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Purification of mitochondrial RNase P in *A. nidulans*

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Purification of mitochondrial RNase P in *A. nidulans*

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Résumé

La ribonucléase P (RNase P) est une ribonucléoprotéine omniprésente dans tous les règnes du vivant, elle est responsable de la maturation en 5' des précurseurs des ARNs de transfert (ARNts) et quelques autres petits ARNs. L'enzyme est composée d'une sous unité catalytique d'ARN (ARN-P) et d'une ou de plusieurs protéines selon les espèces. Chez les eucaryotes, l'activité de la RNase P cytoplasmique est distincte de celles des organelles (mitochondrie et chloroplaste).

Chez la plupart des espèces, les ARN-P sont constituées de plusieurs éléments structuraux secondaires critiques conservés au cours de l'évolution. En revanche, au niveau de la structure, une réduction forte été observé dans la plupart des mtARN-Ps. Le nombre de protéines composant la RNase P est extrêmement variable : une chez les bactéries, environ quatre chez les archéobactéries, et dix chez la forme cytoplasmique des eucaryotes. Cet aspect est peu connu pour les formes mitochondriales.

Dans la plupart des cas, l'identification de la mtRNase P est le résultat de longues procédures de purification comprenant plusieurs étapes dans le but de réduire au minimum le nombre de protéines requises pour l'activité (exemple de la levure et *A. nidulans*). Cela mène régulièrement à la perte de l'activité et de l'intégrité des complexes ribonucléo-protéiques natifs.

Dans ce travail, par l'utilisation de la technique de BN-PAGE, nous avons développé une procédure d'enrichissement de l'activité RNase P mitochondriale native, donnant un rendement raisonnable. Les fractions enrichies capables de cette activité enzymatique ont été analysées par LC/MS/MS et les résultats montrent que l'holoenzyme de la RNase P de chacune des fractions contient un nombre de protéines beaucoup plus grand que ce qui était connue. Nous suggérons une liste de protéines (principalement hypothétiques) qui accompagnent l'activité de la RNase P.

De plus, la question de la localisation de la mtRNase P de *A. nidulans* a été étudiée, selon nos résultats, la majorité de la mtRNase P est attachée á la membrane interne de la mitochondrie. Sa solubilisation se fait par l'utilisation de différents types de détergent. Ces derniers permettent l'obtention d'un spectre de complexes de la RNase P de différentes tailles.

Abstract

RNase P is a ribonucleo-protein complex (an RNA enzyme or ribozyme) that cleaves 5' leader sequences of precursor tRNAs and a few other small RNAs. It occurs in all three domains of life, Bacteria, Archaea and Eukarya, with the latter containing distinct nuclear and organellar (mitochondrial or plastid) activities. In most instances, the complex contains a single, well-conserved RNA subunit that carries the active center of the enzyme. Yet, compare to bacterial and nuclear P RNA, most mtP RNAs are structurally highly reduced. The number of P proteins is highly variable: one in Bacteria, about four in Archaea, and ten in the cytoplasmic form of Eukarya. Much less is known in the case of mitochondria. MtRNase P is usually purified by using numerous separation steps that include unphysiological conditions, leading to complexes having a minimum number of subunits (e.g., in yeast and *Aspergillus nidulans*), that often loose their activity. Here, using BN PAGE, we have developed an enrichment procedure for *A. nidulans* mtRNase P that avoids some of the most disruptive conditions. The protein composition of active fractions was identified with LC/MS/MS, indicating that the RNase P holoenzyme is much larger than previously thought.

Finally, the question of mtRNase P localization within mitochondria was investigated, by tracing its RNA subunit by RT PCR. We found that mtRNase P of *A. nidulans* is a predominantly membrane-attached enzyme; it is in part solubilized by detergents such as digitonin and Triton.

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2BME	2-mercaptoethanol
A. nidulans	*Aspergillus nidulans*
A-type	Ancestral type
AcCoA	Acetyl coenzyme A
Asp	Aspartic acid
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate hydrolase
AT rich	Adenine-Thymine rich
B-type	*bacillus* type
BNC	Blue native preparative polyacrylamide gel column
BN-PAGE	Blue native polyacrylamide gel electrophoresis
bp	Base pairs
C5	RNase P protein subunit of *E. coli*
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CoA	Coenzyme A
CR	Conserve region
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid

FA	Formic acid
fmol	Femtomole
G+C	Guanine + cytosine
Glu	Glutamic acid
His	Histidine
J	Joints
kb	Kilobase
kDa	Kilodalton
kg	kilogram
LC/MS/MS	Liquid chromatography tandem mass spectrometer
Lys	Lysine
M	Molar
M1 RNA	RNA subunit of *E. coli* RNase P
mtDNA	Mitochondrial DNA
Mitosol	Mitochondrial soluble matrix
mM	millimolar
Mth	*Methanothermobacter thermoautotrophicus*
mtP RNA	RNA subunit of mitochondrial RNase P
mtRNase P	Mitochondrial RNase P
ng	nanogram
nt	nucleotides
OAA	Oxaloacetate

pmol	picomole
P protein	Protein subunit of RNase P
P RNA	RNA subunit of RNase P
pre-tRNA	tRNA precursor
PSI-BLAST	Position specific iterative basic local alignment search tool
RNA	Ribonucleic acid
RNase P	Ribonuclease P
RNA Pol (I, II, II)	RNA polymerase (I, II, III)
rDNA	Ribosomal DNA
rnpB	Gene encoding P RNA
rRNA	Ribosomal RNA
S-domain	Specificity domain
SDS	Sodium dodecyl sulfate
S_N2	Nucleophilic substitution
TCA	trichloroacetic acid
TCEP	Tris (2-carboxyethyl) phosphine
TOM #	Translocase of outer membrane # kDa subunit
tRNA	Transfer RNA

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To my Mother and Father,
Who, thousands kilometers away,
have made my heart warm,
when it was too cold out there!

