E (Rne). Antibodies were generated against PNPase, initially against fusion proteins expressing two highly antigenic sites predicted to exist in PNPase, and later against a His(6)-PNPase fusion protein. These antibodies, along with a previously generated anti-RNase E antibody, were used to detect Rne or PNPase at various stages during the partial purification of RNase E. Rne and PNPase were found to remain in a stable complex, in association with other unidentified proteins, after several purification steps, and in particular after anion exchange chromatography. Over-expression and partial purification of Rne deletion mutants revealed that loss of the N-terminal portions of Rne did not prevent the mutant from binding PNPase independently, highlighting the importance of the C-terminal portion of Rne in associating with PNPase. Co-chromatography experiments could not determine whether the N-terminal region of Rne bound directly or indirectly to PNPase. A Far-Western experiment, which separates partially purified proteins in cell lysates and assesses their binding individually, demonstrated that derivatives of Rne retaining the C-terminal acidic tail of Rne were competent to bind PNPase. These experiments illustrating the binding of PNPase to the C-terminus of Rne complement the findings that PNPase binding is lost when the Rne C-terminus is missing (Kido *et al.*, *J. Bact.*, **178**, 3917-3925 (1996)).

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2D	two dimentional
3D	three dimentional
AS26	26% (w/v) ammonium sulfate fraction
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
°C	degrees Celcius
C-terminal	carboxy terminal
CTP	cytidine 5'-triphosphate
dd	dideoxy
dATP	deoxyadenosine 5'-triphosphate
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemoluminescent
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetate
FPLC	fast protein liquid chromatography
g	gravity
Hepes	4(-2-hydroxyethyl)-1-pierazineethanesulfonic acid
His(6)	oligo(6) histidine
IPTG	isopropyl-β-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
kg	kilogram
LB	Luria-Bertani
M	molar
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mRNA	messenger RNA
MW	molecular weight
µg	microgram
µL	microlitre
NDP	nucleoside diphosphate
ng	nanogram
NMP	nucleoside monophosphate
NMR	nuclear magnetic resonance
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
PAP	poly(A) polymerase

PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
PMSF	phenylmethylsulfonylfluoride
PNPase	polynucleotide phosphorylase
poly(A)	polyadenylate
PTBN	sodium phosphate-Tween 20-bovine serum albumin-Na azide
RBD	RNA binding domain
REP	repetitive extragenic palindrome
RNase	ribonuclease
RNase E	ribonuclease E
rne	rne/ams/hmp gene
Rne	rne/ams/hmp gene product
rRNA	ribosomal RNA
S1	purported antigenic site 1 of PNPase
S2	purported antigenic site 2 of PNPase
S200	200,000 x g supernatant
SDS	sodium dodecyl sulfate
TAE	Tris-sodium acetate-NaEDTA
TBE	Tris-Boric acid-NaEDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	tri(hydroxymethyl) aminomethane
tRNA	transfer RNA
V	volts
w/v	weight per volume

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Chapter 1

INTRODUCTION

1.1 OVERVIEW

Much of our understanding of the biological processes that constitute cellular metabolism has been gleaned from the gram-negative bacterium *Escherichia coli* (Neidhardt et al., 1987). The ease of manipulation of *E. coli* combined with its rapid replication has made it an important model organism for the study of synthesis, maturation, function and decay of RNA (D'Alessio and Riordan, 1997). The extensive genetic and biochemical data currently available, combined with the sequencing and analysis of the *E. coli* genome should provide the first full description of the enzymes involved in RNA metabolism, and their functional roles.

RNA has long been established as the link between transcription and translation in all organisms; therefore, all cells are obliged to make a major commitment to RNA synthesis. Approximately 20% of the dry cell mass in prokaryotes is RNA, most of which is ribosomal RNA (approximately 81%) and transfer RNA (approximately 14%) (Neidhardt et al., 1990). Both of these classes of RNA are considered 'stable' in relation to cellular growth rates, whereas messenger RNA (mRNA), constituting only 4% of the RNA, is considered metabolically 'labile'. In *E. coli*, typical mRNA half-lives are 60-120 seconds, although a few mRNAs (e.g. ompA mRNA) display half-lives of up to 15 minutes (Belasco and Higgins,

1988).

The instability of mRNA plays a number of roles in cellular metabolism. First of all, it has a direct affect on the maximal steady-state concentration of mRNA in the cell which is entirely independent of the promoter strength at the transcriptional level. Secondly, rapid mRNA decay allows the amplification of negative regulatory signals leading to an accelerated repression of gene expression. This has been observed in a number of instances, including the selective decay of some ribosomal protein mRNAs during translational repression in *E. coli* (Singer and Nomura, 1985) and the rate of degradation of RNAI, an anti-sense repressor that is a key element of control in the replication of ColE1-type plasmids (Lin-Chao and Cohen, 1991). Similar findings are found in eukaryotes in the down-regulation of c-fos and other eukaryotic "immediate early" mRNAs following induction (Greenberg and Ziff, 1984), and the eukaryotic autoregulation of β-tubulin synthesis (Yen *et al.*, 1988). Third, differential expression of distal gene products in some polycistronic mRNAs can be accounted for by selective decay of the transcript (Newbury *et al.*, 1987). Fourth, recycling of the degraded ribonucleotides within the cell serves to conserve metabolic energy. Finally, antisense nucleic acids often activate mRNA decay to control the levels of gene expression (Inouye, 1988).

1.2 A "CONSENSUS" MODEL FOR PROKARYOTIC mRNA DECAY

In the early 1970s, the work of Kepes, Apirion and Kennell on the mRNA decay in *E. coli* culminated into what is best described as

a "consensus" model proposed by David Apirion (Apirion, 1973). This model predicted that the initial step in mRNA decay was an endonucleolytic cleavage(s) in the transcript, followed by scavenging of the newly generated fragments by 3'-exonucleases. The model suggested the involvement of the exoribonucleases RNase II and PNPase, but did not identify the specific endonuclease. Moreover, it did not explain individual differences in degradation rate among mRNA species or the role of translation in influencing stability. However, it did provide a framework from which studies of mRNA decay could embark. In the ensuing 20 years, many endo- and exonucleolytic enzymes have been identified and studied in an attempt to refine the "consensus" model of prokaryotic mRNA degradation (Belasco and Higgins, 1988; Higgins *et al.*, 1992).

1.3 THE ENZYMES OF PROKARYOTIC mRNA DECAY

In *E. coli*, there is an abundance of ribonucleases that target RNA and almost half of these RNases function in tRNA metabolism: RNase P being required for 5' end formation and RNases D, PH, T, and BN in 3' end formation and maintenance (Deutscher, 1993a). The enzymes directly linked to mRNA decay may be divided into two categories, the endonucleases and the exonucleases.

1.3.1 THE ENDORIBONUCLEASES:

1.3.1.1. RNase III

This enzyme has been characterized as a double-stranded, RNA-specific endoribonuclease (Robertson and Dunn, 1975) that exists as

a homodimer of 25 kDa subunits. Its main role in the cell appears to be in the maturation of ribosomal RNA, although it has been demonstrated to act on phage, on cellular mRNAs, and plasmid transcripts, including sense-antisense RNA duplexes (Nicholson, 1995). Cells that lack RNase III are still viable (Babitzke *et al.*, 1993), indicating that alternate processing pathways can provide functional rRNA, and that RNase III cleavage is unlikely to play a central role in general mRNA decay.

1.3.1.2 RNase E

RNase E was originally identified in a temperature-sensitive mutant of *E. coli* as an activity which cleaved 9S rRNA *in vitro*, yielding the immediate precursor to 5S rRNA (Ghora and Apirion, 1979). A temperature-sensitive mutant (*rne-3071*) that failed to produce p5S RNA was discovered, and extracts from these mutant bacteria contained a thermolabile enzyme that cleaved 9S RNA *in vitro* at two sites (Misra and Apirion, 1979). Independent work on the *ams-1* (altered mRNA stability) *E. coli* mutant strain showed temperature sensitive growth and impaired ability to degrade bulk RNA at the non-permissive temperature (Ono and Kuwano, 1979). This mutation was later discovered to be allelic to *rne-3071* (Mudd *et al.*, 1990b; Babitzke and Kushner, 1991; Taraseviciene *et al.*, 1991; Melefors and von Gabain, 1991). Together, these results clearly indicated that RNase E activity plays a central role in bulk mRNA decay.

The cloning of the *rne/ams* gene initially proved problematic

(Chanda *et al.*, 1985). Later, sequencing errors (Chauhan *et al.*, 1991; Claverie-Martin *et al.*, 1991) led to misunderstandings regarding the identity of the endonuclease component and the size of the Rne polypeptide. Ultimately, the *rne/ams* gene was cloned (Casarégola *et al.*, 1992) and sequenced correctly (Mackie, 1993). The *rne/ams* gene maps at 23.5 minutes on the *E. coli* chromosome (Casarégola *et al.*, 1992) and its expression is autoregulated by RNase E cleavage of the *rne/ams* transcript (Mudd and Higgins, 1993; Jain and Belasco, 1995). The gene encodes a protein of 1061 amino acids with a predicted molecular mass of 118 kDa (Cormack *et al.*, 1993). Mobility of the Rne/Ams protein in SDS-polyacrylamide gels has been observed to be anomalously slow (equivalent to about 180 kDa)(Casarégola *et al.*, 1992) due to three proline-rich regions within the protein (McDowall and Cohen, 1996) . The mobility of the Rne/Ams protein in 2D gels indicates that the pI of the polypeptide is 5.0 (Taraseviciene *et al.*, 1994).

Initial attempts to purify RNase E were also troublesome because of aggregation and its extreme sensitivity to proteolysis (Roy and Apirion, 1983). This led to the identification of RNase K, once claimed to be a specific endonuclease for *ompA* mRNA (Lundberg *et al.*, 1990), but later found to be a proteolytic fragment of the catalytic subunit of RNase E which lacks any physiological significance (Mudd and Higgins, 1993). The problem of isolating the enzyme responsible for RNase E activity was eventually solved when the over-expressed Rne/Ams protein was

renatured after partial purification and separation on an SDS gel (Cormack *et al.*, 1993). It was found that the renatured Rne/Ams retained the activity and specificity attributed to RNase E, demonstrating that the *rne/ams* gene encoded the catalytic subunit of RNase E (Cormack *et al.*, 1993). Subsequently, the positions of the *ams-1* (G66S) and *rne-3071* (L68F) mutations were mapped to codons 66 and 68, respectively (McDowall *et al.*, 1993). Both create subtle changes at the N-terminal domain which conferred thermolability to the RNase E activity (McDowall *et al.*, 1993). Likely, these mutations disrupt the substrate binding site at the N-terminal region (Carpousis *et al.*, 1994; Bycroft *et al.*, 1997).

The Rne/Ams protein specifically targets single-stranded regions of its substrates, and performs its endonucleolytic activity as a phosphodiesterase, requiring a divalent metal ion (Mg^{2+}, Mn^{2+}) to cleave RNA, leaving 5'-phosphate, 3'-hydroxyl termini (Misra and Apirion, 1979). Early efforts to determine RNase E specificity focused on identifying a specific nucleotide recognition sequence (Tomcsányi and Apirion, 1985). This putative consensus recognition sequence was not conserved as more cleavage sites were characterized. Studies correlating RNA secondary structure in known substrates to the site of cleavage were also performed (Mackie, 1991; Cormack and Mackie, 1992; Mackie, 1992; Mackie and Genereaux, 1993). These experiments combined with the properties of a number of mutants designed to strategically disrupt folding (Mackie and Genereaux, 1993) showed that RNase E is a

single-strand specific enzyme lacking any strict sequence specificity. This was also confirmed by similar studies on RNA I (McDowall, *et al.*, 1994; Lin-Chao, *et al.*, 1994).

From the cleavage sites characterized thus far, it appears that Rne/Ams prefers to cleave 5' to AU dinucleotides in an A-U-rich context. Often these sites are preceded or followed by a stable stem-loop structure. Conflicting evidence continues to obscure the role of the stem-loops in site identification. Despite the fact that many mRNA cleavage sites are in close proximity to stem-loop structures, studies with synthetic oligonucleotide substrates (McDowall *et al.*, 1995) and more complex RNAs (Mackie and Genereaux, 1993) suggest that these adjacent stem-loops reduce the efficiency of cleavage. Thus, it appears that the stem-loops could serve to stabilize the local secondary structure to ensure an easily cleavable single-stranded site, or could cause the helical stacking of residues in the cleavage site to inhibit attack (Mackie and Genereaux, 1993). Since RNase E can accurately process short, single-stranded RNA oligonucleotides that lack flanking stem-loops, such secondary structures are not strictly required, at least for *in vitro* reactivity (McDowall *et al.*, 1995). *In vivo* RNase E may require at least three unpaired residues at the 5'-end of a substrate for efficient initiation of mRNA decay (Chen *et al.*, 1991; Bouvet and Belasco, 1992; Hansen *et al.*, 1994). This appears to be an essential element in RNase E recognition, since a variety of substrates whose 5'-ends have been sequestered by a secondary structure are resistant to cleavage by both crude RNase E and the

purified Rne/Ams protein, although the cleavage site remains single-stranded (Mackie *et al.*, 1997). It appears that the accurate prediction of RNase E cleavage sites will depend on a greater knowledge of the RNase E-substrate interaction at the atomic level.

Analysis of the amino acid sequence of Rne/Ams has identified several potentially important functional motifs (see Fig. 6). There are two putative nucleotide binding domains, two domains resembling an *E. coli* protein involved in cell division (McDowall *et al.*, 1993), a region resembling dynamin (Casarégola *et al.*, 1992), and a high affinity RNA binding domain (RBD) whose role in RNase E is debated (Cormack *et al.*, 1993; Casarégola *et al.*, 1992; McDowell and Cohen, 1996; Taraseviciene *et al.*, 1995; Miao *et al.*, personal communication). Deletion analysis studies have narrowed the RBD to residues 608-622 (Taraseviciene *et al.*, 1995; Miao *et al.*, personal communication), which exhibit sequence similarities to a basic region in RNase L, the 2-5-A activated endonuclease induced by interferon treatment of mammalian cells (Zhou *et al.*, 1993). Some studies have demonstrated that deletion of the region severely hampers RNase E activity (Tarasevisiene *et. al*, 1995; Miao *et. al*, personal communication), while others have noticed no effect in conditions of enzyme excess (McDowell and Cohen, 1995). Part of the active site appears to map within the first 150 residues in the N-terminal region, along with a sequence homologous to the S1 RNA binding domain (Bycroft et al, 1997). An acidic C-

terminal tail (residues 850-1061) does not appear to be important in catalytic function *in vitro*, but may be necessary for activity *in vivo* (Wang and Cohen, 1994), although some found it unnecessary *in vivo* as well (Chanda *et. al*, 1985; Kido *et al.*, 1996). Overall, it seems that the Rne/Ams protein is comprised of a number of distinct functional modules.

An RNase E-like activity has been identified in the extreme halophile *Haloarcula marismortui* which has the same substrate specificity as *E. coli* RNase E and cross-reacts with monoclonal antibodies raised against *E. coli* RNase E (Franzetti *et al*, 1997). There are two reports of RNase E-like activities in mammalian cells. The human *ard-1* gene encodes a basic, proline-rich polypeptide of 13.3 kDa which has very limited sequence similarities to the Rne/Ams protein in *E. coli* (Wang and Cohen, 1994; Claverie-Martin *et al.*, 1997). The Ard-1 protein is a Mg^{2+}-dependent endoribonuclease that binds and cleaves RNA in a manner identical to RNase E (Claverie-Martin *et al.*, 1997). Expression of the *ard-1* gene in *E. coli* is able to complement *rne* mutants: bulk mRNA decay rates are restored to wild-type and the site-specific cleavages produced *in vivo* and *in vitro* are essentially the same as those of RNase E (Wang and Cohen, 1994). Another study described an activity in human cell extracts which cleaves 9S RNA and *ompA* mRNA *in vitro* with the same specificity as RNase E (Wennborg *et al.*, 1995). The enzyme responsible has a molecular mass of 65 kDa

and is recognized by anti-RNase E antibodies (Wennborg, *et al.*, 1995). The enzyme cleaves within the 5'-AUUUA-3' sequence (Shaw and Kamen, 1986), which is reminiscent of the A,U-rich RNase E cleavage "consensus" sequence (Wennborg *et al.*, 1995).

1.3.2 THE EXORIBONUCLEASES:

1.3.2.1 PNPase

All the exoribonucleases discovered thus far in *E. coli* are enzymes that act in the 3'->5' direction. Two of them, polynucleotide phosphorylase (PNPase) and RNase PH are phosphorolytic phosphodiesterases, while the rest are hydrolytic in activity (Deutscher, 1993b). PNPase (and RNase PH) are distinct among the exoribonucleases in utilizing inorganic phosphate to carry out phosphorylytic cleavage of RNA, creating 5'-ribonucleoside diphosphates (Littauer and Soreq, 1982). In contrast to the hydrolytic reactions, the PNPase-catalyzed reaction conserves free energy in the 5'-rNDP products, which may be important to the cell under energy-poor conditions (Deutscher, 1993b). PNPase efficiently degrades unstructured RNAs, including homoribopolymers, but can be impeded by RNA secondary structure (Guarneros and Portier, 1991; Causton *et al.*, 1994). PNPase can also catalyze the polymerization of 5'-rNDPs forming RNA chains with the release of phosphate, and can catalyze inorganic phosphate exchange with 5'-rNDPs (Littauer and Soreq, 1982).

PNPase consists of three α-subunits of 85 kDa, which generate the catalytic site, and often three β-subunits of 48 kDa (Littauer

and Soreq, 1982; Py *et al.*, 1996). The α-subunit is encoded by the *pnp* gene, mapping at 69 minutes, and is co-transcribed with *rpsO*, which encodes ribosomal protein S15 (Régnier *et al.*, 1987). The α-subunit contains a 69 amino acid sequence at its carboxyl terminus, similar to a sequence motif in ribosomal protein S1 which also binds RNA (Régnier *et al.*, 1987). Biochemical and genetic studies indicate that the RNA binding and catalytic functions of the α-subunit are separable (Littauer and Soreq, 1982) as observed in the Rne/Ams protein. A recent NMR investigation has confirmed the RNA binding domain as the S1 domain, a five-stranded antiparallel β barrel which is present in RNase E, RNase II and other enzymes (Bycroft *et al.*, 1997). Conserved residues on one face of the barrel and adjacent loops form the putative RNA binding site (Bycroft *et al.*, 1997). The β-subunit has been shown to be identical to enolase (Py *et al.*, 1996; Miczak *et al.*, 1996) and is devoid of any RNA binding ability (Py *et al.*, 1996).

An $(\alpha)_3(\beta)_2$ form of PNPase has also been isolated from cells (Littauer and Soreq, 1982), but it has yet to be determined if other complexes of PNPase subunits are present and functionally distinct. PNPase is capable of autoregulating the translation of its message, in cooperation with the action of RNase III and RNase E (Hajnsdorf *et al.*, 1994a; Robert-LeMeur and Portier, 1994), and can be isolated in a complex with RNase E (Carpousis *et al.*, 1994; Py *et al.*, 1994; see below).

1.3.2.2 RNase II

Ribonuclease II (RNase II) is the major exoribonucleolytic activity in cell-free extracts, and acts by hydrolytically degrading RNA from the 3' end releasing 5'rNMPs (Shen and Schlessinger, 1982; Cannistraro and Kennell, 1994; Coburn and Mackie, 1996a). It is encoded by the *rnb* gene which maps at 29 minutes on the *E. coli* chromosome (Donovan and Kushner, 1983; Zilhao *et al.*, 1995). The *rnb* gene has been cloned and sequenced, and the RNase II enzyme over-expressed and purified to near homogeneity (Coburn and Mackie, 1996a). The DNA sequence predicts a protein of 644 amino acids, and the electrophoretic mobility of purified RNase II is approximately 70 kDa, which corresponds to the predicted molecular mass of 72.5 kDa (Coburn and Mackie, 1996a). The enzyme is active in its monomeric form, and requires divalent metal ions (Mg^{2+}, Mn^{2+}) and monovalent cations (K^{+}, NH_4^{+}) for maximal activity (Gupta *et al.*, 1977; Ghosh and Deutscher, 1978; Cudny and Deutscher, 1980). The enzyme is most reactive against the homoribopolymer poly(A) (Shen and Schlessinger, 1982).