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

1.1. RNase P, a ubiquitous and ancient ribozyme

In "the central dogma of molecular biology" (Crick 1958; Crick 1970), DNA (the genetic material) and proteins (biocatalysts) take central, primary roles in living organisms. According to this view, RNA molecules occupy only intermediate helper functions in protein translation (transfer, ribosomal and messenger RNAs). This dogma was for the first time disputed by Fraenkel-Conrat and Williams (Fraenkel-Conrat and Williams 1955), suggesting a new role of RNA in tobacco mosaic virus[1]. Its genetic material consists of RNA that during infection is transmitted to the host's genome. The second postulate (that biocatalysis is always based on proteins) was corrected much later, by finding RNAs with catalytic properties (called ribozymes), namely the self-splicing intron in the cytoplasmic rRNA of *Tetrahymena thermophila* (Kruger et al. 1982), and the RNA subunit (M1 RNA) of RNase P in *E. coli* (Guerrier-Takada and Altman 1984a). The groundbreaking discoveries of RNAs with biocatalytic activity, in the absence of proteins, earned Cech and Altman the 1989 Nobel prize in chemistry.

According to a hypothesis that was developed at about the same time by Walter Gilbert, evolution of life started out with RNAs, followed only thereafter by the invention of DNA and proteins (Gillbert 1986). In such an ancient "RNA World", life would be exclusively based on RNAs, including the storage of genetic information and catalytic reactions. Following this idea, catalytic RNAs of modern organisms would be remnants of an ancient "RNA world". In fact, the universal presence of RNase P in all three domains of life, Bacteria, Archaea and Eukarya is somehow consistent with an ancient origin of this ribozyme. A few recently detected exceptions, such as Archaea without RNase P, and the absence of P RNA in human mtRNase P, are interpreted as secondary loss (Holzmann et al. 2008; Randau, Schroder, and Soll 2008). Additional members of the ribozyme family are group I and II introns (Michel and Cummings 1985; Michel and Lang 1985; Cech and Bass

[1] A group IV RNA virus (single stranded positive-sense RNA) that infects plants, in particular tobacco.

1986; Peebles et al. 1986; Michel and Jacquier 1987; Michel et al. 1990; Michel and Ferat 1995), hammerhead (Forster and Symons 1987a; Forster and Symons 1987b), hairpin (Feldstein, Buzayan, and Bruening 1989; Hampel and Tritz 1989; Haseloff and Gerlach 1989), hepatitis delta virus (Wu et al. 1989) and Varkud satellite (Saville and Collins 1990) catalytic RNAs.

1.2. Cellular functions of RNase P

Numerous ribonucleases have essential cellular functions, catalyzing the precise processing of precursor RNA molecules into components of mature size. In most instances, the catalytic RNAs are associated with one or more proteins into ribonucleoprotein complexes. Among them, RNase P is a key enzyme in tRNA biosynthesis. Its main function is the processing of tRNA 5′ ends, but it is also known to cleave other RNAs like the tRNA-like pseudo-knotted structures in viral RNAs (Mans et al. 1990), 4.5S RNA (Peck-Miller and Altman 1991), 10Sa RNA (Komine et al. 1994), C4 antisense RNA from bacteriophages P1 and P7 (Hartmann et al. 1995), and a polycistronic pre-mRNA (Alifano et al. 1994). With few exceptions, RNase P is present in all cells and organelles that carry out tRNA synthesis, in all three domains of life: Bacteria, Archaea, and Eukarya. RNase P has an essential function (Krupp et al. 1986) as inactivation of genes coding for RNase P RNA results in cell death (Hollingsworth and Martin 1986; Cherayil et al. 1987; Waugh and Pace 1990; Lee et al. 1991).

Despite substantial variation in RNase P RNA structure and the number of proteins in RNase P of various species, its catalytic specificity remains virtually unchanged. It is the only known endonuclease that removes the 5’ extensions of tRNA-precursors, by site-specific hydrolysis of a phosphodiester bond, leaving mature tRNAs with a 5’ phosphate (Figure 1-1a). RNase P activity requires divalent metal ions, optimally magnesium (Mg^{2+}), for folding of the RNA, for binding of protein and substrate, and for catalytic activity (Beebe, Kurz, and Fierke 1996). The metal and pH dependence of the cleavage rate is consistent with Mg^{2+} dependent nucleophilic attack on the substrate phosphodiester, to produce 5’ phosphate and 3’ hydroxyl termini. Available data are consistent with an S_N2-like mechanism, a nucleophilic substitution, where a hydroxide ion (apparently magnesium-bound) performs an

in-line attack on the leaving group of a scissile[1] phosphodiester (Haydock and Allen 1985)(Figure 1-1b). The 2'-OH serves as a ligand for Mg^{2+} at the substrate cleavage site. 2'-deoxyribose substitution at the cleavage sites (positions −2, −1, and +1) of P RNA reduces the apparent number of bound Mg^{2+} from three to two, and increases the apparent dissociation constant for Mg^{2+} from the micro-molar to the milli-molar range (Smith and Pace 1993).

However, the function of RNase P is not restricted to catalyzing precise RNA cleavage. Recent studies elucidate that human cytoplasmic RNase P plays an additional role as a transcription factor for RNA Pol III, and in transcription of tRNAs and other small non-coding RNAs (Jarrous and Reiner 2007), thus linking transcription of these molecules with RNA processing. RNase P exerts this role through association of Pol III with the chromatin of active tRNA and 5S rRNA genes, as demonstrated by ChIP analysis[2]. Protein subunits of cytoplasmic RNase P preferentially bind to chromatin of tRNA and 5S rRNA genes in dividing cells, but dissociate from chromatin in mitotic cells (Reiner et al. 2006).