Although mutants in RNase II exhibit a mild phenotype, double mutants deficient in PNPase and RNase II are inviable (Donovan and Kushner, 1986). This has been interpreted to mean that these exonucleases are functionally redundant. RNase II has been implicated along with PNPase and RNase E as one of the principal agents of mRNA decay, although discrete mRNA decay intermediates that accumulate in the absence of these three enzymes suggest that

other degradative enzymes may also exist (Arraiano *et al.*, 1988). Unexpectedly, both RNA-OUT (Pepe *et al.*, 1994) and the *rpsO* mRNA (Hajnsdorf *et al.*, 1994b) are stabilized in strains containing active RNase II relative to otherwise isogenic *rnb* mutants. This led to the hypothesis that RNase II, or other RNA binding proteins, could act as repressors of degradation by remaining bound to the substrate when they encountered a stable stem-loop structure, as found in the 3' end of RNA-OUT, rpsO mRNA, and others (Causton *et al.*, 1994). However, recent *in vitro* studies have shown that RNase II procesively removes mononucleotides from the 3' ends of RNAs until a stem-loop is encountered; it then dissociates leaving up to 10 unpaired residues at the 3' end (Coburn and Mackie, 1996b). Such RNAs cannot bind either RNase II or PNPase efficiently and thus become "resistant" to exonuclease action (Coburn and Mackie, 1996b). It remains to be seen if RNase II associates with other proteins *in vitro* or *in vivo*.

1.4 THE "DEGRADOSOME" COMPLEX

Along with the evidence that the Rne/Ams protein is central in prokaryotic mRNA decay, several findings have suggested that it is part of a larger functional protein complex that has been termed the "degradosome". First, gel filtration chromatography of Rne/Ams from crude extracts in mild conditions has revealed that it is part of a protein complex of approximately 2500 kDa molecular weight (Niguma and Mackie, unpublished observations). Secondly, efforts

to purify RNase E by conventional methods led to the finding that RNase E activity co-purified with PNPase (Carpousis *et al.*, 1994), which is commonly thought to scavenge the mRNA fragments at the terminal stages of mRNA decay. Third, a study attempting to characterize the protein complex bound to the REP motif, a stable stem-loop structure which is a barrier to mRNA decay, identified both Rne/Ams and PNPase in the complex (Py *et al.*, 1994). Together these results implied a strong association between Rne/Ams and PNPase. Additional components of the complex have been identified subsequently, including the putative "DEAD-box" RNA helicase, RhlB (Py *et al.*, 1996; Miczak *et al.*, 1996), enolase (Py *et al.*, 1996; Miczak *et al.*, 1996), and the heat shock protein, DnaK (Miczak *et al.*, 1996). The functional significance of the latter components are unclear, and their relevance to mRNA decay *in vivo* is uncertain. The components of the degradosome appear to exist as multimeric proteins: densitometric analysis of Coomassie Brilliant Blue-stained SDS-PAGE gels reveal that the degradosome is 16% RNase E, 28% PNPase, 11% RhlB and 18% enolase (Py et al., 1996).

1.4.1 RNA helicases

The presence of an RNA helicase in combination with the endoribonuclease Rne/Ams and the exoribonuclease PNPase immediately presents the satisfying scenario of a protein degradation complex in which the RNase E activity cleaves the substrate into small fragments, and PNPase digests the remnants with the aid of RhlB to unwind inhibitory secondary structure(s). However, there is no

direct evidence that RhlB does possess helicase activity. *E. coli* encodes a surprisingly large number of potential RNA helicases, including at least five in the "DEAD-box" family (Linder *et al.*, 1989; Kalman *et al.*, 1991), a group of RNA helicase-like proteins containing a conserved Asp-Glu-Ala-Asp (DEAD) sequence motif (Linder *et al.*, 1989). Three of these DEAD-box helicases appear to function in ribosomal assembly or function (Nicol and Fuller-Pace, 1995). Since rRNA precursors are one of many substrates of the Rne/Ams complex, this may implicate RhlB in another role. Two other putative helicases, DeaD and SrmB, have been reported to stabilize mRNA decay (Iost and Dreyfuss, 1994), but whether this is a consequence of an inherent helicase activity or other properties is unclear.

1.4.2 Poly(A) polymerase

E. coli contains a poly(A) polymerase activity (PAP I) which catalyzes the template-independent addition of adenylate residues to 3' ends of RNA (Deutscher, 1978; Cao and Sarkar, 1992a,b; He *et al.*, 1993; Xu *et al.*, 1993). PAP I is a monomeric enzyme, with a molecular weight of approximately 55 kDa (Cao and Sarkar, 1992b), and is encoded by the *pcnB* gene, mapping at 3 minutes (Lopilato *et al.*, 1986). PAP I is active *in vivo*, as mRNAs can be isolated with 3'-poly(A) tails (Cao and Sarkar, 1992a).

PAP I has not been shown to associate with the "degradosome" complex, but may be integral to the rate of mRNA decay. Mutations in *pcnB* cause a reduction in the plasmid copy number because of the

slower turnover of RNA I, the anti-sense regulator of plasmid replication (Lopilato *et al.*, 1986). The addition of a poly(A) tail to RNA I stimulates its rate of degradation by PNPase 4-5 fold (Xu and Cohen, 1995). The attack of RNase II on otherwise resistant mRNAs has also been shown to be assisted by polyadenylation of the substrate (Coburn and Mackie, 1996b). Currently, there are no quantitative measurements of the affinity of either exonuclease for polyadenylated mRNA substrates because of the lability of the poly(A) tail. It has been suggested that the poly(A) tail provides an unstructured 3' end which assists the binding of 3' exonuclases to the mRNA (Coburn and Mackie, 1996a and 1996b; Littauer and Soreq, 1982), or it may also facilitate the binding of RNA helicases to the stem-loops structures impeding exo- and endonucleolytic digestion. Thus, there may be a parallel between ubiquitin which tags proteins for degradation (Ciechanover, 1994), and polyadenylation which marks mRNA for disposal.

1.4.3 Other enzymes

Rnase P, which processes the 5' end of tRNA precursors, has been shown to also process the *his* operon mRNA in *Salmonella* and stabilize it (Alifano *et al.*, 1994). RNase M has been proposed to produce endonucleolytic cleavages in A-U rich sequences (Cannistraro and Kennell, 1989), although this enzyme may in fact be RNase I, a non-specific periplasmic ribonuclase that is known to redistribute to the cytoplasm after cell lysis (Deutcher, 1985). Clearly, the ribonucleolytic enzymes of *E. coli* need to be

haracterized better to understand their action and interaction in RNA metabolism.

1.5 NEW PERSPECTIVES ON mRNA DECAY

Mature mRNAs usually contain a hairpin or related secondary structure at their 3' ends, which blocks the digestive activities of RNase II and PNPase. Since there are apparently no 5'->3' exonucleases in *E. coli*, mRNAs are generally stable until endonucleolytic cleavages upstream from the 3' terminus allow access of exonucleases to the body of the mRNA (Higgins *et al.*, 1993) or until PAP I polyadenylates the 3' end to facilitate digestion past secondary structure (Xu and Cohen, 1995; Coburn and Mackie, 1996a and 1996b). In prokaryotes, the effect of translation on mRNA must also be considered because bacterial transcription and translation are often coupled events. Endonucleolytic cleavage sites can be blocked by translating ribosomes, and mRNA decay rates can be influenced by the frequency of translational initiation (Petersen, 1993). When transcription and translation are uncoupled, mRNA synthesis may be largely completed before appreciable translation occurs, leading to reduced stability presumably since it would be more exposed to degradative endonucleases (Iost and Dreyfus, 1995). The "consensus" model implies that the endonuclease digestions occur in a random fashion; however, there is evidence of primary endonucleolytic cleavage sites near the 5' end (Bechhofer, 1993), which triggers a 5'->3' propagated "wave" of endonucleolytic cleavages (Hansen *et al.*,

1994). Several *E. coli* mRNAs with prolonged physical and functional lifetimes, such as *ompA*, exhibit stable RNA secondary structures at their 5' ends which confer resistance to degradation (Emory and Belasco, 1990; Emory *et al.*, 1992; Hansen *et al.*, 1994). Adding a 5' single-stranded extension to the 5' hairpin neutralizes RNase E resistance (Emory *et al.*, 1992), supporting evidence that RNase E dependent degradation at *E. coli* mRNA 5' ends appears to require a single-stranded region (Hansen *et al.*, 1994). The idea of random endonucleolytic digestion has been dispelled further by the observation that longer mRNAs do not necessarily have shorter half lives (Belasco, 1993), which would not be possible in a random digestion scenario.

A "tethering" model for the degradosome (G.A. Mackie, personal communication) explains the current findings as follows: the multi-enzyme degradosome complex initiates mRNA decay by binding at a site proximal to the 5' end in a rate-limiting step, inhibited by secondary structure at the extreme 5' end (Chen *et al.*, 1991; Bouvet and Belasco, 1992; Hansen *et al.*, 1994) and by competition from translational initiation (Petersen, 1992; Rapaport and Mackie, 1994). The initiated RNase E complex is tethered to the substrate by the strong RNA binding domain of the Rne protein (Miao, personal communication). The first single-stranded A+U rich mRNA sequence would then migrate, without significant dissociation, to a second Rne subunit in a form of "pseudo-processivity". Each endonucleolytic cleavage catalyzed by Rne would leave mRNA

fragments with 3' ends suitable for PNPase or RNase II digestion, in some cases only after prior oligoadenylation of the new 3' end to facilitate their binding (Coburn and Mackie, 1996b; Xu and Cohen, 1995). When the RNase E complex encounters an internal stem-loop structure on the substrate, it may either skip over it or unwind it, possibly with the aid of the putative RNA helicase, RhlB. Tightly folded structures at the 3' end of mRNA may also be unfolded by the RhlB associated with the degradosome, or polyadenylation of the 3' end may be sufficient to allow PNPase degradation. In an alternative theory, Sidney Kushner proposed that polyadenylation of the 3' end of the mRNA allowed 'loading' of PNPase into the degradation complex which was already present on the substrate (O'Hara *et al.*, 1995). PNPase shortening of the poly(A) tail would be accompanied by upstream endonucleolytic cleavage by RNase E, generating a new 3' end which could be polyadenylated, and the cycle repeated (O'Hara *et al.*, 1995). The latter theory fails to explain the extended stability of substrates such as *ompA*, which has a highly structured 5' untranslated region impeding RNase E-dependent degradation (Hansen *et al.*, 1994). An alternative pathway may apply to smaller mRNAs and the fragments digested by RNase E cleavage, where decay is largely from the 3'->5' direction, mediated by exonucleases and PAP I activity. This pathway can be demonstrated *in vivo* (Mackie, 1989) and *in vitro* (Coburn and Mackie, 1996b) for the S20 mRNA and implied in others (Meyer and Schottel, 1992).

Significant recent progress has been made in the understanding of the mRNA decay process in *E. coli*, particularly in defining the macromolecules involved. However, little is known about the interaction of the enzymes involved in the degradosome. The purpose of this study was to identify a binding region on Rne/Ams for the best characterized of the degradosome enzyme interactions, the Rne/Ams and PNPase association (Carpousis *et al.*, 1994; Py *et al.*, 1994; Py *et al.*, 1996; Miczak *et al.*, 1996). The temperature sensitive mutants at the 5' end of Rne/Ams have been shown to disrupt interactions with PNPase (Carpousis *et al.*, 1994), while others have found that C-terminal deletions of Rne/Ams also interfere with PNPase binding (Kido *et al.*, 1996). In these studies, I attempted to define a specific PNPase binding domain within the Rne/Ams protein by characterizing a series of Rne/Ams deletion mutants, and monitoring their ability to associate with PNPase under various conditions *in vitro*.

Chapter 2

MATERIALS AND METHODS

2.1 REAGENTS

Bacto-tryptone, bacto-yeast extract and bacto-agar were purchased from Difco Laboratories. Ampicillin, carbenicillin, aprotinin, pepstatin A, leupeptin, PMSF and lysozyme were obtained from Sigma. Agarose, acrylamide, bis-acrylamide, ammonium persulfate, TEMED, urea and EDTA were purchased from Bio-Rad Laboratories. β-mercaptoethanol, DTT and Triton X-100 were obtained from Fisher Scientific. Deoxy and dideoxy-ribonucleotides were bought from Pharmacia. [α-^{32}P]-CTP, [α-^{32}P]-ATP, ^{35}S-dATP and ^{3}H-poly(A) were purchased from Amersham. IPTG was obtained from Promega. All other reagents were of reagent grade or higher and were obtained from Fisher, Bio-Rad, BDH, Pharmacia or Sigma.

Taq DNA polymerase, T4 DNA ligase, T7 RNA polymerase, *Eco RI, Hind III, Hinc II, Bam HI, Nde I, Xho I,* and DNase I were purchased from either Pharmacia, Promega, New England Biolabs or Boehringer Mannheim and used according to each manufacturer's instructions.

Biogel A1.5m (100-200 mesh) and Biogel A15m (100-200 mesh) were obtained from Bio-Rad. Protein molecular weight standards for gel filtration (Blue dextran, thyroglobulin, ferritin, catalase, aldolase, and bovine serum albumin) were from Pharmacia.

Freund's Incomplete Adjuvant was purchased from Sigma. Immobilon P transfer membrane was bought from Millipore, while ECL

reagents were obtained from Amersham. Protein A affixed to Sepharose beads were from Pharmacia.

Kodak X-Omat AR film was used for autoradiography.

2.2 VECTORS, STRAINS AND MEDIA

2.2.1 Vectors

The plasmid pET-3xc (Appendix 1) has an ampicillin resistance gene, a T7 promoter, and fuses a 12 amino acid T7 *gene 10* leader peptide to the N-terminus of the protein of interest (Studier, *et al.*, 1990). pET-24b (Appendix 2) has a kanamycin resistance gene, a T7*lac* promoter, and permits a C-terminal fusion of a cloned open reading frame to a C-terminal His-Tag.

The plasmid pEPα18 contains the coding sequence for a His-tag-PNPase fusion protein, and was obtained from C.F. Higgins' laboratory. It is comprised of the full *pnp* gene cloned into pET-14b, which contains an ampicillin resistance gene, and a T7 promoter (Py *et al.*, 1994).

All pET vectors are derived from pBR322 and use the T7 RNA polymerase to direct expression of cloned genes (Studier, *et al.*, 1990).

2.2.2 Bacterial Strains

Immediately after ligation, all clones were transformed into the *E. coli* strain JM109 (F' *traD36, lacI^q, lacI^{q}ZΔM15, proA$^+$B$^+$* / e14$^-$, *Δ(lac-proAB), thi, gyrA96,* (Nal^r), *endA1, hsdR17, relA1, supE44,*

recA1). This strain is capable of being transformed by non-supercoiled DNA efficiently.

All plasmids used for protein over-expression were transformed into the *E. coli* strain BL21(DE3) (F^- *ompT*, *hsdS*$_B$, r_B^-, m_B^- (λDE3)). BL21(DE3) lacks both the *lon* and *ompT* proteases, and is lysogenic for λDE3, which carries the T7 RNA polymerase gene under *lacUV5* promoter control. Expression of T7 RNA polymerase is inducible by the addition of IPTG.

2.2.3 Media

M9ZB medium (10g bacto-tryptone, 6g Na_2HPO_4, 3g KH_2PO_4, 1g NH_4Cl, 5g NaCl, per 1 litre; $MgCl_2$ added to 1 mM after autoclaving) was used to support the growth of GM402, an over-expressor of the Rne protein (Cormack, *et al.*, 1993).

LB broth (10g bacto-tryptone, 5g bacto-yeast extract and 5g NaCl per 1 litre; supplemented after sterilization with 0.2% glucose, 1 mM $MgSO_4$, 10 µg/mL thiamine, and 50 µg/mL of the appropriate antibiotic (ampicillin, carbenicillin, or kanamycin)) was used to grow other cells containing a plasmid of interest. LB agar plates were used to support the growth of transformed cells. It was prepared exactly as the LB broth prior to autoclaving, with the addition of 15g/litre of bacto-agar. After autoclaving, the mixture was cooled to about 50°C, prior to supplementation as needed.

2.3 OLIGONUCLEOTIDES

The oligonucleotides were synthesized on an Applied Biosystems 391 DNA Synthesizer or equivalent and were resuspended in dH_2O and stored at -20°C.

2.4 RECOMBINANT DNA METHODS

2.4.1 Isolation of plasmid DNA

Plasmids were prepared from 35 mL saturated cultures grown in LB broth using a modification of the alkaline lysis method (Birnboim and Doly, 1979) and purified either by cesium chloride isopycnic centrifugation or ethanol precipitation, as outlined in Sambrook *et al.* (1989). 10 mL plasmid purifications were also performed using the Wizard *Plus* Minipreps Purification System by Promega.

2.4.2 Restriction enzyme digestions

For every µg of DNA to be digested, approximately 10 units of restriction enzyme was added. The DNA and restriction enzyme were mixed with appropriate buffer according to the enzyme manufacturer's specifications.

2.4.3 Separation of DNA by gel electrophoresis

Small DNA and RNA fragments (<500 bp or nucleotides) were separated by electrophoresis in 6% polyacrylamide gels (29:1 ratio of acrylamide:bis-acrylamide), containing 1X TBE buffer (90mM Tris, 90 mM boric acid and 2 mM NaEDTA). 6×10^{-4} % Ammonium persulfate and 2.4×10^{-3}% TEMED was added to crosslink the acrylamide.

Electrophoresis was performed at 150 V using a Pharmacia Gene Power Supply (GPS 200/400).

Separation of larger DNA fragments was performed in 0.8% agarose dissolved in 1X TAE buffer (40 mM Tris, 20 mM sodium acetate and 1 mM NaEDTA). Electrophoresis was performed with a Pharmacia GPS 200/400 set to approximately 0.12 amps.

DNA and RNA was visualized in both types of electrophoresis by staining with 1 µg/mL ethidium bromide and viewing under ultraviolet light.

2.4.4 In Vitro amplification of DNA by the Polymerase Chain Reaction

100 pmol each of forward and reverse primers were combined with approximately 20 ng of a linear template, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, Mg-free buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.3), 0.1% gelatin) and 5 units/µL Taq polymerase according to the manufacturer's specifications. A Hypercell Biologicals Programmable Thermal Controller was used to perform the following thermal cycle: 5 min @ 94°C, then 30 cycles of 1 min @ 48°C, 3 min @ 72°C and 1 min @ 94°C. Products of PCR were purified by extraction with equal volumes of chloroform/isoamyl alcohol (24:1 ratio) solution and phenol/chloroform/isoamyl alcohol (25:24:1 ratio) solution, followed by an ethanol precipitation. The QIAquick PCR Purification Kit from Qiagen was also used as instructed by the manufacturer.

2.4.5 Ligations

60 ng of pET-3xc was mixed with equimolar amounts of the

appropriate DNA fragment, along with 1 mM ATP, 1X One-Phor-All buffer and 0.1 Weiss unit of T4 ligase to a volume of 10 µL. The incubation was performed at 15°C overnight.

2.4.6 Transformations into *E. coli* competent strains

E. coli strains were rendered competent by a calcium chloride method described by Bio-Rad Inc. The cells, stored as a 200 µL glycerol stock at -70°C, were thawed and mixed with plasmid DNA and kept on ice for 60 min., followed by a 3 min shock at 42°C. Approximately 1 mL of LB medium was added and the samples were incubated at 37°C for 1 hour. Cells were then spread onto 100-mm diameter LB agar plates containing 50 µg/mL of the appropriate antibiotic (ampicillin, carbenicillin, or kanamycin) and incubated at 37°C overnight.

2.4.7 DNA Sequencing

All clones were sequenced using the dideoxy method developed by Sanger *et al.* (1977) and Biggin *et al.* (1983). Approximately 2-3 µg of plasmid DNA and 20 ng of oligonucleotide primer were used in accordance with the method of the Pharmacia T7 Sequencing Kit. The ^{35}S-labelled samples were heat-denatured in 40% (v/v) formamide and separated in 8% acrylamide (19:1, acrylamide:bis-acrylamide) sequencing gels containing 8 M urea in TBE buffer.