Similarly in yeast, a nuclear portion of the protein component of mitochondrial RNase P (Rpm2p) has a transcriptional activation domain. It defines the steady state mRNA levels of several nucleus-encoded mitochondrial proteins (TOM40, TOM6, TOM20, TOM22, and TOM37). It also plays the role of transcription activator for nuclear genes and functions in translation of mitochondrially encoded subunits of cytochrome c oxidase (Stribinskis et al. 2001; Stribinskis et al. 2005). Moreover, biochemical and reverse genetic studies reveal that human nuclear RNase P subunits co-purify and associate with components of Pol I and its transcription initiation factors through association at the promoter and coding region of rDNA. Hence, RNase P is also required for efficient transcription by Pol I (Reiner et al. 2008). Taken together, it is likely that at least the cytoplasmic form of eukaryotic RNase P is associated in a larger complex that regulates both transcription and RNA processing.

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[1] The bond of a substrate that is subject to enzymatic cleavage.

[2] Chromatin Immuno-precipitation is a method to identify the association of proteins with specific regions of a genome utilizing specific antibodies.

1.3. Varying structure of RNase P ribonucleo-protein complexes

RNase P is a ribonucleo-protein complex, an RNA enzyme (ribozyme) that is associated with protein subunits. In most instances, it contains a single, well-conserved RNA subunit that carries the active center of the enzyme (Figure 1-2), and one or several protein subunits (Altman 1995; Frank and Pace 1998; Hartmann and Hartmann 2003). Bacterial, archaeal and eukaryotic P RNAs are similar in size, but are associated with a varying number of proteins: a single (small) protein in Bacteria (Kole and Altman 1981), four to five in Archaea (LaGrandeur et al. 1993; Andrews, Hall, and Brown 2001), and up to ten in the cytoplasmic RNase P of Eukarya (Garber and Altman 1979; Akaboshi, Guerrier-Takada, and Altman 1980; Bowman and Altman 1980; Kline, Nishikawa, and Soll 1981; Gold and Altman 1986; Krupp et al. 1986; Lee and Engelke 1989).

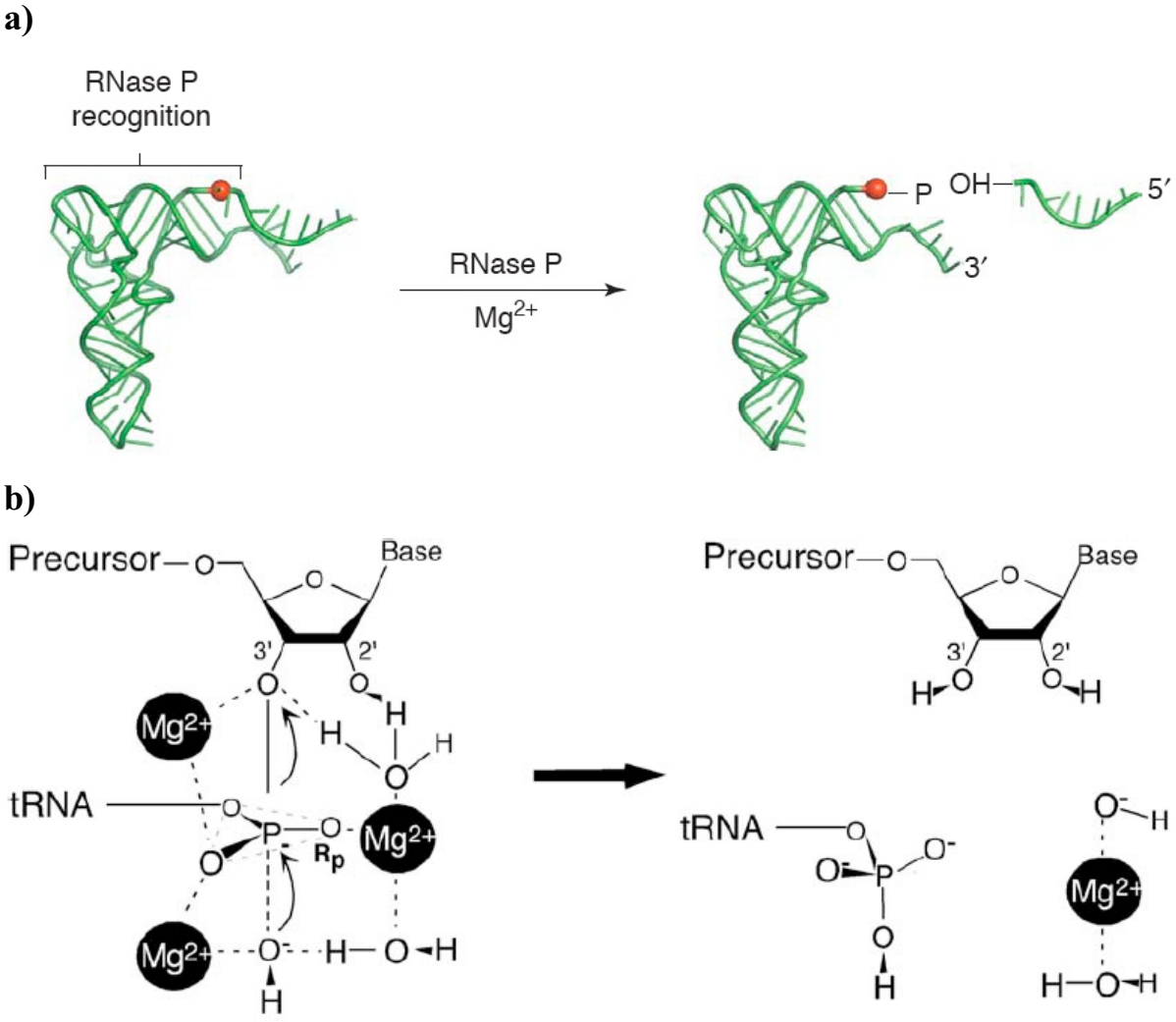

Figure 1-1- The RNase P reaction

(a) 5' maturation or pre-tRNA (substrate) catalyzed by bacterial RNase P holoenzyme in the presence of magnesium. The two products of the reaction are the mature tRNA 5' terminus and the 5' precursor sequence (Evans, Marquez, and Pace 2006). **(b)** Postulated mechanism of the Mg^{2+}-dependent phospho-diester cleavage reaction catalyzed by RNase P. Possible S_N2 type reaction showing stabilization of the attacking hydroxide nucleophile and the transition states of Mg^{2+} ions (Smith and Pace 1993). Figure taken from (Smith and Pace 1993; Evans, Marquez, and Pace 2006)

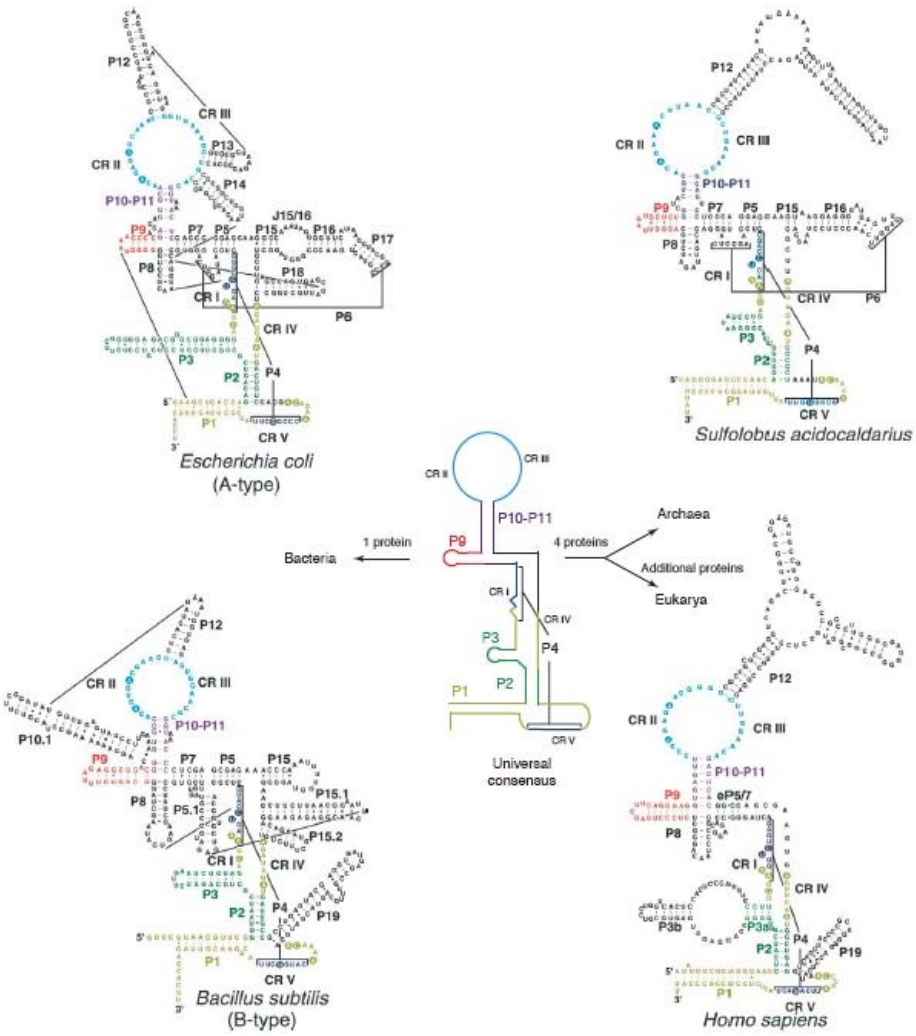

Figure 1-2- Secondary structures of P RNAs in the three domains of life.

Structural elements are labeled from 5' to 3' as 'P' for paired region, 'L' for loop and 'J' for joining region, and elements that are not found in all P RNA's are in black. Base pairs represented by dots indicate a non-canonical base interaction. Long-range tertiary interactions (in the P4 helix) are shown as brackets and/or lines (Chen and Lambowitz 1997; Evans, Marquez, and Pace 2006; Marquez et al. 2006). Paired regions (P) are numbered from the 5' end. Universally conserved nt are highlighted. CRI - V represents conserved regions. **Left:** Secondary structure of bacterial P RNAs; type -A (*E. coli*) and type -B (*B. subtilis*). Both types are composed of two domains designated as specificity domain and catalytic domain that are believed to fold independently. **Middle:** Minimum consensus structure. **Right:** An example of archaeal and Eukaryotic P RNA secondary structure. Figure taken from (Evans, Marquez, and Pace 2006).

RNA subunit

The RNase P RNA subunit (P RNA) is known to carry the catalytic center of RNase P, initially based on the observation that most of the bacterial and some of the archaeal and eukaryotic RNAs are active *in vitro* without proteins (Stark et al. 1978; Kole and Altman 1981; Guerrier-Takada et al. 1983). More recent investigations of RNA crystal structures have confirmed this view, from a 3D structural perspective (Kazantsev et al. 2005).

Molecular and bioinformatics probing has identified hundreds of P RNA sequences from all three domains of life, which are about 150-500 nt in length (Morales et al. 1989; Brown and Haas 1995; Chen and Pace 1997; Brown and Doolittle 1999; Frank et al. 2000). Their secondary structure is usually compact and highly conserved. For instance, all bacterial P RNAs share a core structure of approximately 200 nt, 13 of which are universally conserved in a minimum consensus structure (Chen and Pace 1997). This RNA core region is sufficient for RNase P activity *in vitro*, but the full length RNA is a more efficient catalyst (Waugh, Green, and Pace 1989)(Figure 1-2). Similarities in primary and secondary structures of known P RNAs suggest that they all evolved from a common ancestral RNA.