2.5 OVER-EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

2.5.1 Sodium Dodecyl Sulfate Polyacrylamide Gels (SDS-PAGE)

Samples were combined with protein sample buffer (60 mM Tris-

HCl (pH 6.8), 1.5% (w/v) SDS, 25 mM DTT, 5% (v/v) glycerol and Bromophenol Blue) then boiled to denature the proteins. Protein preparations were separated by electrophoresis in 10%, 12.5% or 15% polyacrylamide gels (36:1 ratio of acrylamide:bis-acrylamide) containing 400 mM Tris-HCl (pH 8.8) and 0.1% (w/v) SDS. An upper 4.5% polyacrylamide (36:1 ratio of acrylamide:bis-acrylamide) stacking layer containing 125 mM Tris-HCl (pH 6.8) and 0.1% (w/v) SDS was poured above the separating layer. Electrophoresis was performed in Laemmli's buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) at 70 volts through the stacking portion and 150-200 volts through the separating gel.

Proteins separated on SDS-PAGE gels were visualized by staining with Coomassie Brilliant Blue R250, followed by destaining in a 5% (v/v) ethanol, 5% acetic acid solution. Dilute samples were concentrated to a usable volume by precipitation with 5 volumes of cold acetone.

2.5.2 Protein over-expression assay

Protein over-expression from the pET plasmid-BL21(DE3) system was confirmed by growing cultures of single clones in 10 mL of LB broth, and inducing them with IPTG as stated previously. 1 mL aliquots of culture were taken at 0, 1, 2 and 3 hours after induction. The cells were collected by centrifugation and lysed by boiling in 200 µL of 2X SDS sample buffer (0.1 M Tris-HCl, pH 6.8, 3% (w/v) SDS, 0.05 M DTT, 10% (w/v) glycerol). A brief sonication with a microprobe tip was performed to break DNA viscosity, then the proteins were separated on an SDS-PAGE gel as described in

2.5.1.

2.5.3 Culture and induction of recombinant proteins

All target genes were cloned into pET plasmids and placed under the control of T7 transcription and translation signals. Cultures were grown in 125 mL of M9ZB media to an optical density of 0.5 at 600 nm. They were then diluted with an equal volume of M9ZB containing 1mM IPTG to achieve induction and growth was continued with vigorous aeration for 3 hr. The cells were collected by centrifugation and washed in 25 mL of Buffer A (60 mM Tris (pH 7.6), 10 mM $MgCl_2$, 60 mM NH_4Cl, 0.5 mM EDTA, 5% (v/v) glycerol). The cells were again collected by centrifugation, then resuspended in 4 mL Buffer A. After supplementation with protease inhibitors and DNase (0.1 mM DTT, 0.2 mM PMSF, 2 µg/mL aprotinin, 0.8 µg/mL leupeptin, 0.8 µg/mL pepstatin A, and 20 µg/mL DNase I), the cells were disrupted by passage through an Aminco French pressure cell at 8,000 p.s.i. The lysate was centrifuged at 30,000 x g for 45 min, and the supernatant (S30) was fractionated by precipitation with 26% (w/v) $(NH_4)_2SO_4$ to yield the AS26 fraction as described by Mackie (1991). The pellet containing the over-expressed protein was resuspended in 4 mL of Buffer A with the aforementioned protease inhibitors, and stirred with 3% Triton X-100 and 1.2 M NH_4Cl, in a manner similar to Carpousis *et al.* (1994). A 200,000 x g centrifugation yielded a supernatant (S200) containing the proteins of interest.

2.5.4 Cleveland Mapping

The protein bands of interest were excised from a 10%

polyacrylamide gel and inserted into the wells of a 15% polyacrylamide gel. Each slice was overlayed with 1.0, 0.1, or 0.01 µg of crude chymotrypsin in protein sample buffer (see section 2.5.1). The sample and protease were electrophoresed through 90% of the stacking gel, followed by a current stoppage for 30 min to allow protease digestion. The current was then resumed, and the polyacrylamide gel examined as described in section 2.5.1.

2.5.5 Anion and cation exchange chromatography on the Pharmacia FPLC system

Native or deleted Rne protein purified as described in section 2.5.3, was diluted 1:10 in Buffer A (60 mM Tris, pH 7.6, 10 mM $MgCl_2$, 60 mM NH_4Cl, 0.5 mM EDTA, 5% (v/v) glycerol plus protease inhibitors as described in section 2.5.3) then loaded onto the FPLC SuperLoop. Chromatography through the anion exchange Mono Q column and cation exchange on the Mono S column were performed identically, at a flow rate of 1 mL/min. Injection of 10 mL of sample was followed by 13 mL of buffer 1 (10 mM Hepes, 1 mM EDTA, 0.1 mM PMSF, 0.5% (v/v) Genapol X-080, 40 mM NaCl). For the next 28 mL, buffer 2 (identical to Buffer 1 except 1 M NaCl) was injected as an increasing gradient until the salt concentration ended at 0.75 M NaCl. These conditions are a modification of standard FPLC programs outlined by Pharmacia.

2.5.6 Size Exclusion Chromotography

Two Pharmacia gel filtration columns (1 cm x 50 cm) were packed with Biogel A1.5m (100-200 mesh) or Biogel A15m (100-200 mesh), respectively, according to procedures outlined by Bio-Rad.

The columns were equilibrated in Buffer A and a standard curve for elution was determined using Blue Dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and BSA (67 kDa) as markers.

2.5.7 Immobilized metal ion chromatography

Cultures of pEPα18 in BL21(DE3) were grown in 125 mL of LB media, induced with IPTG and harvested as outlined in section 2.5.3. Cell lysates were taken to the S30 stage as previously stated, then purified on His-Bind resin using the Novagen His-Bind Kit, following a protocol described by the manufacturer and Py, *et al.* (1994) with a slight modification. Initial adsorption was formed batchwise and the bound protein collected by centrifugation of the resin-protein complex at 600 x g in a 50 mL plastic centrifuge tube. The supernatant containing unbound proteins was removed with a pipette. The subsequent washes, elution and stripping were performed in a 5 mL capacity disposable plasic column as recommended.

2.6 IMMUNOLOGICAL METHODS

2.6.1 Preparation of antigenic protein for rabbit immunization

Up to 1 mg of total protein was separated on a 10% or 12.5% SDS-PAGE gel. The protein of interest was identified by staining with Coomassie Brilliant Blue R250 in water, and that portion of the gel was removed and lyophilized. The dried gel slice was crushed in a mortar and pestle and the equivalent of 250 µg of

protein was suspended in 1 mL water. An equal volume of Freund's Incomplete Adjuvant was slowly added with agitation on a benchtop shaker until a smooth emulsion was achieved. 1 mL of this emulsion was injected into 8 week old New Zealand white rabbits (approximately 4-5 kg) for the initial immunization, and subsequent boosts were conducted every 3-4 weeks.

2.6.2 Rabbit bleeds

All bleeds were performed by the staff at the Animal Care Centre, University of British Columbia. A minimum of 10 mL of rabbit blood was removed prior to the initial immunization, and after each boost. After three immunizations, a full body bleed was obtained.

All bleeds were allowed to clot at 37°C for 2 hours. The clot was then separated from the sides of the collection vessel and the bleed stored at 4°C overnight. The following day, the serum was removed from the clot and any remaining insoluble material removed by centrifugation at 10,000 x g for 10 min at 4°C, as described by Harlow & Lane (1988). The serum was prepared for storage by heat inactivation at 56°C for 35 min, followed by the addition of an equal volume of glycerol. The prepared serum was stored at -20°C or -70°C.

2.6.3 Western blots

Separation of proteins on 10% or 12.5% SDS-PAGE gels, and wet electrophoretic transfer to nitrocellulose or Immobilon P in alkaline carbonate buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% (v/v) methanol) for 2 hrs was performed in a manner similar to that of

Harlow & Lane (1988). The membrane was blocked with a solution of 5% casein in PTBN (0.002 M sodium phosphate (pH 7.0), 5 x 10^{-4}% (v/v) Tween 20, 0.1% (w/v) BSA, 1 mM Na azide, 0.85% (w/v) NaCl). Exposure to primary antibody was a minimum of 1.5 hours, and to the secondary antibody (goat α-rabbit IgG (H+L)-horseradish peroxidase conjugate) a minimum 0.5 hours. All washes were done in Phosphate Buffered Saline (PBS) (Sambrook *et al.*, 1989). Visualization of antibody binding was performed by ECL using the Western Blotting Analysis System Kit from Amersham.

2.6.4 Antibody stripping

Membranes were submerged in a solution of 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, and 0.1 M β-mercaptoethanol warmed to 50°C for 30 min, with agitation. The stripped membranes were washed 2 times in PBS, then reblocked with PTBN + 5% casein for 1 hour.

2.6.5 Far-Western blotting

Far-Western blotting was performed as in section 2.6.3, with the following exceptions: after membrane blocking with 5% casein in PTBN, the membrane was washed twice with PBS, then exposed to highly purified PNPase (approximately 25 µg/mL), supplied by G.A. Coburn, for 1 hour at 37°C. The membrane was again washed twice with PBS, then exposed to antibodies as described for Western blots.

In the initial Far-Western blotting experiment, exposure to the highly purified PNPase was performed in 2 mL of PTBN + 5% casein (see section 2.6.3), PBS (see Sambrook *et al.*, 1989), Buffer

A (see section 2.5.5) or Denhardt's solution (see Sambrook *et al.*, 1989). Subsequent Far-Westerns were performed in 2 mL of PBS.

TABLE 1

Oligonucleotides

Oligo Name	Orient-ation	Sequence[1]	Co-ord[2]
KN1f	For-ward	5' GGAAACCGGCA**GGATCC**CTCGTCAGGCT 3'	*pnp* 54
KN1b	Rev-erse	5' TTGTTTGTCGG**GGATCC**GGTAAGCATCG 3'	*pnp* 792
KN2f	For-ward	5' CGTGCTGGCAG**GGATCC**CGGATATGGAC 3'	*pnp* 1230
KN2b	Rev-erse	5' TTGAGAGATGT**GGATCC**GACCTTCTTTAC 3'	*pnp* 1959
T7 Prom.	For-ward	5' TTAATACGACTCACTATAGGG 3'	
Rne C216a	Rev-erse	5' TGACGTAAGTA**CTCGAG**GAATGCGCGAAC 3'	*rne* 671
Rne C216b	Rev-erse	5' CGTAAGTACTCGAG**GAATTC**GCGAACGATTACG 3'	*rne* 668

1. The bases marked in **bold** are the engineered restriction sites and the bases underlined differ from the native sequence.

2. The co-ordinates given are complementary to the 5' residue of the particular oligonucleotide.

Chapter 3

RESULTS

3.1 ANTIBODY GENERATION

3.1.1 Generation of antibodies against antigenic sites in PNPase

The first objective of these studies was to create polyclonal antibodies directed against specific regions of the PNPase protein. By optimally over-expressing the two most antigenic sites in PNPase, I attempted to generate high titres of antibodies that were highly specific.

The lack of any three dimensional structure information forced us to predict antigenic sites based on the PeptideStructure and PlotStructure modelling program (Jameson and Wolf, 1988) (Fig. 1). The first antigenic site selected (S1) is close to the amino-terminal end of the protein, and spans amino acids 23 to 260. The second antigenic site (S2) is towards the carboxy terminal, and covers amino acids 415 to 649. The nucleotide sequences of the *pnp* gene corresponding to these antigenic sites were amplified by PCR using oligonucleotides that add flanking *Bam HI* restriction sites (Table 1). The amplified sequences were ligated into the polycloning site of pET-3xc (Novagen; Appendix 1) at its *Bam HI* site, and insertion of sequences into the proper orientation was confirmed by DNA sequencing (data not shown). The clones were named pS1 and pS2, respectively.

Figure 1. Antigenic site predictions in PNPase from the primary structure. The nucleotide sequence of the α-subunit of PNPase (Régnier *et al.*, 1987) was analysed by the Sequence Analysis Software Package (GCG Package, Version 7) by Genetics Computer Group Inc. The PeptideStructure and PlotStructure programs (Jameson and Wolf, 1988) used the sequence information to predict a secondary structure for the protein. Antigenic sites are predicted based on the hydrophilicity and the secondary structure of the polypeptide chain in a given region, and are shown as a region surrounded by an octagon. α-helix regions are indicated by a sine wave, and β-sheet regions by a sharp, sawtooth wave. Coiled regions are represented as a dull, sawtooth wave, and turns in the polypeptides are shown as a 180° turn in the line.

PLOTSTRUCTURE of: khnpnp.pep ck: 3520

TRANSLATE of: khnpnp.nts check: 1353 from: 1 to: 2136

Chou-Fasman Prediction
February 4, 1994 14:29

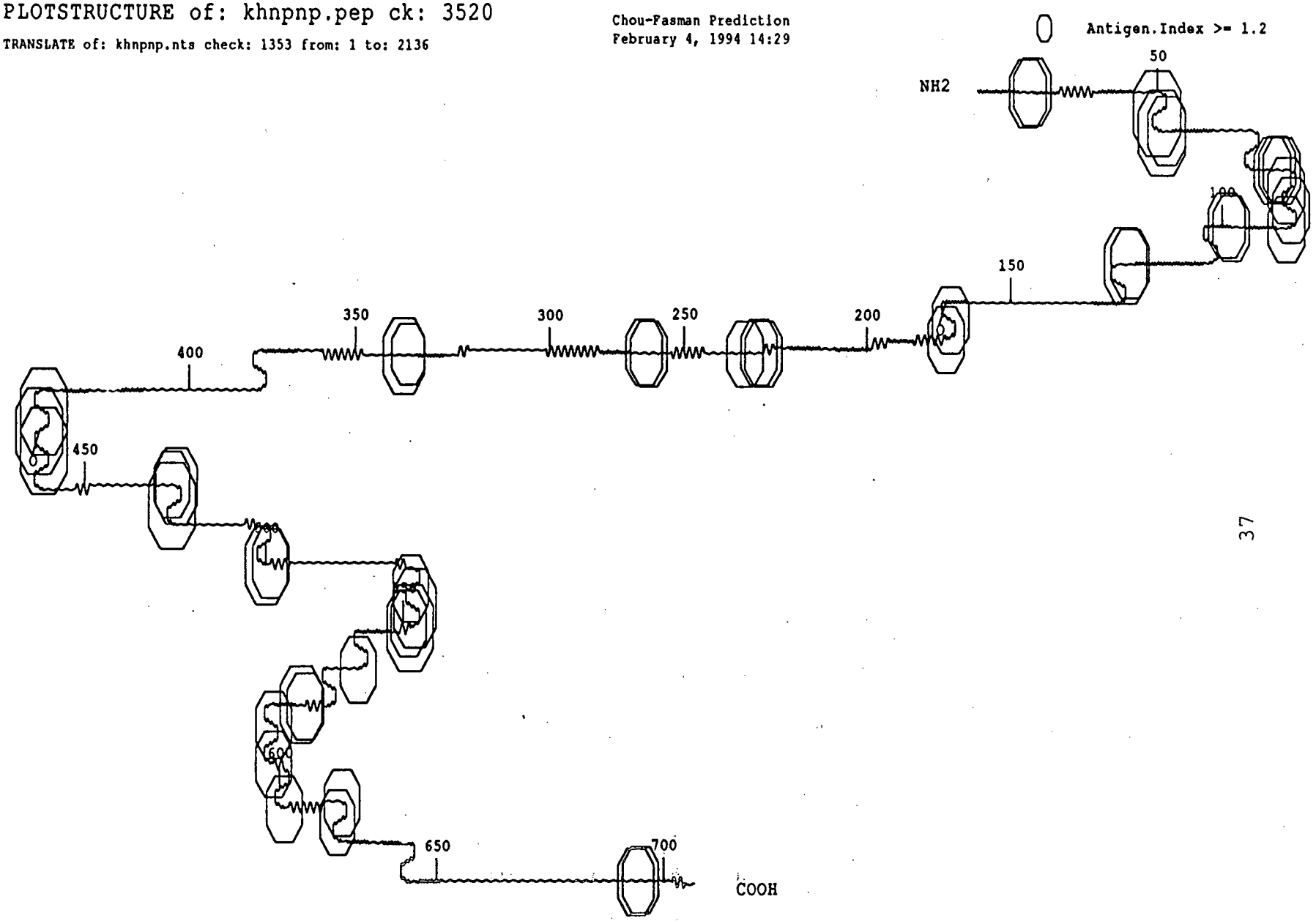
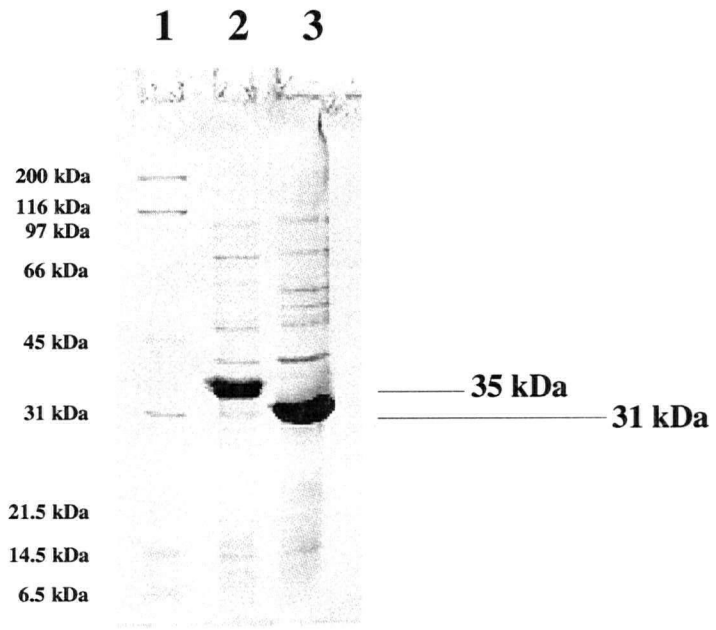


Figure 2. Over-expression and identification of proteins containing the two antigenic regions of PNPase. (a) 1.0 μL of extracts of BL21(DE3) containing pS1 (lane 2) and pS2 (lane 3) were separated on a 10% polyacrylamide gel after induced over-expression of PNPase antigenic site 1 (S1) and site 2 (S2), respectively (see Sec. 2.5.3). The approximate molecular weights of the over-expressed proteins are indicated on the right, based on the migration of the standard proteins in lane 1. (b) Analysis of the components of the S1 doublet by Cleveland mapping (See Sec. 2.4.4). Lanes 2-5 contain the upper band of the S1 doublet combined with 0, 1.5, 1.0 and 0.1 μg of chymotrypsin, respectively. Lanes 6-9 contain the lower band of the S1 doublet exposed to 0, 1.5, 1.0 and 0.1 μg of chymotrypsin, respectively. Products were separated by electrophoresis on a 15 % polyacrylamide gel. A, B and C indicate the common migration point of three polypeptides.