Protein subunits

The function of the various RNase P – associated protein subunits remains little understood, in part because most do not carry known conserved functional motifs. In addition, the number of P protein subunits is highly variable. The *E. coli* (and other bacterial) RNase P has a single, small 14 kDa protein subunit with RNA binding properties, but as already mentioned, the RNA subunit is catalytically active in the absence of this protein *in vitro*, demonstrating that the RNA is directly involved in catalysis (Stark et al. 1978; Guerrier-Takada and Altman 1984a; Franklin, Zwick, and Johnson 1995; Pace and Brown 1995; Doudna and Cech 2002). Archaea have 4-5 different protein subunits (Lawrence et al. 1987; Hall and Brown 2002), and Eukarya (cytoplasmic RNase P) up to ten (Jarrous and Altman 2001). Curiously, it is unknown which of the archaeal and eukaryotic P proteins (if any) is homologous to the single, bacterial P protein. In fact, the potential roles of protein subunits appear to be all structural, for instance stabilization of the active tertiary structure of RNA (Guerrier-Takada et al. 1983; Westhof, Wesolowski, and Altman 1996; Kim et al. 1997),

mediation of holoenzyme dimer formation (Fang et al. 2001), and enhancement of RNase P specificity (Crary, Niranjanakumari, and Fierke 1998; Kurz, Niranjanakumari, and Fierke 1998; Niranjanakumari, Kurz, and Fierke 1998; Sun et al. 2006).

RNase P activity also exists in mitochondria and chloroplasts. Following the endosymbiont theory[1], which invokes a bacterial origin of mitochondria and plastids, organelle RNase P is expected to be bacteria-like. Yet, evidence for this idea is limited. Mitochondrial RNase P was first examined in *Saccharomyces cerevisiae*, demonstrating the presence of a mtDNA-encoded RNA with most limited similarity to bacterial homologs, and a nucleus-encoded protein subunit (Rpm2p) that has no similarity to the bacterial P protein (Hollingsworth and Martin 1986). Mitochondrial RNase P of the ascomycete fungus *A. nidulans* has also a mtDNA-encoded RNA subunit - which is more bacteria-like than that of yeast (Lee and Engelke 1989; Lee, Lee, and Kang 1996; Martin and Lang 1997) - but has at least seven not further identified proteins (Lee et al. 1996). Surprisingly in human mitochondria, the activity is based on only three proteins, without requirement for an RNA (Rossmanith and Karwan 1998a; Holzmann et al. 2008).

1.4. Bacterial RNase P

Because of the simplicity of bacterial RNase P and the autocatalytic activity of its RNA subunit, most structural and mechanistic studies have focused on bacterial ribozymes, in particular *E. coli* and *B. subtilis* (Robertson, Altman, and Smith 1972).

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[1] According to the endosymbiont theory organelles originate from bacterial endosymbionts. The endosymbionts became increasingly dependent on their host, in particular after massive transfer of the symbiont's genes to the nucleus of its host. Mitochondria originated from α-Proteobacteria (Rickettsiales or its close free-living relatives) and chloroplasts from Cyanobacteria (Blanchard and Lynch 2000; Gray, Burger, and Lang 2001)

A protein-independent RNA subunit

In *E. coli* and *B. subtilis* RNase P, the RNA subunit is highly predominant constituting 90% of the ribozyme by weight. *In vitro*, bacterial P RNAs are active without a protein subunit, at high magnesium concentrations and without requirement for an energy source. Bacterial P RNA sequences contain five regions of primary sequence conservation (CR I to V), and a highly conserved secondary and tertiary structure (Figure 1-2) that consists of two domains: a specificity or S-domain that binds pre-tRNAs (Qin, Sosnick, and Pan 2001), and a catalytic or C-domain (Pan 1995; Loria and Pan 1996).

According to comparative analysis, bacterial RNase P structures fall into two distinct groups: the A-type (ancestral) occurs in the majority of bacterial groups, and the B-type (*Bacillus* type) primarily in low G+C gram-positive bacteria. These two prototype structures show remarkable similarity in their catalytic core (P1-P5 and P15) and joining regions (J3/4, J5/15, J15/2 and J2/4), all of which play a role in catalysis. P RNAs of both structure types are formed by coaxially stacked helical domains that, by long-range docking interactions, are joined together to create a compactly folded RNA with a flat surface, for pre-tRNA binding (Kazantsev and Pace 2006). The structural differences between type A and B might be due to differences in docking elements (Figure 1-2). Despite these structural differences between the P RNAs, they are similar enough that they can be mutually replaced *in vivo*: even a single gene copy of the *E. coli* A-type *rnpB* inserted into the chromosome of *B. subtilis* is completely functional, rescuing the growth of a non-functional endogenous *B. subtilis rnpB gene* (Wegscheid, Condon, and Hartmann 2006).

The two types of P RNA architecture come with differences in their biogenesis, biochemical/biophysical properties, and enzyme function (Haas and Brown 1998). For instance, in *B. subtilis,* the mature P RNA 5’ and 3’ ends are generated by autolytic processing *in vitro*, after binding of the P protein to the precursor P RNA (Loria and Pan 2000). In *E. coli*, RNase E is responsible for *in vivo* cleavage of the 3' end (Lundberg and Altman 1995). The type B RNase P forms a specific dimer consisting of two RNA and two protein subunits, which does not exist for type A (Fang et al. 2001) (Figure 1-2).

A sole protein subunit, not essential *in vitro*

A wide variety of bacterial P proteins (and corresponding genes) have been investigated over the years, including in *E. coli* (Hansen, Hansen, and Atlung 1985), *B. subtilis* (Ogasawara et al. 1985), *Proteus mirabilis* (Skovgaard 1990), *Micrococcus luteus* (Fujita, Yoshikawa, and Ogasawara 1990) and *Streptomyces bikiniesis* (Morse and Schmidt 1992). They all have a single, small protein subunit (named C5; ~14 kDa), which carries a large number of charged amino acids. Several roles were proposed for the C5 protein *in vivo*.