(a)


1 2 3
200 kDa
116 kDa
97 kDa
66 kDa
45 kDa
31 kDa
21.5 kDa
14.5 kDa
6.5 kDa
35 kDa
31 kDa

(b)

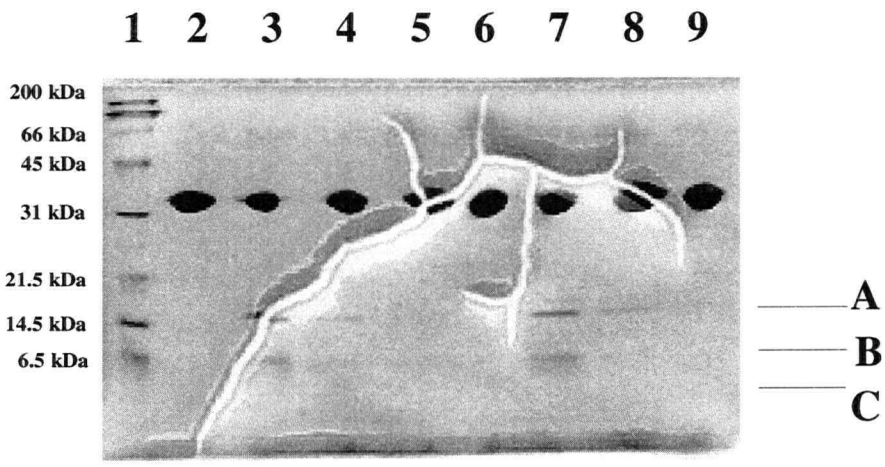
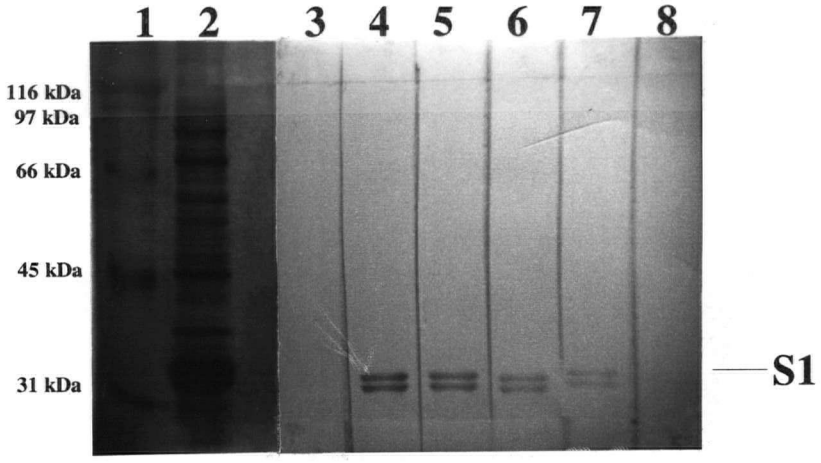
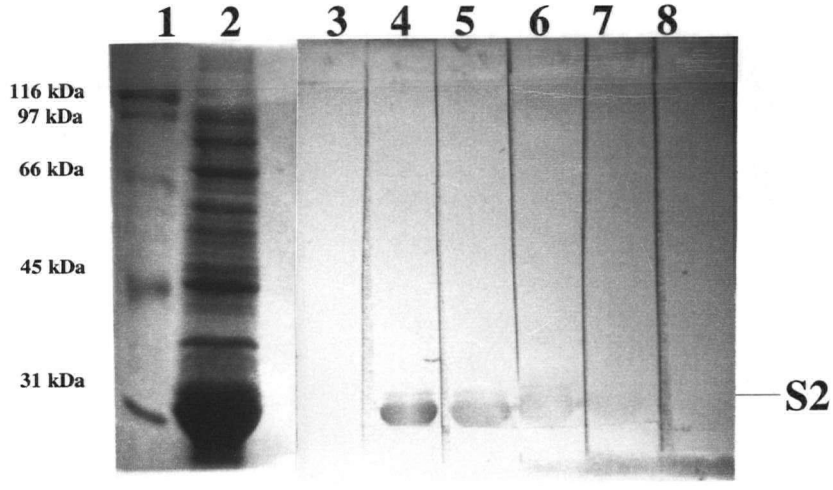

1 2 3 4 5 6 7 8 9
200 kDa
66 kDa
45 kDa
31 kDa
21.5 kDa
14.5 kDa
6.5 kDa
A
B
C

Figure 3. Detection of T7 gene10-PNPase fusion proteins by Western blotting. (a) An extract of BL21(DE3) (pS1) over-expressing PNPase antigenic site 1 (S1) was separated by SDS-PAGE and blotted onto nitrocellulose (See Sec. 2.5.3). Lane 2 shows an SDS-PAGE separation of the cell extract before blotting onto nitrocellulose. Lanes 4-8 show the chromogenic visualization of the S1 protein on the nitrocellulose using a serial dilution of the α-S1 antibody (1:8000, 1:16000, 1:32000, 1:64000 and 1:128000 dilution, respectively). (b) An extract of BL21(DE3) (pS2) over-expressing PNPase antigenic site 2 (S2) was separated by SDS-PAGE and blotted onto nitrocellulose. Lane 2 shows the separated cell extract prior to nitrocellulose blotting, visualized by staining with Coomassie Blue. Lanes 4-8 show the chromogenic visualization of the S2 protein on nitrocellulose by a serial dilution of the α-S2 antibody (1:8000, 1:16000, 1:32000, 1:64000 and 1:128000 dilution, respectively). Lane 3 in (a) and (b) contains extracts of BL21(DE3) that over-express RNase E (i.e. GM402), used as a negative control. Lane 1 in (a) and (b) contains standard protein markers.

(a)


1 2 3 4 5 6 7 8
116 kDa
97 kDa
66 kDa
45 kDa
31 kDa
S1

(b)


1 2 3 4 5 6 7 8
116 kDa
97 kDa
66 kDa
45 kDa
31 kDa
S2

Both pS1 and pS2 were transformed into BL21(DE3) and cultures derived from individual clones grown and induced with IPTG (Sec. 2.4.6). Extracts were separated on 10% acrylamide gel (Fig. 2(a)). Fig. 2(a) shows strong bands of the anticipated size of T7 gene10-PNP fusions detected from clones harbouring pS1 and pS2. The S1 antigenic protein was over-expressed as a doublet, which raised concerns about whether both bands were derivatives of S1. To address this point, a Cleveland mapping experiment using the protease chymotrypsin was performed. As seen in Fig. 2(b), both the upper band (lanes 2-5) and lower band (lanes 6-9) yielded partial digestion products (A, B, C) of low mobility. The common digestion profile of both over-expressed bands indicate that they are comprised of essentially the same amino acid sequence.

The over-expressed antigenic proteins were prepared and used for immunization of rabbits as outlined in Section 2.6. Fig. 3 shows that the serum from rabbits immunized against S1 and S2 contained high titres of α-S1 and α-S2, respectively, after two boosts. Other proteins from the cell extracts of a Rne over-expressor did not cross react with the antibodies (Fig. 3, lane 3). Data provided later (e.g. Fig. 8(d)) show that α-S1 recognizes native PNPase.

3.1.2 Generation of antibodies against a His(6)-PNPase fusion protein

During the course of these studies, members of Christopher F. Higgins' laboratory were able to purify a His(6)-PNPase fusion protein to near homogeneity using Novagen's His-Tag purification

Figure 4. Purification of over-expressed His(6)-PNPase by metal ion chelate chromatography. Extracts of BL21(DE3) (pEPα18) containing over-expressed His(6)-PNPase were purified to the S30 stage (see Sec. 2.4.3), then exposed to activated Ni^{2+} His-Bind resin (Sec. 2.4.6). Unbound His(6)-PNPase was removed by gravitational elution from a column (Lane 2), initially with 5 mM imidazole ["Binding Buffer" wash] (Lane 3) and subsequently with 60 mM imidazole ["Wash Buffer" wash] (Lane 4). This was followed by 15 mL of 1M imidazole ["Elution Buffer" wash] (Lanes 5-19), and removal of the Ni^{2+} by 100 mM EDTA ["Strip Buffer" wash] (Lanes 20-25). The proteins in each lane were precipitated with acetone from 25 μL (Lane 2), 134 μL (Lane 3), 85 μL (Lane 4), 26 μL (Lanes 5-19) and 200 μL (Lanes 20-25) of eluant, then separated on a 10% polyacrylamide gel.

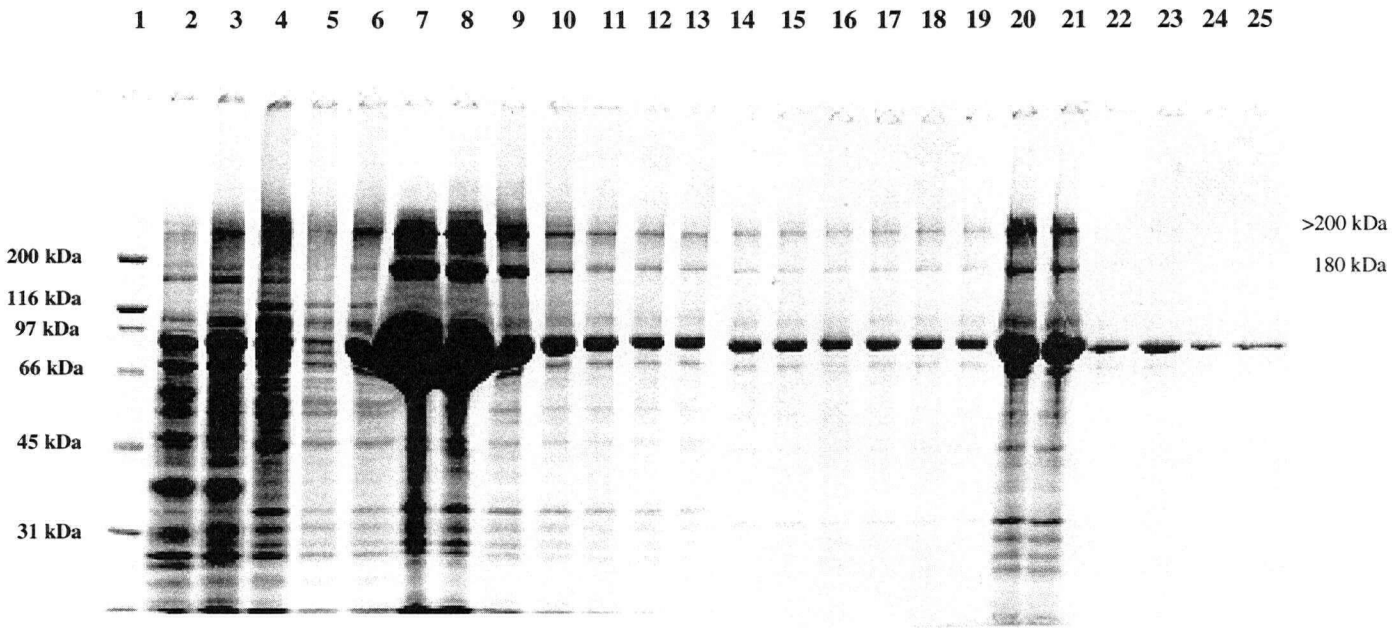
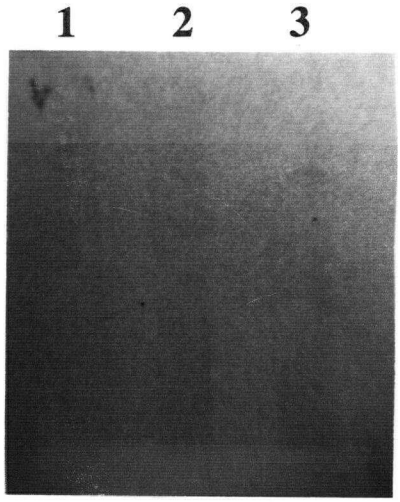
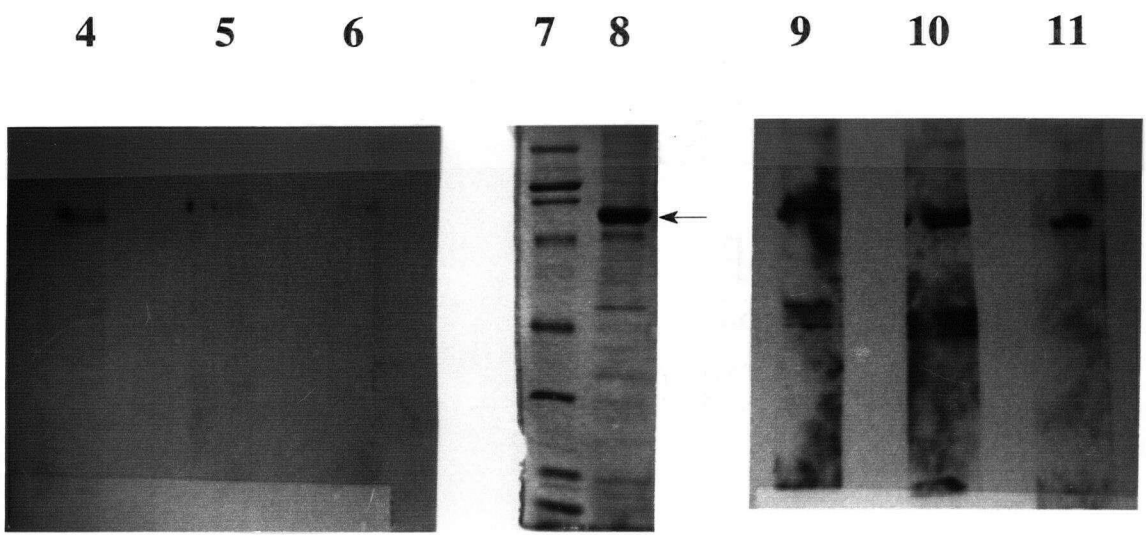

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
200 kDa
116 kDa
97 kDa
66 kDa
45 kDa
31 kDa
>200 kDa
180 kDa

Figure 5. Polyclonal antibodies raised against purified His(6)-PNPase detected by Western blotting. Extracts of strain BL21(DE3) (pEPα18) containing His(6)-PNPase were separated by SDS-PAGE, blotted to Immobilon P, and probed with rabbit pre-immune sera (Lanes 1-3), post-first immunization sera (Lanes 4-6), and post-second immunization sera (Lanes 9-11), as described in Sec. 2.5.3. Lanes 1, 4, 9 were exposed to a 1:500 antibody dilution, lanes 2, 5, 10 were exposed to a 1:1000 dilution, and lanes 3, 6, 11 were exposed to a 1:2000 dilution. Lane 8 shows the extract of BL21(DE3) (pEPα18) containing over-expressed His(6)-PNPase (arrow) separated by SDS-PAGE and stained with Coomassie Blue, while lane 7 contains protein size standards.


1
2
3


4
5
6
7
8
9
10
11

protocol (Py, *et al.*, 1994). I attempted to duplicate the purification, and use this fusion protein to generate specific antibodies directed against the entire PNPase polypeptide.

The plasmid coding for the His(6)-PNPase fusion protein, pEPα18, was obtained from Christopher F. Higgins' laboratory (Py, *et al.*, 1994). It includes the full length *pnp* gene ligated into the BamHI cloning site of pET-14b (Novagen; Appendix 2), a vector carrying bacteriophage T7 transcription and translation signals as well as an N-terminal His-tag. In these experiments, the pEPα18 was transformed into BL21(DE3) and the fusion protein over-expressed (Sec. 2.5). The His(6)-tag sequence served as an affinity tag for purification of the fusion protein by metal ion (Ni^{2+}) chelation chromatography (Novagen). Fig. 4 depicts the profile of elution of bound proteins, and their analysis on a 10% polyacrylamide gel. Most of the His-PNPase had eluted after approximately 3-5 mL of elution buffer had been passed through the affinity column. A number of proteins are associated with His-PNPase and they eluted in roughly equimolar amounts, including a 85 kDa protein that may be PNPase, a 180 kDa protein that may be Rne and a protein band >200 kDa. The final buffer, containing EDTA, chelates the Ni^{2+} and removes it from the column matrix. Fractions 20-25 in Fig. 4 show that a small portion of the His(6)-PNPase and associated proteins remained bound to the metal after the elution buffer wash.

As described in Sec. 2.6, the purified His(6)-PNPase was separated by SDS-PAGE and 250 μg of protein affixed to acrylamide

was mixed with Freund's Incomplete Adjuvant to form an emulsion. This was used to immunize a rabbit, and the sera obtained after two boosts contained high titres of α-His-PNPase (Fig. 5). Moreover, these sera lacked antibodies that cross-reacted with other *E. coli* proteins, since these sera did not detect (or bind to) any other proteins in the cell extracts.

3.2 Rne DELETION MUTANT PROTEINS

A collection of rne deletion mutant constructs was created in an attempt to locate important domains, including those capable of binding PNPase, within the Rne polypeptide.

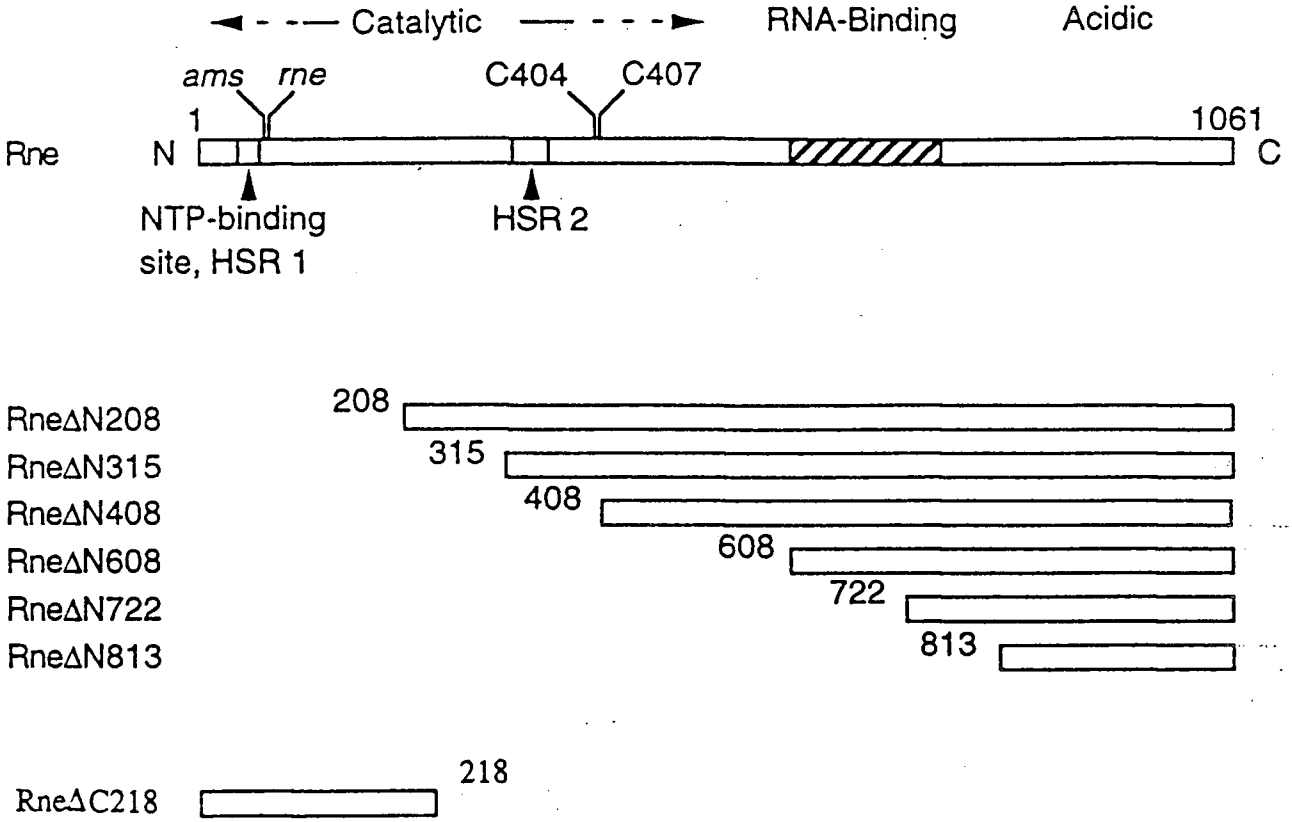
3.2.1 Rne N-terminal deletion mutants

Oligonucleotides that introduce an *Xho I* restriction site at the 3' end of the *rne* gene, and a *Nde I* site at the 5' end of interest were used to amplify *rne* gene deletion mutants by PCR. These were ultimately ligated into pET 24b, and over-expressed in BL21(DE3). Fig. 6 depicts the set of N-terminally deleted Rne proteins, constructed by Xin Miao (Miao, personal communication).

3.2.2 Rne C-terminal deletion mutants

Fig. 6 also shows the C-terminal deletion RneΔC218 that was constructed using an oligonucleotide that introduced an *Nde I* restriction site in the 5' end of the *rne* gene at the ATG initiation codon, and another oligonucleotide that created a *Eco RI* site in the *rne* sequence corresponding to amino acid 218. The oligonucleotides were used in a PCR reaction to amplify the *rne*

Figure 6. A map of deleted Rne proteins. The complete Rne protein is pictured on top with its postulated functional domains indicated. Xin Miao's N-terminal deletion mutants are denoted as RneΔNxxx, while the C-terminal deletion mutant discussed in Sec. 3.2.2 is denoted as RneΔC218.


Catalytic
RNA-Binding
Acidic
ams
rne
C404
C407
1
1061
Rne
N
C
NTP-binding
site, HSR 1
HSR 2
RneΔN208
RneΔN315
RneΔN408
RneΔN608
RneΔN722
RneΔN813
208
315
408
608
722
813
RneΔC218
218

sequence coding for the first 218 amino acids (Sec. 2.3). This amplified DNA was digested appropriately, ligated into pET 24b, and transformed into *E. coli* strain BL21(DE3) (Appendix 2, Fig. 8). The accuracy of this construction was confirmed by DNA sequencing (data not shown).