It is known that the *B. subtilis* and *E. coli* P proteins may be exchanged without significant loss of function (Guerrier-Takada et al. 1983; Waugh and Pace 1990), implying that P RNA - P protein interactions are well conserved. Considering the low primary sequence identity of RNase P proteins, the shape and surface-charge distribution remains the most important element for protein-RNA recognition. Indeed, structure-mapping experiments of A and B type P RNAs reveal that both little conserved residues and highly conserved regions of the RNA core are recognized by the proteins (Biswas et al. 2000; Sharkady and Nolan 2001; Rox et al. 2002).

Substrate Recognition

RNase P has to process complete sets of pre-tRNAs plus a few other RNA substrates in a particular cell or organelle; it must therefore be capable of recognizing a common structural feature. Experiments reveal that both protein and RNA subunits of the ribonucleo-protein are imperative for substrate recognition, *in vivo*.

In *E. coli*, mutagenesis and chemical-modification experiments show that the S domain, which includes P7 and P12 (Harris and Christian 2003), attaches to the T stem and loop of pre-tRNA (Knap, Wesolowski, and Altman 1990; Nolan, Burke, and Pace 1993; LaGrandeur et al. 1994). The catalytic domain consisting of helixes P1-P5 and P15 interacts with the NCCA motif at the 3' of pre-tRNAs, and the nucleotide at position -1 relative to the cleavage site (Kirsebom and Svard 1994; LaGrandeur et al. 1994; Oh, Frank, and Pace 1998). According to photo-crosslinking experiments, the protein subunit enhances catalytic

efficiency and alters substrate recognition by directly contacting the single-stranded 5′ leader sequence of pre-tRNA (Niranjanakumari et al. 1998) (Figure 1-3). Crosslinking and mutation analysis identified a series of conserved nucleotides located in P11, J5/15, J18/2, and in the P15–P16 internal bulge that participates in substrate binding (Kirsebom and Svard 1994; Oh and Pace 1994; Easterwood and Harvey 1997). Following phylogenetic comparisons, additional specificity elements that were previously suspected in the 5'-leader sequence of pre-tRNAs have not been identified (Chang et al. 1975; Mao, Schmidt, and Soll 1980; Schmidt et al. 1980; Engelke, Gegenheimer, and Abelson 1985). In fact, substrates with non-natural 5'-leaders (as small as one nucleotide) are processed effectively (Kline, Nishikawa, and Soll 1981; Surratt et al. 1990; Smith and Pace 1993). The 3' terminal CCA sequence found in all tRNAs, considered to be an important recognition determinant in bacterial RNase P, improves the efficiency of pre-tRNA processing by RNase P RNA. Yet, the presence of this motif it is not absolutely required. Mutations of the 3'-CCA motif merely reduce substrate binding (Km effects, (Kirsebom and Svard 1994; Oh and Pace 1994; Hardt et al. 1995)) and catalysis (K2-effects, (Oh and Pace 1994; Busch et al. 2000)). *In vivo*, protein cofactors attenuate the effect of 3'-CCA deletion (Guerrier-Takada and Altman 1984b; Oh and Pace 1994).

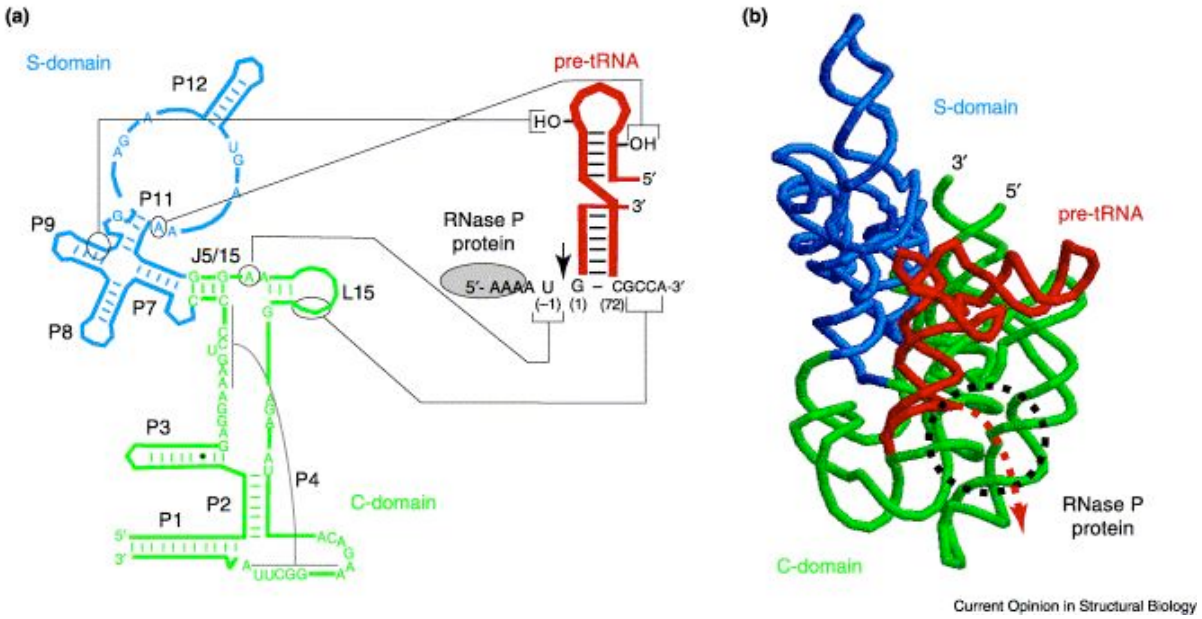

Figure 1-3- Schematic structure of RNase P in *E. coli*.