3.3 NATIVE AND MUTANT Rne-PNPase INTERACTIONS ASSESSED BY CO-CHROMATOGRAPHY

3.3.1 Fractionation of Rne-PNPase on an anion exchange column.

In an effort to purify the native Rne protein to near homogeneity, it was over-expressed in BL21(DE3) and precipitated from cell lysates by 26% (w/v) $(NH_4)_2SO_4$ (Sec. 2.5). The Rne was solubilized by 3% Triton X-100 and 1.2 M NH_4Cl, and ribosomes, membrane fragments and other unwanted proteins sedimented by centrifugation at 200,000 x g. The resulting supernatant (S200) was diluted in Buffer A supplemented with protease inhibitors, then fractionated on an anion exchange matrix (Resource Q, Pharmacia) with a 40 mM to 750 mM NaCl gradient (Sec. 2.5.5).

Fig 7(b) shows an SDS-PAGE separation of the proteins eluted from the anion exchange matrix. A protein migrating at the 180 kDa distance, corresponding to Rne, elutes from the matrix through almost all of the salt gradient, accompanied by an 85 kDa protein in approximate equimolar amounts. Fig 7(c) is a Western blot utilizing α-RNase E. The immunoreactive species of approximately 180 kDa in lanes 32-60 is clearly the Rne protein. Fractions 34-46

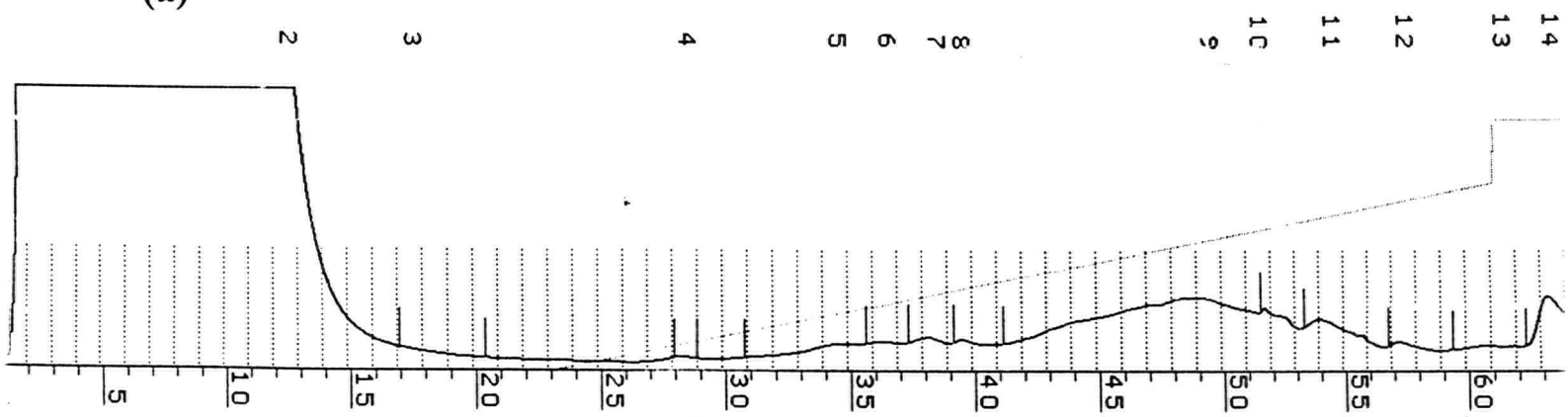
Figure 7. Fractionation of enriched extracts of GM402 on an anion exchange column (Resource Q). The Rne protein was over-expressed in cultures of GM402 and precipitated from cell lysates using 26% (w/v) $(NH_4)_2SO_4$ (Sec. 2.5). The protein was resolubilized by 3% Triton X-100 and 1.2 M NH_4Cl, centrifuged at 200,000 x g, diluted 10-fold, then fractionated on an anion exchange matrix with a 40 mM to 750 mM NaCl gradient (Sec. 2.5.5).

(a) Ultraviolet absorption profile of the anion exchange column fractionation. 1.0 mL fractions were collected from the fractionation, numbered 1-64. The broad band of absorption in fractions 2-15 is largely due to Triton X-100, not protein.

(b) Approximately 200 ng of protein from selected fractions was precipitated with 5 volumes of acetone, separated by SDS-PAGE and visualized with Coomassie Blue staining (Sec. 2.4.1). Lane S contains protein size standards used to identify Rne (migrating at the 180 kDa position) and PNPase (migrating at 85 kDa).

(c,d) Western blotting using polyclonal antibodies raised against Rne (c) and PNPase S1 (d) (Sec. 2.6). The selected fractions from panel (b) were separated by SDS-PAGE and blotted onto Immobilon P. The blot was probed first with α-Rne antibody (c), stripped of bound antibody (Sec. 2.6.4), then reprobed with α-PNPase S1 antibody (d). The arrows in panels (c) and (d) indicate the migration point of Rne and PNPase, respectively.

(a)


2
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35
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45
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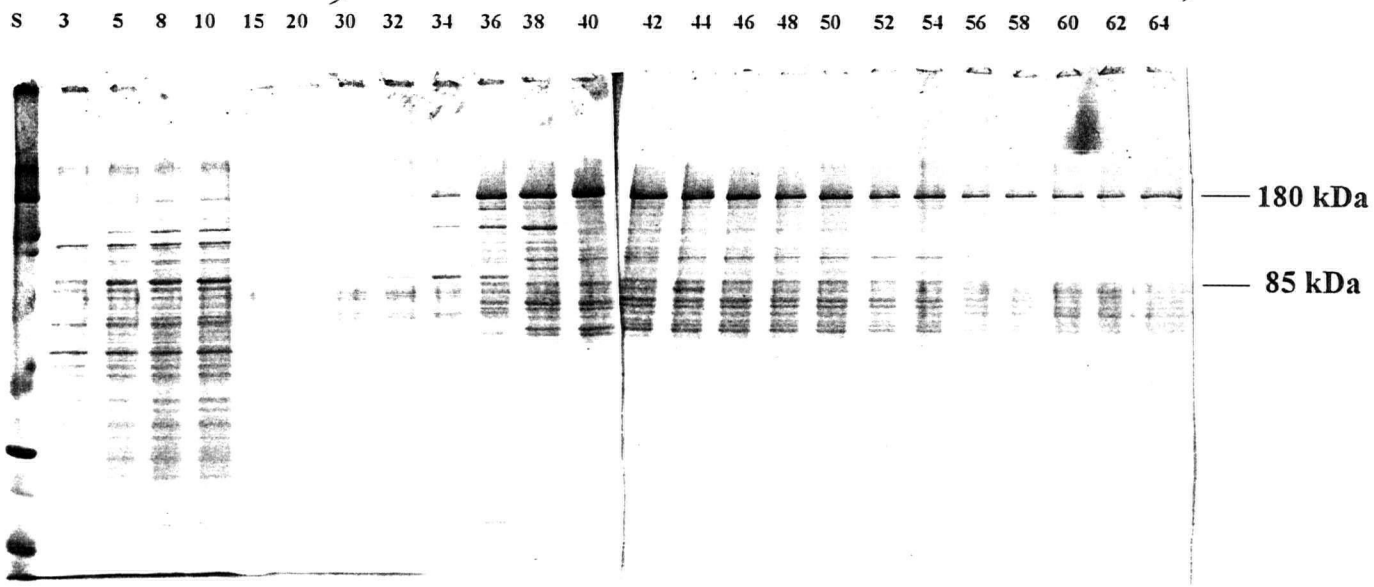
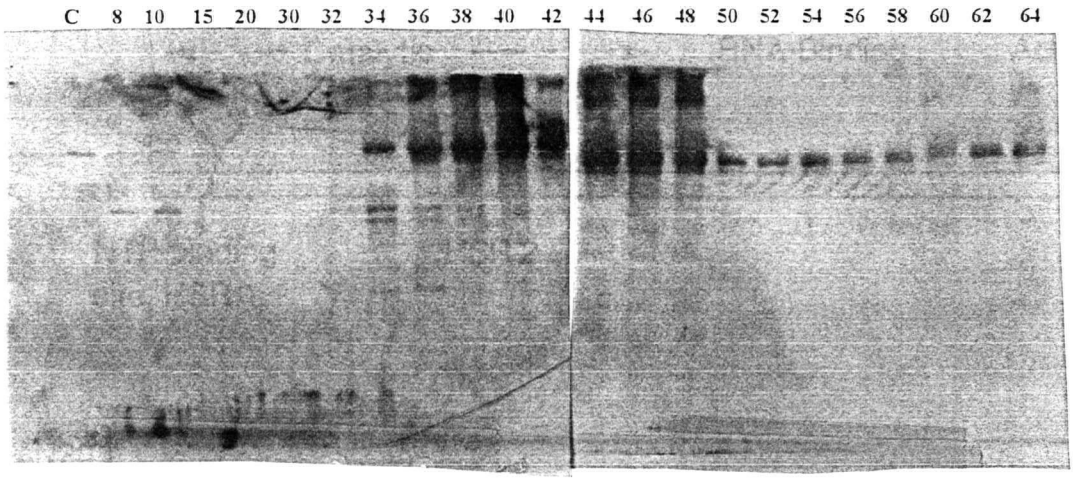
55
60

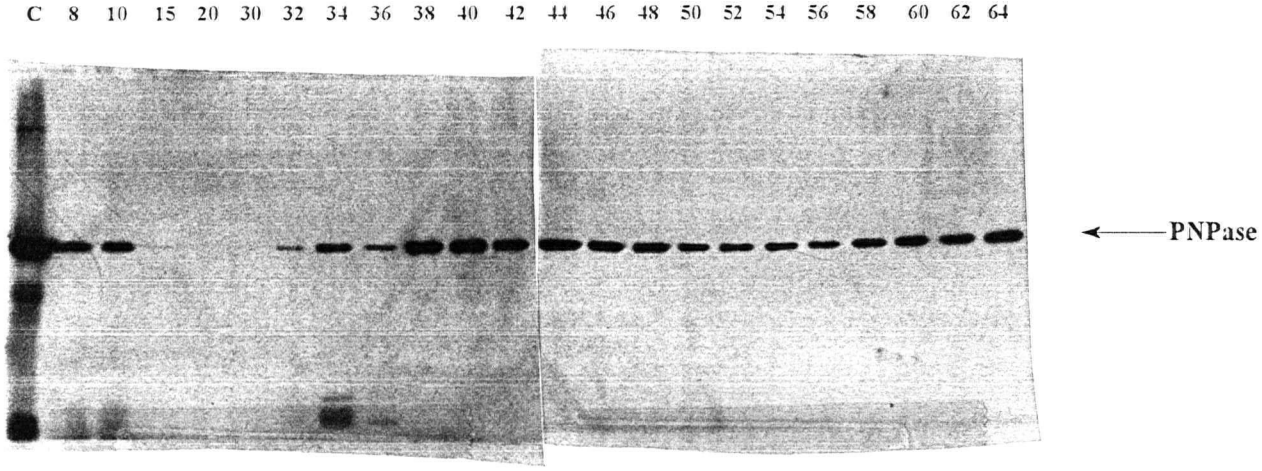
(b)


S 3 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64
180 kDa
85 kDa

(c)


C 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64
Rne

(d)


C 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64
PNPase

contain a >200 kDa protein that reacts with α-RNase E; this protein may be an oxidation dimer of Rne. In Fig 7(d), α-S1 (which recognizes PNPase) was used to confirm that the 85 kDa protein from Fig 7(b) was PNPase.

3.3.2 Fractionation by anion exchange chromotography of Rne N-terminal deletion mutants

In an attempt to fractionate PNPase and other proteins co-purifying with the Rne protein, N-terminal deletion mutants of Rne were purified as described in Sec. 2.5 and Sec. 3.3.1. Since the native Rne protein is normally associated with PNPase after partial purification, a loss of PNPase association by a truncated Rne would suggest that an essential PNPase binding site was deleted.

An N-terminal deletion mutant missing the first 608 amino acids of Rne (RneΔN608) was fractionated in a manner identical to that of the native protein (Fig.8). In contrast to Fig 7(a), Fig 8(a) shows a sharp absorbance peak in fractions 34-35, corresponding to an abundant 100 kDa protein. Fraction 39-40 represents a small peak of absorbance, and corresponds to a 85 kDa protein in the SDS-PAGE gel. Western blotting using α-Rne (Fig 8(c)) and α-S1 (Fig 8(d)) confirmed that the 100 kDa protein was the deletion mutant RneΔN608, and the 85 kDa protein was PNPase. Fig 8(c) also shows a possible oxidation dimer of RneΔN608 that is >200 kDa and crossreacts with α-Rne, as well as a smaller protein in fractions 5-10 and 33-36 that could be a RneΔN608 degradation product. Fig 8(d) reveals α-S1 reacting with an 80 kDa protein in fractions 42-48 that may be a PNPase digestion product.

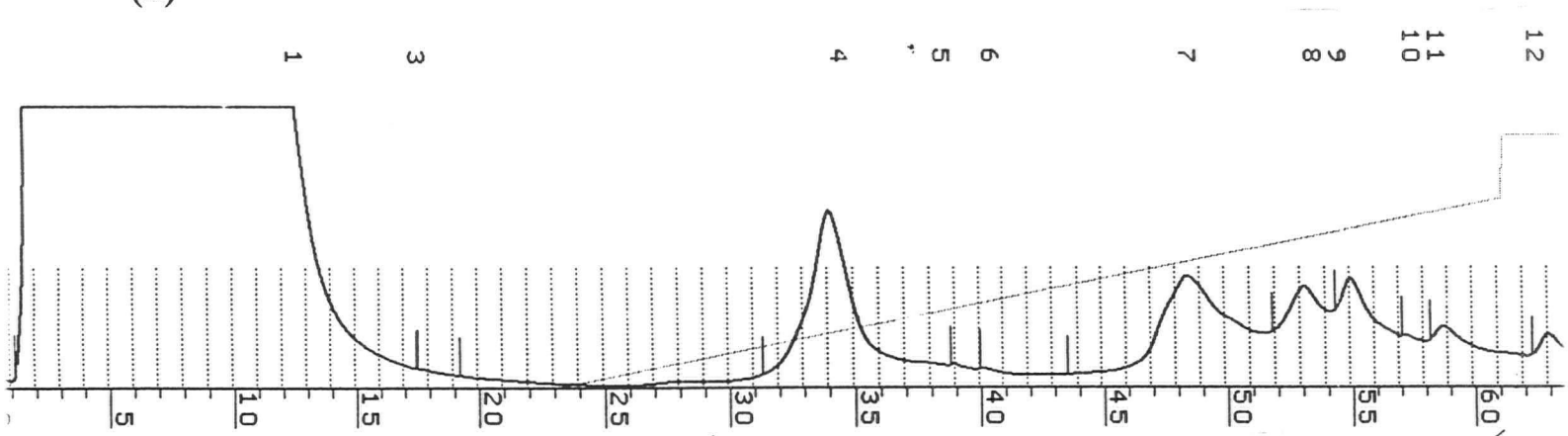
Figure 8. Fractionation of partially purified extracts of RneΔN608 on an anion exchange column (Resource Q). The RneΔN608 protein was over-expressed in cultures of BL21(DE3) and precipitated from cell lysates using 26% (w/v) $(NH_4)_2SO_4$ (Sec. 2.5). The protein was resolubilized in 3% Triton X-100 and 1.2 M NH_4Cl, centrifuged at 200,000 x g, diluted by 1/10th, then fractionated on an anion exchange matrix with a 40 mM to 750 mM NaCl gradient (Sec. 2.5.5).

(a) Ultraviolet absorption profile of the anion exchange column fractionation. 1.0 mL fractions were collected from the fractionation, numbered 1-64. The broad band of absorption in fractions 2-15 is largely due to Triton X-100, not protein.

(b) Approximately 200 μg of protein from selected fractions were precipitated with 5 volumes of acetone, separated by SDS-PAGE and visualized with Coomassie Blue staining (Sec. 2.5.1). Lane S contains protein size standards, used to determine the location of Rne (migrating at the 180 kDa position) and PNPase (migrating at 85 kDa).

(c,d) Western blotting using polyclonal antibodies raised against Rne (c) and PNPase S1 (d) (Sec. 2.6). The selected fractions from panel (b) were separated by SDS-PAGE (10% gel) and blotted onto Immobilon P. The blots were probed first with α-Rne antibody (c), stripped of bound antibody (Sec. 2.6.4), then reprobed with α-PNPase S1 antibody (d). The arrows in panels (c) and (d) indicate the migration point of RneΔN608 and PNPase, respectively.

(a)


1
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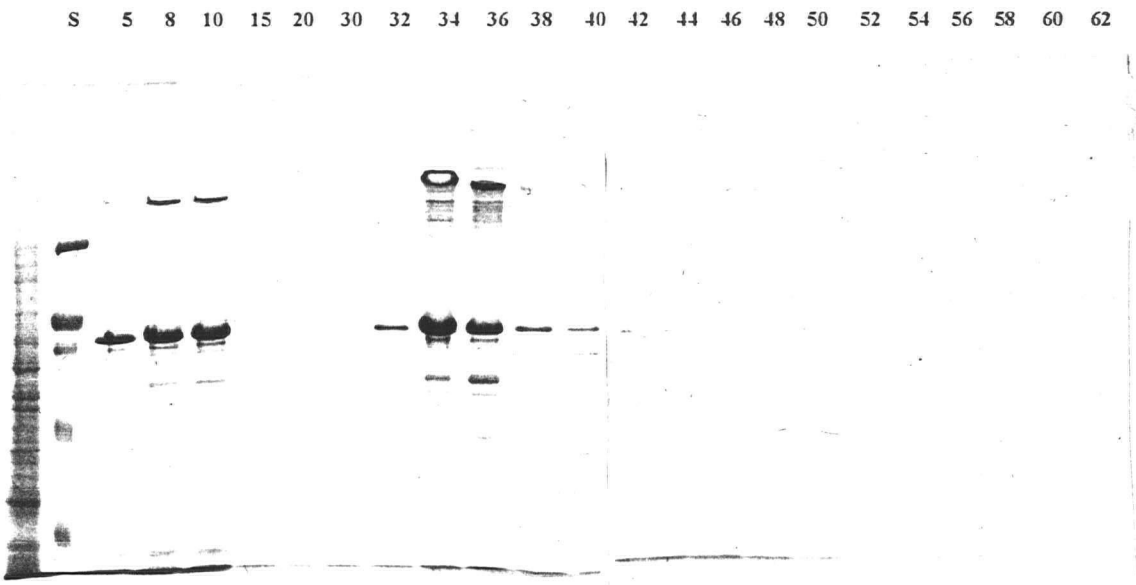
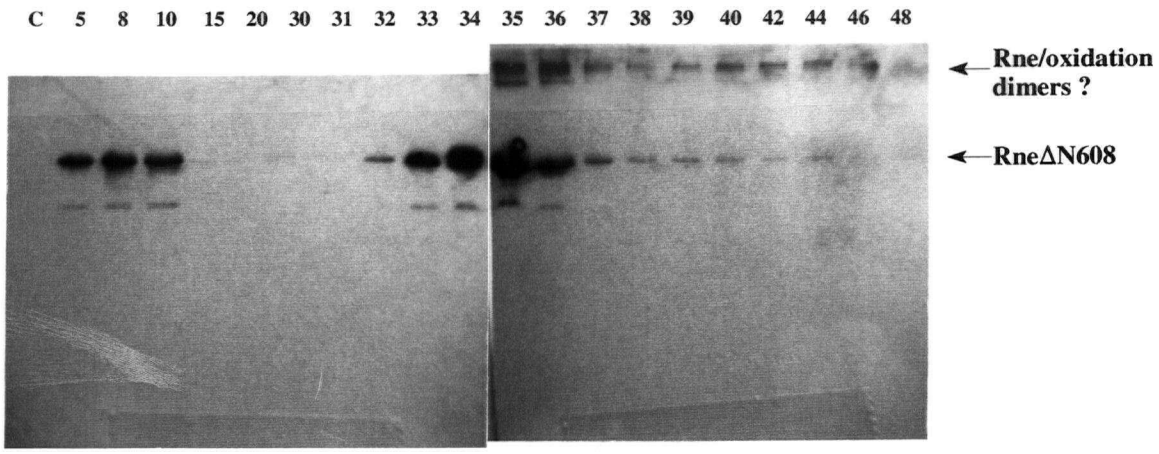
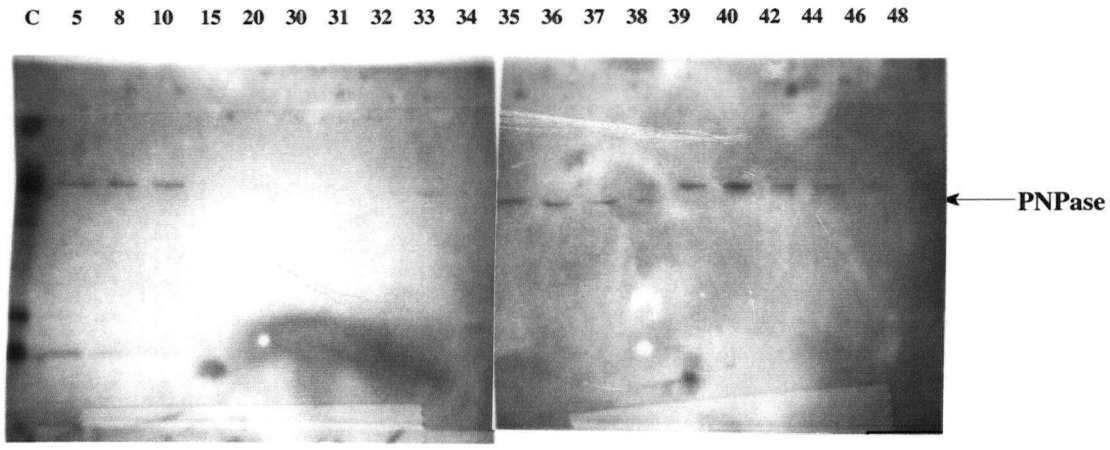
(b)


S 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62
180 kDa
100 kDa
85 kDa

(c)


C 5 8 10 15 20 30 31 32 33 34 35 36 37 38 39 40 42 44 46 48
Rne/oxidation dimers ?
RneΔN608

(d)


C 5 8 10 15 20 30 31 32 33 34 35 36 37 38 39 40 42 44 46 48
PNPase

These results indicated that, in contrast to native Rne, the majority of RneΔN608 elutes from the anion exchange column in fractions 34-35, not evenly over the entire salt gradient. It is also apparent that little PNPase is present in fractions 34-35 (Fig 8(d)); rather, most of the PNPase elutes at fractions 39-40, and a small amount of it appears from fractions 41 onwards associated with a small amounts of native Rne and RneΔN608 (Fig 8(c)). Nearly identical protein elution patterns were observed for all of the N-terminal Rne deletion mutants (RneΔN208, RneΔN315, RneΔN408, RneΔN608, and RneΔN813) as detailed in Figures 11-14.

3.3.3 Rne C-terminal deletion mutant fractionation by anion (Mono Q) and cation (Mono S) exchange chromatography

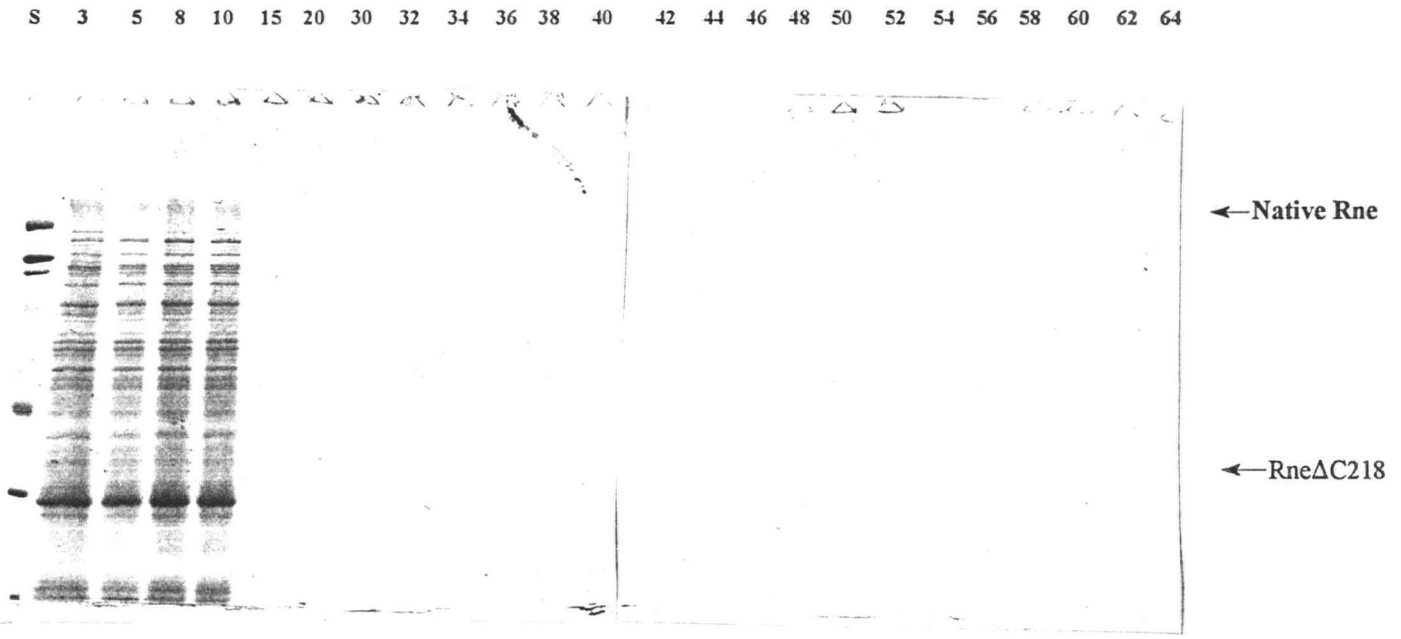
To determine the role of the first 200 amino acids of the Rne protein in associating with PNPase, a C-terminal deletion mutant, pRneΔC218, was constructed as described in Sec. 3.2.2. The corresponding protein was over-expressed and partially purified as in Sec. 2.5 and Sec. 3.3.1.

Passage of the partially purfied RneΔC218 through an anion exchange matrix was ineffective, since all the mutant Rne, along with a number of other proteins, failed to bind (Fig 9(a)). Fractionation through a cation exchange matrix (Mono S) resulted in significant retention of RneΔC218 in a highly purified state (Fig 9(b), lanes 32-36). Nonetheless, a large proportion of the RneΔC218, along with native Rne and a number of other proteins, did not bind to the matrix.

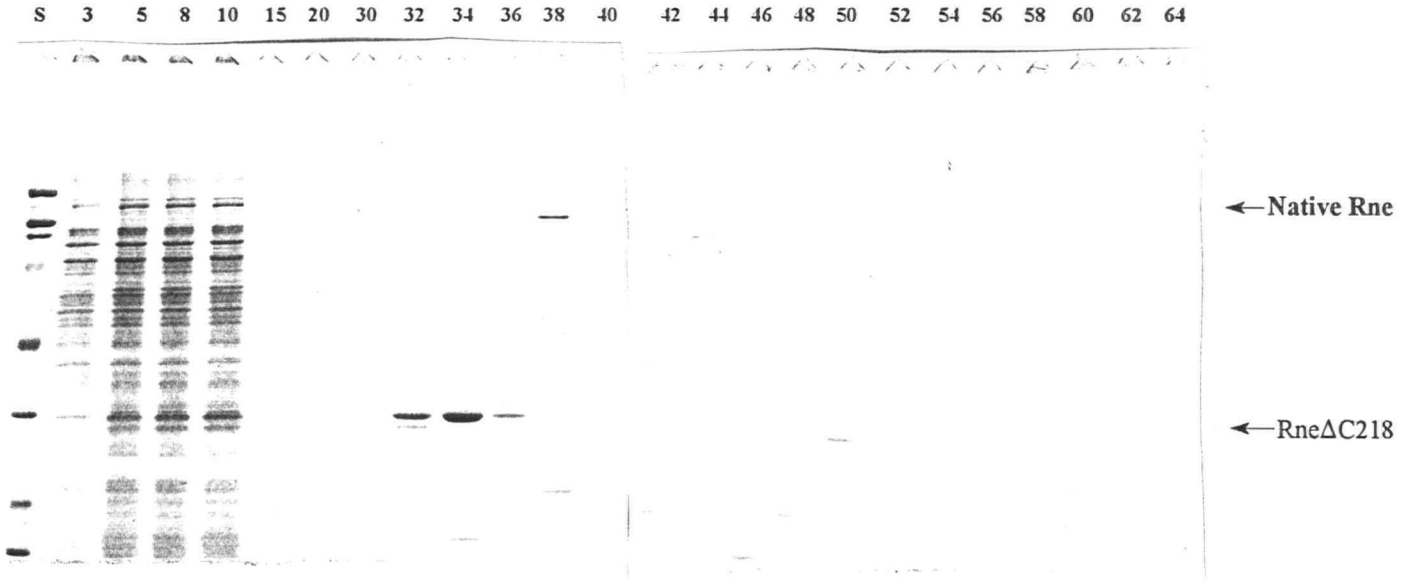
To confirm that RneΔC218 is able to bind cation, but not

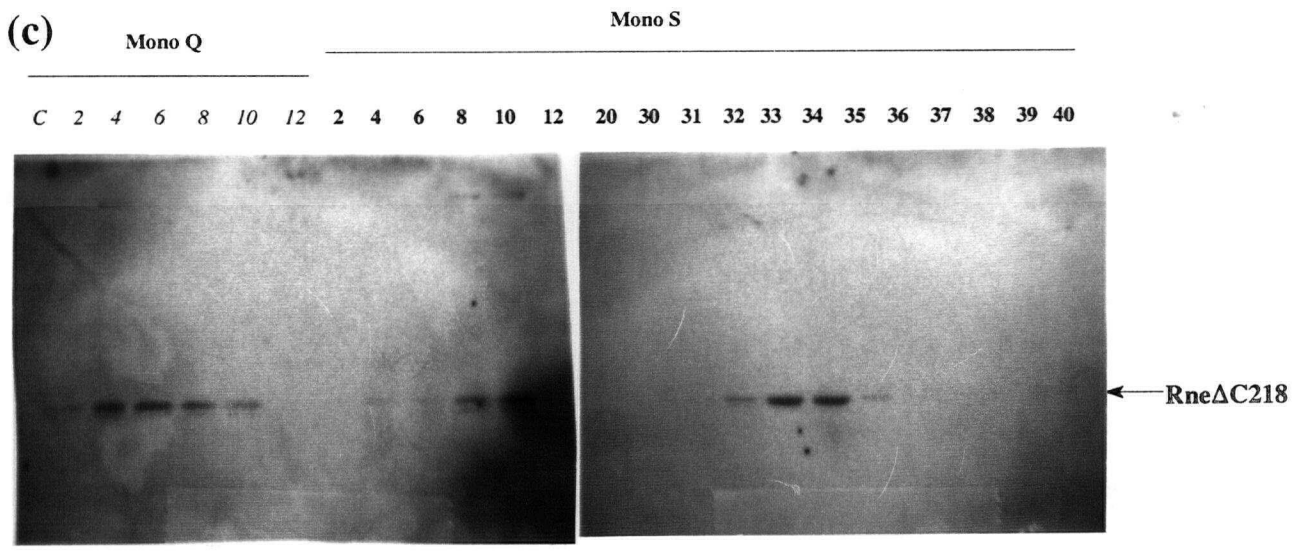
Figure 9. Fractionation of partially purified RneΔC218 by ion exchange chromatography. (a) Anion exchange (Resource Q) fractionation. Approximately 200 μg of protein from selected fractions was precipitated with acetone, separated by SDS-PAGE and visualized with Coomassie Blue staining (Sec. 2.5.1). Lane 0 contains protein size standards. (b) Cation exchange (Resource S) fractionation was conducted as outlined above for (a). (c,d) Western blotting using polyclonal antibodies raised against Rne (c) and PNPase S1 (d) (Sec. 2.6). The flow-through fractions from the anion exchange column are indicated in italics and the selected fractions from the cation exchange column are in standard numerals. All fractions were separated by SDS-PAGE and blotted onto Immobilon P. The blots were probed first with α-Rne antibody (c), stripped of bound antibody (Sec. 2.6.4), then reprobed with α-PNPase S1 antibody (d). The arrows in panels (a), (b), (c) and (d) indicate the migration point of RneΔC218 and PNPase as denoted.

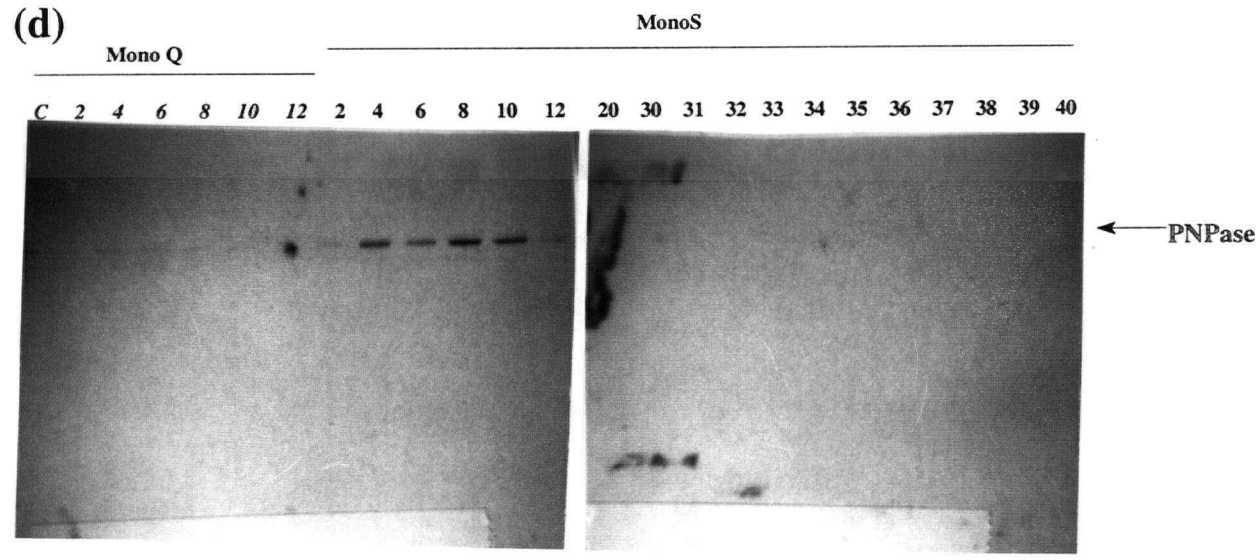
(a)


S 3 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64
←Native Rne
←RneΔC218

(b)


S 3 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64
←Native Rne
←RneΔC218


(c)
Mono Q
Mono S
C 2 4 6 8 10 12 2 4 6 8 10 12 20 30 31 32 33 34 35 36 37 38 39 40
RneΔC218


(d)
Mono Q
MonoS
C 2 4 6 8 10 12 2 4 6 8 10 12 20 30 31 32 33 34 35 36 37 38 39 40
PNPase

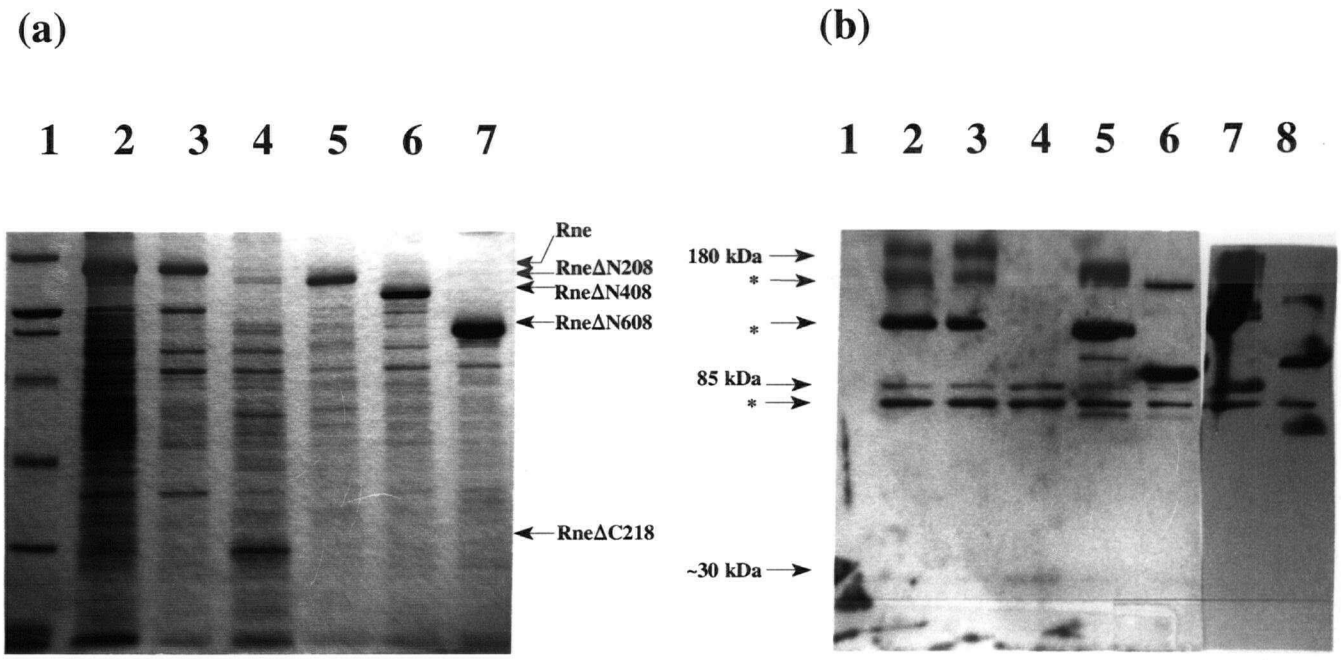
anion, exchange matrices, fractions from each column were analysed by Western blotting using α-Rne (Fig 9(c)). None of the immunoreactive RneΔC218 binds to the Mono Q column, while more than half of the RneΔC218 can bind the Mono S column. The flowthrough fractions of the Mono Q and Mono S separation both contain native Rne protein. The same fractions were also probed with α-His(6)-PNPase antisera (Fig 9(d)). No immunoreactive PNPase could be detected in fractions 32-36 of the Resource S eluate, although it could be detected in flowthrough fractions from both matrices.

3.4 ASSESSMENT OF Rne-PNPase INTERACTIONS BY FAR-WESTERN BLOTTING

Copurification of RNase E and PNPase through several chromatographic steps implies that there is a PNPase binding domain within the Rne protein (Carpousis, *et al.*, 1994; Py *et al.*, 1996; this work). To substantiate this possibility further, native and mutant Rne proteins were assessed for their ability to bind free, purified PNPase in a Far-Western blot experiment. Native, C-terminal and N-terminal deletion mutants of Rne were partially purified, separated by SDS-PAGE, transferred to an Immobilon P membrane, and exposed to a solution of free, highly purified His(6)-PNPase (Sec. 2.6.5). Unbound PNPase was removed by washing, and any bound PNPase was identified by using α-His(6)-PNPase as a primary antibody.

Fig 10(a) depicts an SDS-PAGE gel containing over-expressed native and mutant Rne proteins. Lanes 2 and 3 have the native and

Figure 10. Far-Western blotting of native and mutant Rne protein with free PNPase. (a) Extracts of strains over-expressing native and mutant Rne were separated on a 10% SDS - polyacrylamide gel and stained with Coomassie brilliant blue. Lane 1, protein markers; Lane 2, native Rne; Lane 3, Rne-3071 mutant protein; Lane 4, RneΔC218; Lane 5, RneΔN208; Lane 6, RneΔN408; Lane 7, RneΔN608. (b) Far-Western blot. The same proteins from panel (a) were transferred from a 10% SDS-polyacrylamide gel to Immobilon P (see Sec. 2.6.3). The immobilized proteins were exposed to free PNPase then washed (Sec. 2.6.5). Physical association between PNPase and the immobilized proteins was detected by reaction with polyclonal α-His(6)-PNPase antibodies and chromogenic detection. The samples in lanes 1-7 are identical to those in panel (a).