(a) Bacterial P RNA consensus secondary structure (from Pace and Brown, 1995). P designates paired helices and J junctions. The indicated nucleotides are conserved in 90% of the bacterial RNase P. The S domain is in blue and the C domain in green. Arrows indicate the cleavage sites in the tRNA substrate. The circled region is the contact site of the C5 protein with the 5′ leader sequence of the tRNA substrate.

(b) 3D structure of the RNase P/pre-tRNA complex; the dotted circle delineates the predicted position of the C5 protein. The color codes are the same as in (a). Figure taken from (Harris and Christian 2003).

1.5. Archaeal RNase P

Compared to bacterial RNase P, the archaeal ribozyme is more complex, containing several proteins. Archaea represent an evolutionarily distinct class of organisms more closely related to Eukarya than to Bacteria (Woese, et al., 1990). Only few archaeal RNase P holoenzymes have been characterized in detail, including that of the thermoacidophilic *Sulfolobus acidocaldarius* (LaGrandeur et al. 1993), the methanogen *Haloferax volcanii* (Lawrence et al. 1987) and *Methanothermobacter thermoautotrophicus* (Hall and Brown 2002).

Less complex P RNAs in Archaea

Based on phylogenetic-comparative sequence analysis, secondary structure models for archaeal P RNAs have been proposed (Haas et al. 1996; Harrier 2001). As in Bacteria, the conserved CRI-CRV elements are present in all archaeal P RNAs, suggesting their common evolutionary origin. Based on secondary structure, archaeal RNase P is categorized into type A (represented in *Pyrococcus furiosus*) and type M (*Methanococcus jannaschii*).

Catalytic domains of the archaeal type A RNA show remarkable similarity to bacterial type A P RNAs. On the contrary, type M RNA lacks the structural elements implicated in substrate binding that is otherwise typical for bacterial P RNAs (Nolan, Burke, and Pace 1993; Svard, Kagardt, and Kirsebom 1996; Tallsjo, Kufel, and Kirsebom 1996; Loria and Pan 1997). Archaeal type A P RNA (but not M) is catalytically active in the absence of proteins, however, requiring 4 M NH_4OAc and 300 mM $MgCl_2$ for activity (Pannucci et al. 1999)(Figure 1-4). The high ionic strength is likely required to stabilize the folds for substrate binding, a role taken over by proteins in type M P RNAs.

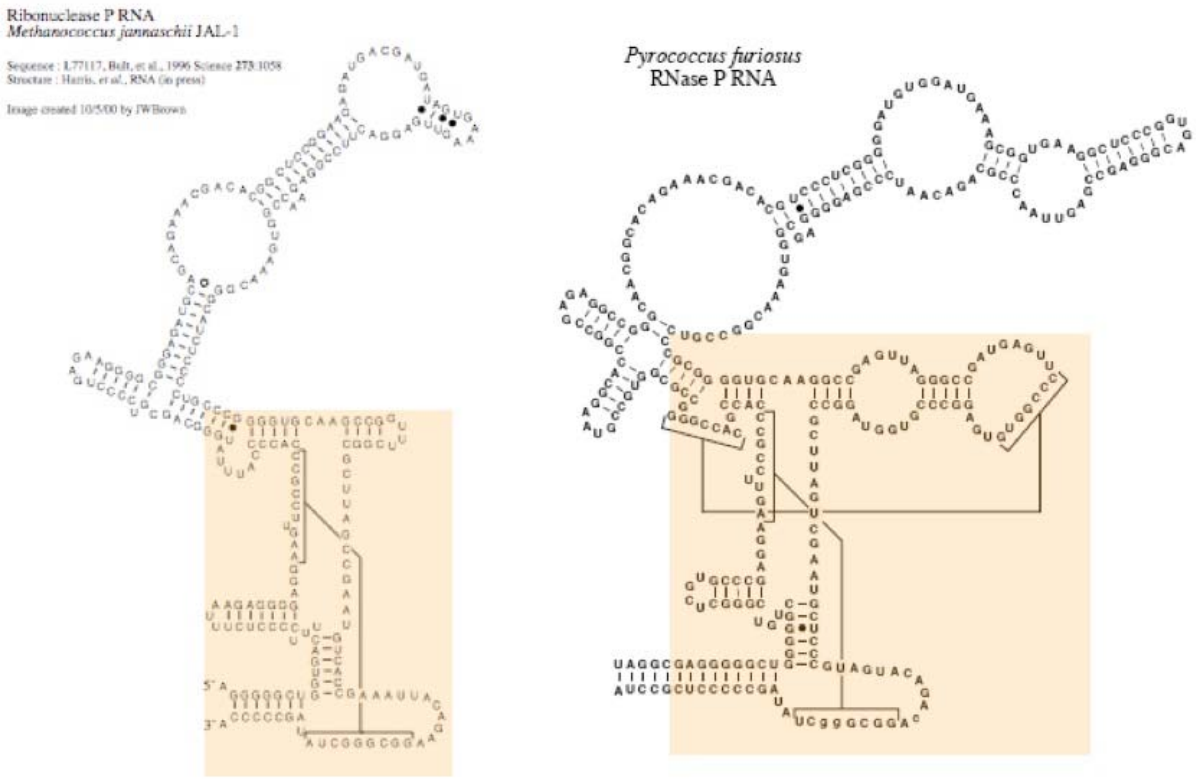

Figure 1-4- Secondary structure models of type A (*Pfu*) and M (*Mth*) archaeal P RNAs
Secondary structures were derived by phylogenetic comparative sequence analysis (Brown and Doolittle 1999). Universally conserved nucleotides in P RNAs are highlighted (adapted from the RNase P database at http://www.mbio.ncsu.edu/RNaseP/home.html).