(a)
1 2 3 4 5 6 7
Rne
RneΔN208
RneΔN408
RneΔN608
RneΔC218
(b)
1 2 3 4 5 6 7 8
180 kDa
*
*
85 kDa
*
~30 kDa

Rne-3071 polypeptides, respectively, while lanes 4 to 7 contain over-expressed RneΔC218, RneΔN208, RneΔN408 and RneΔN608 proteins, respectively (See Fig. 6). All over-expressed proteins were abundant in relation to other endogenous proteins, and were approximately equal to each other in concentration.

Fig 10(b) shows the Far-Western blot used to determine the ability of native and mutant Rne to bind free PNPase. Wild-type and temperature-sensitive mutant Rne proteins in lanes 2 and 3, respectively, display significant PNPase binding to a protein of approximately 180 kDa (Rne). RneΔN208, RneΔN408, RneΔ608 and RneΔN813 also bind significant amounts of PNPase in lanes 5-8, respectively. Lane 3 shows a small amount of PNPase binding to a 30 kDa protein; however, this is also seen to a lesser degree in lanes 2, 3, 5, 6, 7 and 8. All of the samples contain PNPase endogenous to BL21(DE3) and this appears as a 85 kDa band in all of the lanes.

Other prominent bands reactive with α-His(6)-PNPase are denoted by * in Fig. 10b. These are likely degradation products of Rne. Since identical products are obtained with wild-type Rne (lane 2), Rne-3071 (lane 3) and RneΔN208 (lane 5), the degradation is likely occuring in the N-terminal domain of Rne, leaving the C-terminus intact. The ability of such partial degradation products to bind PNPase would support the idea that the C-terminal domain of Rne contains a PNPase binding site. Their prominence in the Far-Western blot (Fig. 10b) is not proportional to the protein visible in Fig. 10a; the efficient protein transfer to Immobilon of smaller

Rne degradation products compared to the full length Rne protein could explain this discrepancy.

The N-terminal Rne deletion mutants showed a strong affinity for PNPase based on this assay; however, RneΔC218 bound far more weakly. This is most likely a non-specific interaction of PNPase with RneΔC218 or a protein of similar size. The weak binding could also reflect a weak non-specific interaction between α-His(6)-PNPase and RneΔC218.

Chapter 4

DISCUSSION

Several researchers have attempted to purify the components of the *Escherichia coli* mRNA degradosome, and all have demonstrated that Rne and PNPase co-purify with the protein complex (Carpousis *et al.*, 1994; Py *et al.*, 1996; Miczak *et al.*, 1996). The data in this work show that this interaction can be disrupted by deleting portions of the Rne protein, and suggests that the acidic carboxy-terminal tail of Rne plays an important role in PNPase binding to the protein, and may be necessary for self-interaction.

First, to identify Rne and PNPase in these protein interaction studies, antibodies that recognize each protein were necessary. Antibodies directed against two highly antigenic sites predicted to exist in PNPase were raised successfully by cloning and over-expressing these portions of PNPase and using those purified proteins to immunize a rabbit and raise antibodies (i.e. α-S1 and α-S2). During the course of these experiments, a plasmid capable of directing the over-expression of a His(6)-PNPase fusion protein was described (Py *et al.*, 1994). This fusion protein was purified and used to raise an α-His(6)-PNPase antibody. The titres of polyclonal α-His(6)-PNPase were much higher than those of α-S1 and α-S2 antibodies, possibly because there were more antigenic sites exposed in His(6)-PNPase allowing more polyclonal antibodies to be directed against it. A high titre α-RNase E antiserum had been

raised previously in this lab.

The initial attempt to purify Rne to near homogeneity was unsuccessful, but did illustrate the strong affinity between Rne and PNPase. The native Rne protein was over-expressed in BL21(DE3) cells and enriched from cell lysates. It was hoped that the acidic carboxy region of Rne would bind tightly to an anion exchange matrix, and that this property could be utilized to separate Rne from contaminating proteins. Rather than eluting from the anion exchange resin at a distinctive salt concentration as expected, Rne eluted over a wide range of salt concentrations, but always associated with approximately equimolar amounts of PNPase (Fig. 7). A number of unidentified associated proteins were removed at various concentrations of salt. The strong association between Rne and PNPase was also observed in the purification reported by Carpousis *et al.* (1994). Rne and PNPase remained in a stable complex after successive steps including chromatography on S-Sepharose and hydroxylapatite, and centrifugation through a 10-20% glycerol gradient (Carpousis *et al.*, 1994). Immunoprecipitation using α-RNase E also revealed that PNPase co-precipitated with Rne (Carpousis *et al.*, 1994). A separate study by Py *et al.* (1994) that attempted to identify an RNA stem-loop binding protein found a stable complex with RNase E and PNPase activity. Their attempts at precipitating PNPase with α-PNPase showed that a 65kDa proteolytic fragment of Rne co-precipitated with the PNPase (Py *et al.*, 1994).

The fact that the Rne, PNPase and associated proteins were

able to associate with the anion exchange matrix at varying concentrations of salt would suggest that there are different populations of protein complexes that include Rne and PNPase, and all of them have differing charges associated with them.

In an attempt to disrupt the association of Rne and PNPase, N-terminal deletion mutants of Rne were constructed, over-expressed, and purified in an identical manner as the native protein (Miao, personal communication). As illustrated in Fig. 8, there are two distinct populations of the RneΔN608 deletion mutant: the majority of the RneΔN608 elutes from the anion exchange matrix in fractions 34-38 of the salt gradient associated with native Rne, presumably in a mixed oligomer. This suggests that the protein complex involving Rne-RneΔN608, but not PNPase, exists as a unit of stable charge. A small portion of the RneΔN608 is present at the higher concentrations of the salt gradient, associated with native Rne and PNPase. The fact that the native Rne and PNPase are found together in equimolar amounts indicates that their physical interaction remains intact. The SDS-PAGE gels also indicate that multiple populations of protein complexes are present involving native and mutant Rne, PNPase and other unidentified proteins. The identical anion exchange elution profiles were seen for the N-terminal Rne deletion mutants RneΔN208, RneΔN315, RneΔN408 and RneΔN813 (see Figures 11-14). This evidence supports the findings of Kido *et al.* who demonstrated that wild-type Rne is capable of binding PNPase, while truncated Rne lacking the C-terminal half did not (Kido *et al.*, 1996). The data also imply that the C-terminal 250 amino

acids are involved in oligomerization of the native Rne protein, since the mutant RneΔN813 is still able to associate with native Rne (Figures 11-14).

A recombinant plasmid capable of expressing a C-terminal Rne deletion was created to assess the ability of the first 200 amino acids of Rne to bind PNPase. The mutant RneΔC218 was over-expressed and purified as was done for the native Rne and the N-terminal mutants, and separated on the anion exchange matrix. The RneΔC218 did not bind this resin at all, supporting the assumption that the acidic tail of native Rne binds the anion matrix (Fig. 9a). Fractionation of these proteins in a cation exchange matrix showed that a significant portion of the RneΔC218 bound to the matrix, while the rest of the RneΔC218 and all the PNPase and native Rne did not bind at all (Fig. 9b). This appears to suggest that the over-expressed RneΔC218 was unable to titrate PNPase away from the native Rne in the degradosome complex, although it appears that a small portion of the RneΔC218 interacts either specifically or non-specifically with the complex. This serves to illustrate the problem with co-chromatography as a method of protein-protein interactions: if a truncated Rne mutant elutes with PNPase, it is never clear whether the Rne mutant is binding directly to PNPase or to another protein that is bound to PNPase.

In an effort to resolve the direct or indirect binding of Rne deletion mutants to PNPase, a Far-Western blot experiment was performed. In this experiment, total cellular proteins are denatured and separated by size. Free PNPase binding to each

protein is assessed individually, thereby excluding the possibility of indirect binding between the Rne mutants and PNPase. Native Rne, along with the N-terminal and C-terminal Rne mutants described previously, were affixed to a membrane, and allowed to associate with free PNPase. As illustrated in Fig. 10, the native Rne and N-terminal deletion mutants bound PNPase extremely well, while the 30 kDa Rne C-terminal mutant appeared to bind PNPase at a very low level. However, this association is probably non-specific since a faint 30 kDa band is present in the native and N-terminal mutant Rne lanes as well. Possible degradation products of native and mutant Rne (denoted by * in Fig 10) could also bind PNPase. Their identity could be confirmed in a control Western blot experiment using α-RNase E; unfortunately, this was not performed.

It is also interesting to note that the mutant Rne-3071 was able to bind free PNPase, which conflicts with the observations of Carpousis *et al.* (1994), who found that glycerol gradient sedimentation at the non-permissive temperature caused separation of Rne-3071 and PNPase. Perhaps the conformational change that the Rne-3071 protein experiences at the non-permissive temperature is negated by the way the protein is presented on the Immobilon blot or by the conditions of probing. This reasoning may also explain why enolase, which co-purifies with PNPase, did not appear as a PNPase binding protein. In addition, enolase may not renature efficiently.

Co-chromatography combined with the Far-Western experiments on

native and mutant Rne-PNPase interactions confirm that the acidic C-terminal of Rne plays a role in the binding of PNPase. The co-chromatography experiments with the N-terminal deletion mutants cannot distinguish between specific or non-specific interaction between the mutants and PNPase, but do clearly illustrate that the over-expressed mutant proteins are unable to titrate PNPase away from an interaction with native Rne. It is also interesting to note that small portions of Rne N-terminal deletion mutants missing up to 813 amino acids are still able to interact with the native Rne-PNPase complex, possibly by binding to native Rne. The co-chromatography experiments with RneΔC218 were unclear in determining a role of the Rne N-terminal end, since anion and cation matrices were unable to separate a RneΔC218-PNPase complex from the other proteins present.

The Far-Western studies suggest that amino acids 608-1061 of Rne are able to bind free PNPase strongly, which supports past evidence that Rne lacking its C-terminus due to partial proteolysis (Carpousis et al., 1994) or deliberately deleted (Kido et al., 1996) is unable to associate with PNPase. The Far-Western blot also indicated low level, non-specific PNPase binding by a 30 kDa protein in all deletion mutants (see Fig. 10b). Whether the blotted proteins used in the Far-Western have the same 3D structure *in vivo* is unknown, since the experiment disrupts protein structure during the separation and blotting phases (See Materials and Methods).

Future work on the relationship between Rne and PNPase would

likely involve the bifunctional cross-linking of these degradosome proteins. Chemical cross-linking of supramolecular complexes containing several polypeptide chains has been well documented in ribosomal research, and has been used to locate protein binding domains (Traut et al., 1980). Bifunctional imido-esters (e.g. 2-iminothiolane) that introduce a disulfide bond as the cross-link are particularly useful in two-dimensional SDS-PAGE gels, since linked proteins can be cleaved by reduction in the second dimension to regenerate monomeric proteins with the same electrophoretic mobilities as the native proteins (Traut et al., 1980). In addition to finding the Rne-PNPase binding site, binding domains for other degradosome components (e.g. RhlB and enolase) will be identified. There may be difficulties in the cross-linking of Rne-PNPase because of the lack of lysines (and other basic residues) in the C-terminus of Rne, but if it does work then identification of the exact binding domain will be simplified.

A radio-labelled cross-linking reagent could be also be used to join proteins in the degradosome, followed by denaturation of the proteins, tryptic digestion, and HPLC fractionation to locate the site of cross-linking (Stone and Williams, 1993). Since sequence data on the proteins of the degradosome are already available, a predictable pattern of tryptic digestion would be seen in the HPLC protein separation, except for the cross-linked species, which would have the additional mass of the cross-link. The purified cross-linked peptides could be microsequenced by conventional methods (Stone and Williams, 1993) to determine the

binding domain.

Recent years have given us an abundance of new insights into the components of the degradosome. These studies described have served as a point of departure in determining the PNPase binding site in Rne, and hopefully will aid in the further understanding of the interaction and function of the degradosome enzyme complex.

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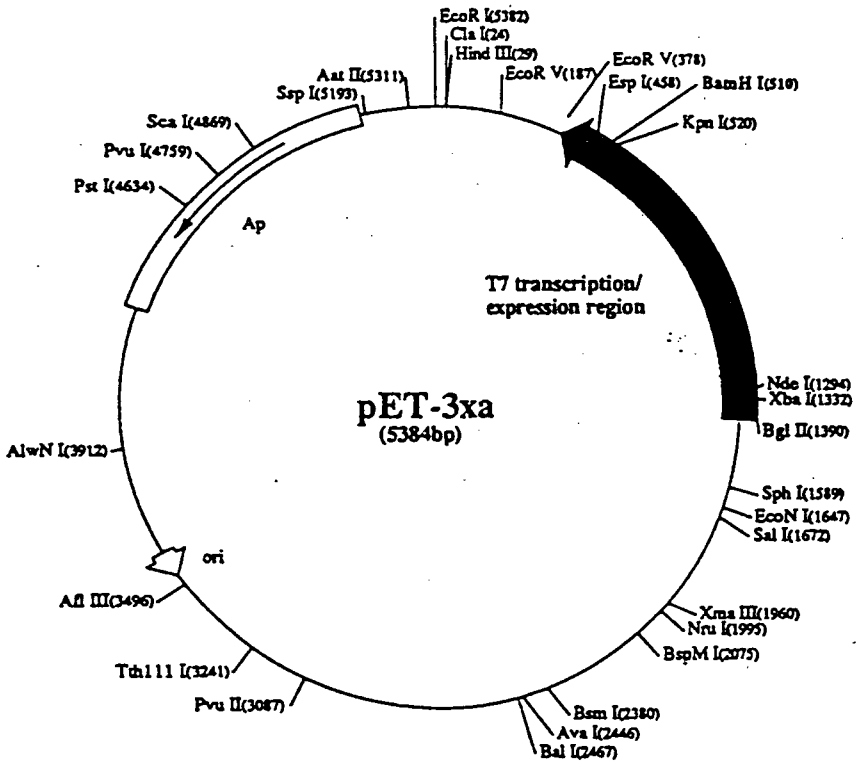
Appendix 1. pET3xc cloning vector used to construct rneΔN208, rneΔN315, rneΔN408, rneΔN608, rneΔN722, and rneΔN813. Restriction sites used for cloning are circled in red.



Appendix 2. pET24b cloning vector used to construct rneΔC218. Restriction sites used for cloning are circled in red.

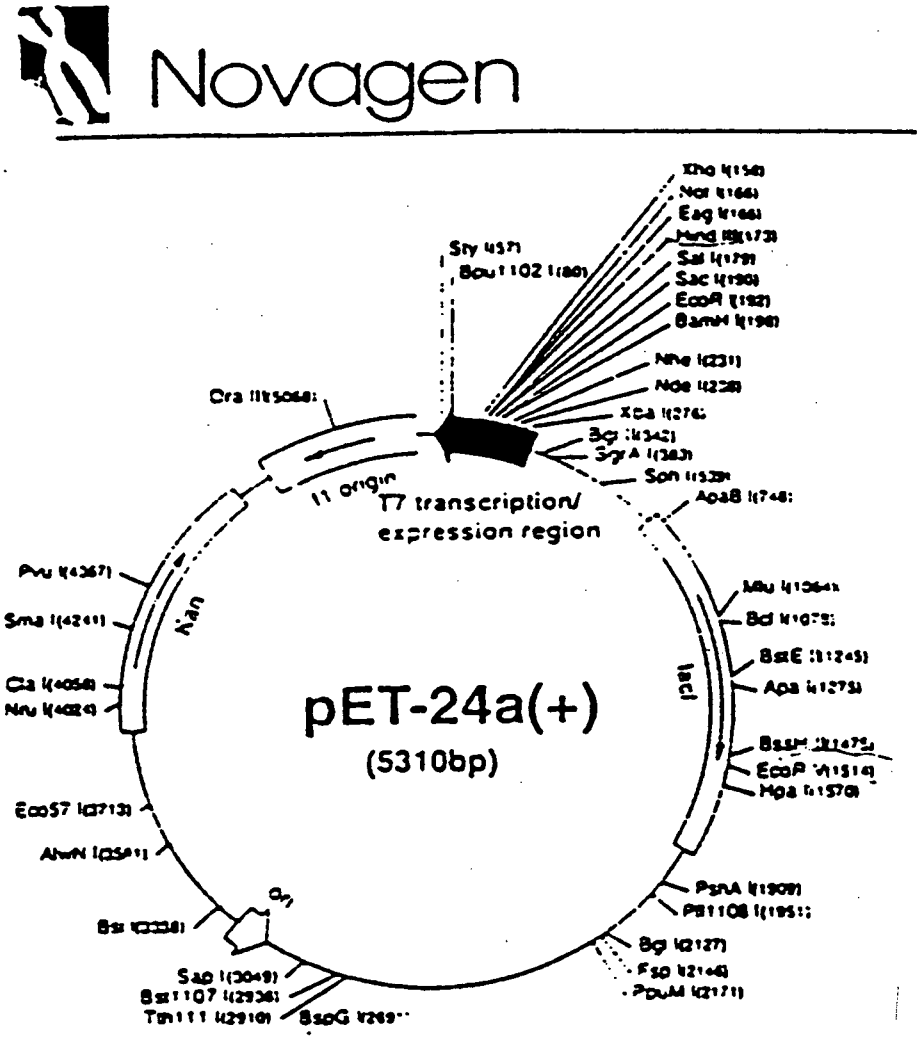
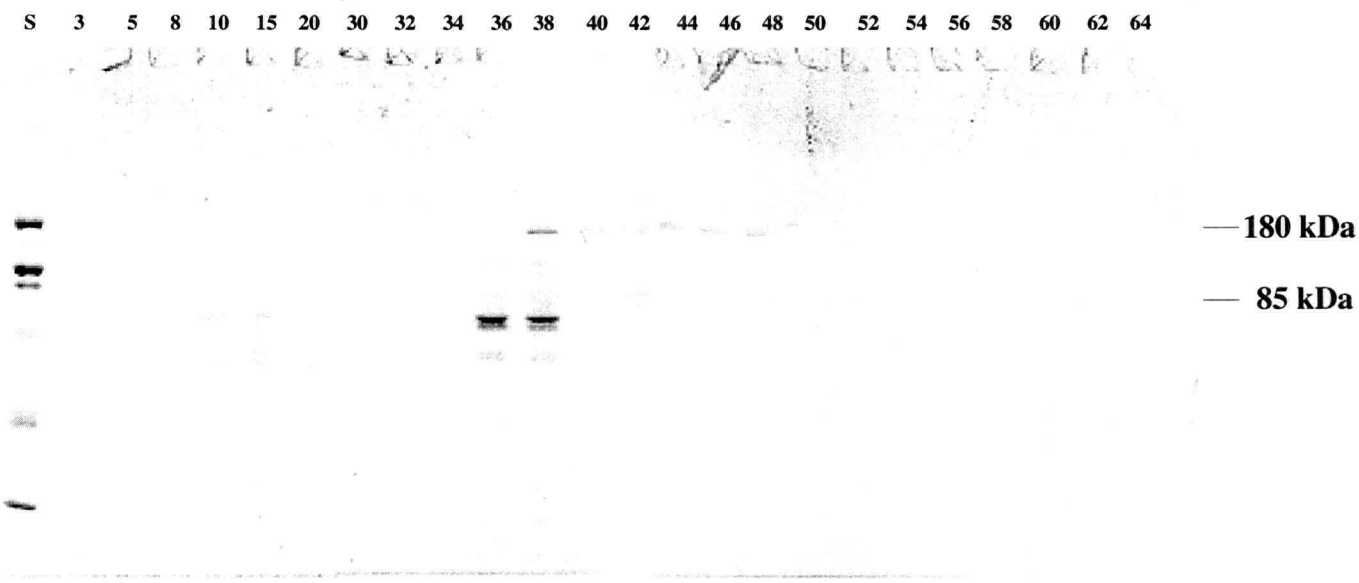


Figure 11: Supplemental Figure - Fractionation of partially purified extracts of RneΔN208 on an anion exchange column (Resource Q). For expermental details, see Figure 8 and the results section.

(a)

(b)


S 3 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64
180 kDa
85 kDa

(c)

C 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60

(d)

C 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60

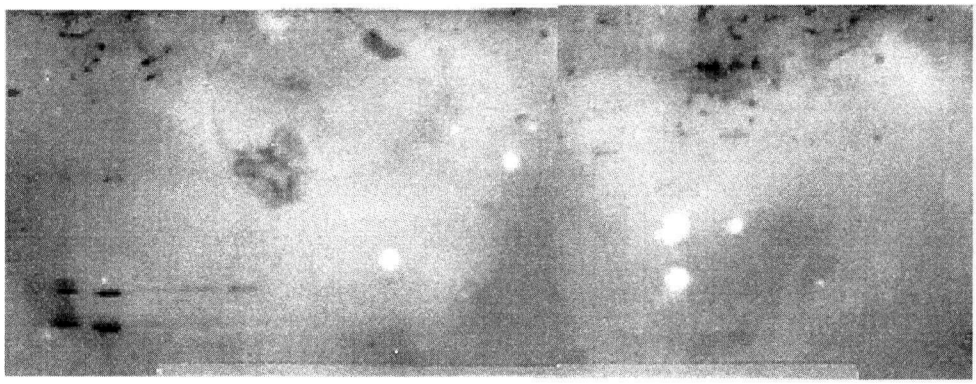
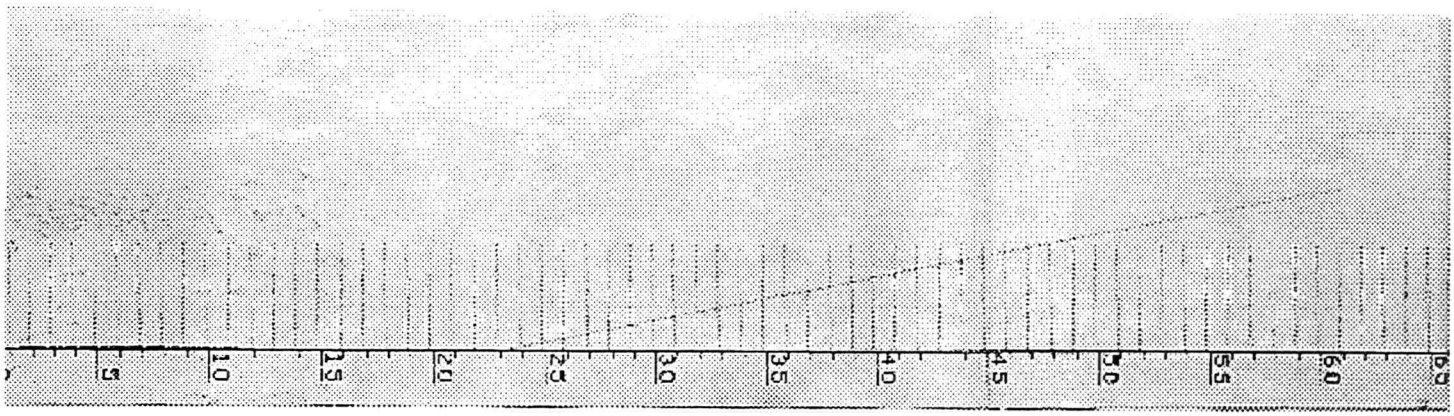
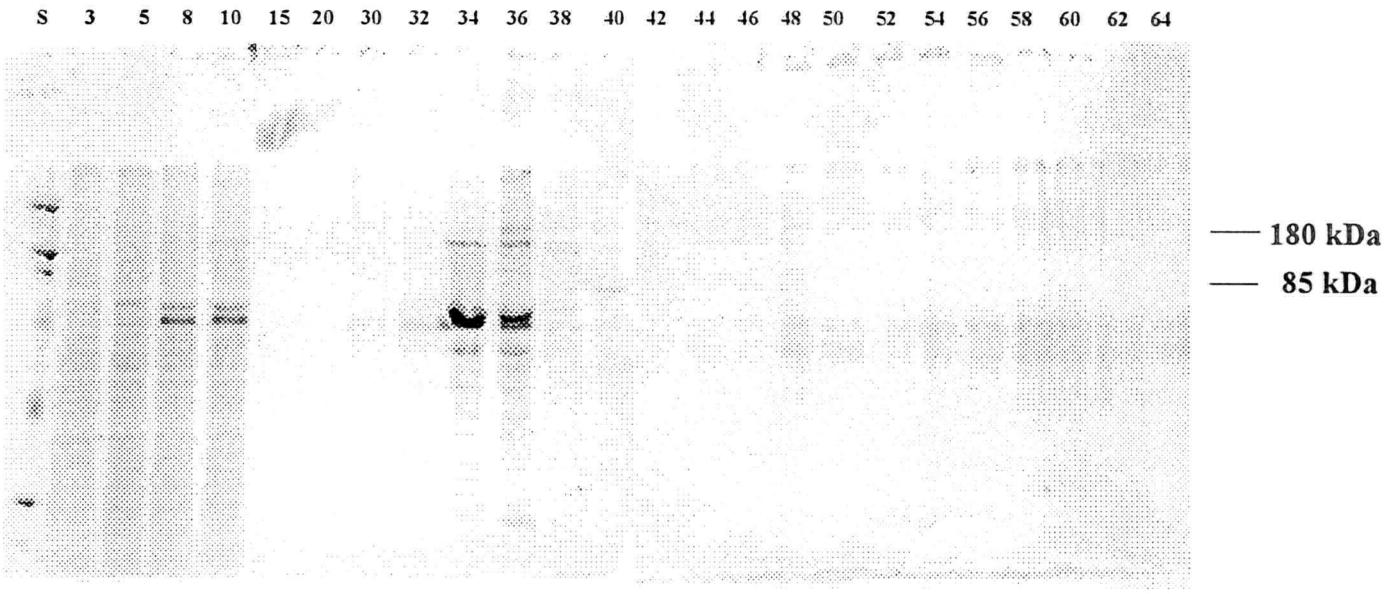


Figure 12: Supplemental Figure - Fractionation of partially purified extracts of RneΔN315 on an anion exchange column (Resource Q). For experimental details, see Figure 8 and the results section.

(a)

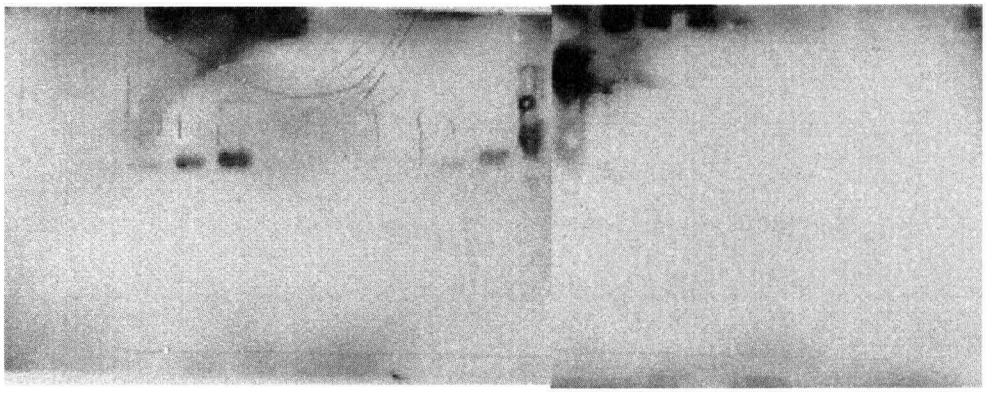


(b)


S 3 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64
180 kDa
85 kDa

(c)

C 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60



(d)

C 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60

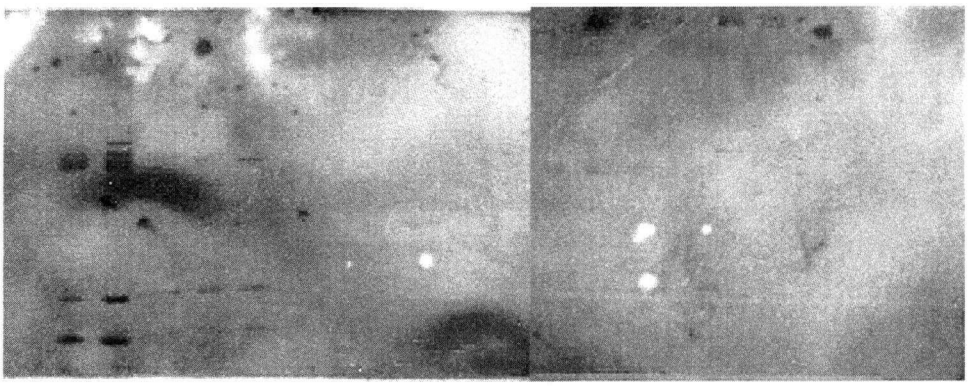
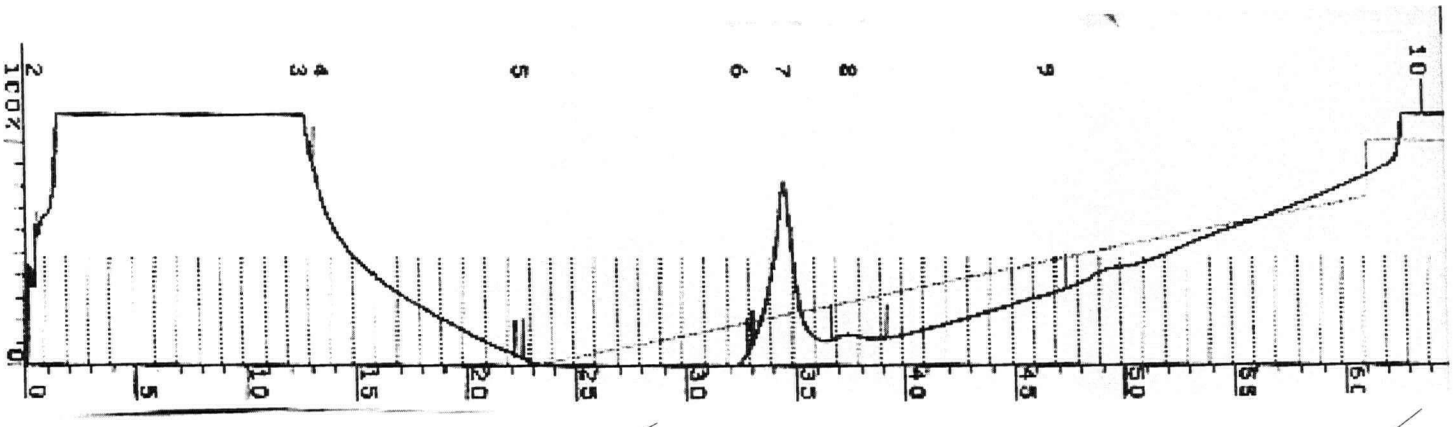


Figure 13: Supplemental Figure - Fractionation of partially purified extracts of RneΔN408 on an anion exchange column (Resource Q). For experimental details, see Figure 8 and the results section.

(a)


2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
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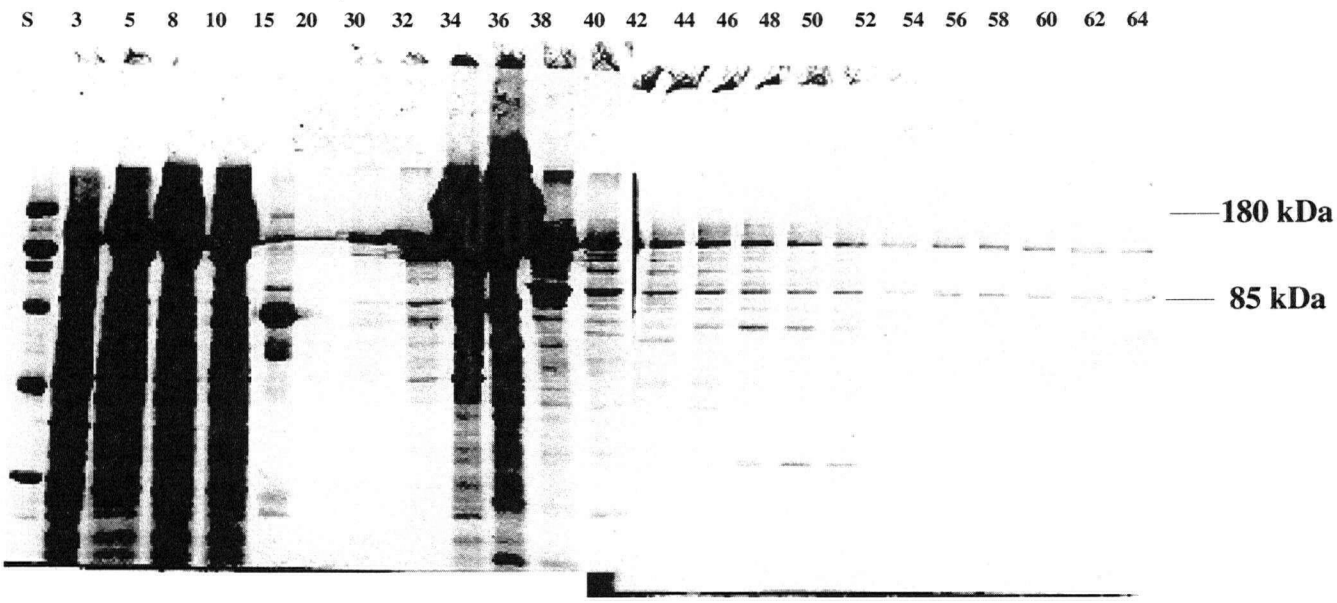
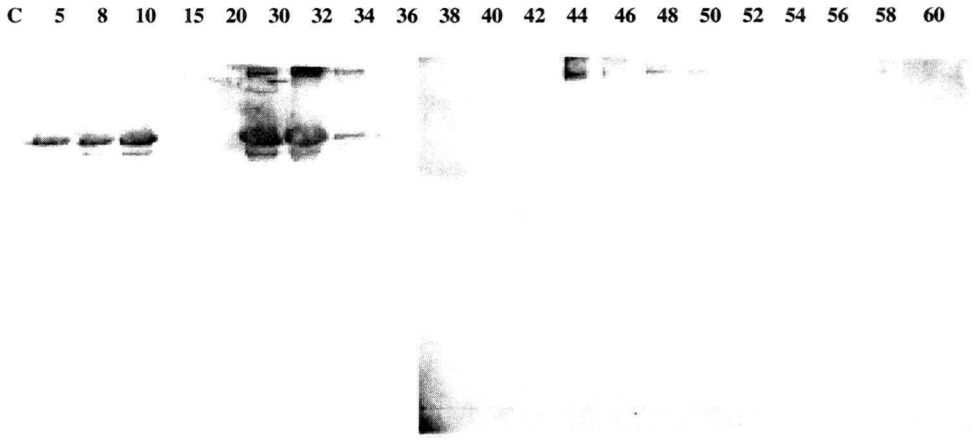
35
40
45
50
55
60

(b)


S 3 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64
180 kDa
85 kDa

(c)


C 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60

(d)

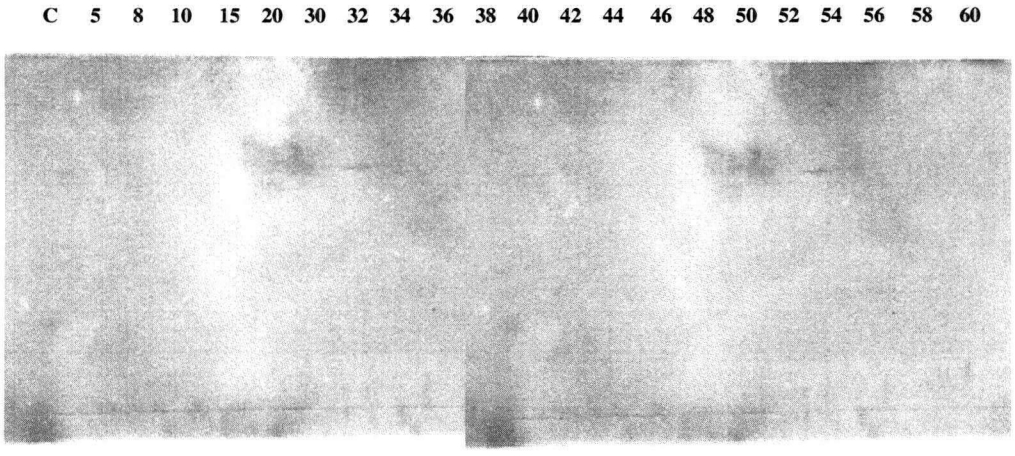
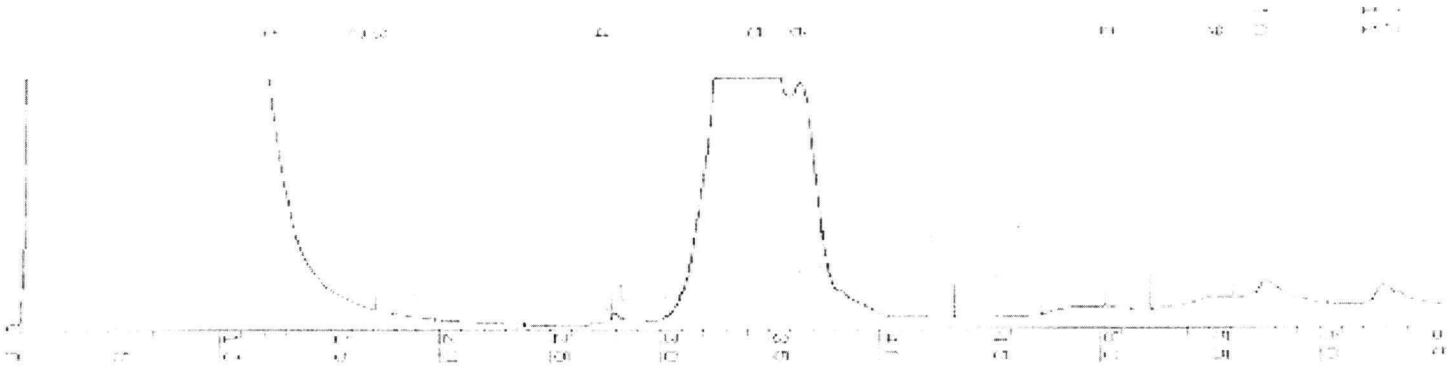

C 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60

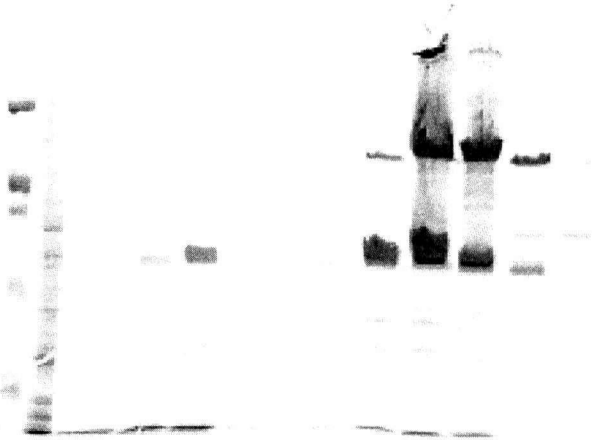
Figure 14: Supplemental Figure - Fractionation of partially purified extracts of RneΔN813 on an anion exchange column (Resource Q). For experimental details, see Figure 8 and the results section.

(a)



(b)

S 3 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64

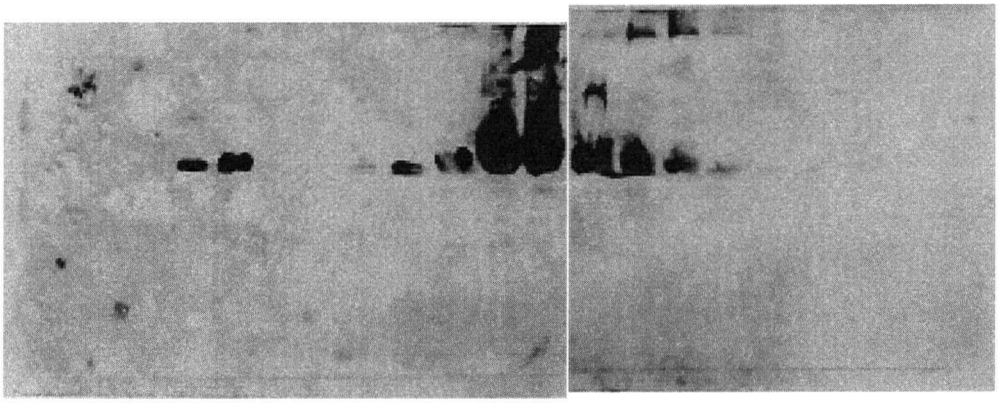


180 kDa

85 kDa

(c)

C 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60



(d)

C 5 8 10 15 20 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60