More protein subunits in archaeal RNase P

Whereas the structure of archaeal P RNA is similar to that of Bacteria, the holoenzyme in Archaea is substantially larger due to additional proteins. Experiments show weak RNase P activity of the chimeric holoenzyme consisting of the archaeal *mth* P RNA and the bacterial P protein of *B. subtilis*, indicating that the archaeal P proteins are at least functional homologs of the bacterial one. Conversely, many archaeal P proteins are conserved and similar to the known eukaryal P proteins (Andrews et al. 2001; Hall et al. 2002; Kouzuma et al. 2003; Boomershine et al. 2003; Tsai et al. 2006). *H. volcanii* RNase P resembles that of bacteria also in term of physical properties, including a high buoyant density and sensitivity to micrococcal nuclease. In contrast, the *S. acidocaldarius* RNase P is different. Although it is sensitive to micrococcal nucleases, its buoyant density is more equal to that of proteins (Darr, Pace, and Pace 1990). In *P. horikoshii* OT3 RNase P, the five protein subunits (*Pho*Pop5, *Pho*Rpp38, *Pho*Rpp21, *Pho*Rpp29, and *Pho*Rpp30) are essential for RNase P activity (Kouzuma et al. 2003; Terada et al. 2006). P RNAs of archaea contain all of the substrate recognition elements required for catalysis but in the absence of proteins, they are structurally defective (Pannucci et al. 1999).

1.6. Eukaryotic RNase P

In eukaryotes, RNase P activity exists in the nucleus, the mitochondrion, and in photosynthetic species in plastids. Eukaryotic RNase P has been initially identified in *S. cerevisiae* (Lee and Engelke 1989), *S. pombe* (Kline, Nishikawa, and Soll 1981; Krupp et al. 1986), *Bombyx mori* (Garber and Altman 1979), chicken (Bowman and Altman 1980), *Xenopus laevis* (Carrara et al. 1989; Doria et al. 1991), beef (Akaboshi, Guerrier-Takada, and Altman 1980), rat (Jayanthi and Van Tuyle 1992) and human (Gold and Altman 1986) Bartkiewicz et al 1989). Eukaryotic RNase P is distinct in structure, size and activity, containing 7-10 protein subunits.

A reduced RNA subunit in eukaryal RNase P

Nucleus-encoded cytoplasmic P RNAs display only moderate similarity (50% between *S. pombe* and *S. cerevisiae*, and 60% between *Homo* and *Xenopus*) (Lee and Engelke 1989; Doria et al. 1991). Secondary structure models of the eukaryal P RNA developed through phylogenetic comparison conform to the bacterial consensus structure, with only slight differences (Chen and Pace 1997; Pitulle et al. 1998; Frank et al. 2000; Marquez et al. 2006). Typically, eukaryotic P RNA has only two-third the length of bacterial P RNA. Among eukaryotes, P RNA sequence conservation is low, but the same distinct block of conserved bacterial and archaeal regions (CR I-V) are clearly discernable. Although most eukaryal RNase P require both RNA and protein subunits for activity, several P RNAs are capable of binding tRNAs specifically and independent of proteins, although with low affinity. It seems that the eukaryal P RNAs hold enough information to fold into a structure similar to that in Bacteria, without the help of proteins (Marquez S,M, 2006). Recent studies reveal that under special conditions, P RNAs from humans and *Giardia lamblia* mediate cleavage of tRNA precursors in the absence of proteins (Kikovska et al. 2006).

Numerous protein subunits in eukaryal RNase P

Unlike bacterial RNase P, RNase P holoenzymes from eukaryal sources are enriched in proteins (70% by mass, compared to 45% and 10% for archaeal and bacterial RNase P holoenzymes, respectively). *S. cerevisiae* nuclear RNase P consists of one RNA (RPR1) and nine essential protein subunits (Pop1p,Pop3p, Pop4p,Rpp1p,Pop5p, Pop6p, Pop7p and Pop8p) with molecular masses ranging from 15.5 to 100.5 kDa (Chamberlain et al. 1998). Pop5p, Pop6p, Pop7p and Pop8p subunits are shared between RNase P and the mitochondrial RNase processing ribozyme (MRP). RNase MRP is a ribonucleoprotein endo-ribonuclease containing RNA with similar sequence and structure to RNase P. It is required for rRNA precursor cleavage in nucleoli (Chang and Clayton 1987a; Chang and Clayton 1987b; Shuai and Warner 1991; Schmitt and Clayton 1993; Lygerou et al. 1996), and is implicated in mitochondrial replication (Stohl and Clayton 1992). Subunits Pop1p, Pop3p, Pop4p, Rpp1p are specific proteins of RNase P. Although the protein subunits are essential for catalytic

activity of the enzyme, little is known about the function of individual protein subunits of the cytoplasmic RNase P in yeast. None of the proteins have recognizable similarity with either 14 kDa bacterial C5 proteins, or the 105 kDa mitochondrial P protein (Dang and Martin 1993).

Purified human RNase P from HeLa cells contains 10 polypeptides designated Rpp14, Rpp20, Rpp21, Rpp25, Rpp29, Rpp30, Rpp38, Rpp40, hPop5, and hPop1. They constitute the core structure of the holoenzyme (Lygerou et al. 1996; Eder et al. 1997; Jarrous et al. 1998; Jarrous et al. 1999; van Eenennaam, Pruijn, and van Venrooij 1999; Jarrous et al. 2001; van Eenennaam et al. 2001a; van Eenennaam et al. 2001b). Six of the protein subunits of human RNase P (Rpp20, Rpp21, Rpp29, Rpp30, Rpp38, hPop5,) show a moderate similarity with *S. cerevisiae* P proteins (Koonin, Wolf, and Aravind 2001; Jarrous 2002). Moreover, conservation of several human Rpp proteins, such as Rpp21, Rpp29, Rpp30, and hPop5 in Archaea suggests that these subunits have essential roles in RNase P function, further supporting the idea of an archaeal-related origin of the eukaryotic nuclear RNA processing machinery (Altman, Gopalan, and Vioque 2000) (Figure 1-5). Another eukaryotic RNase P is purified from a filamentous ascomycete, *Aspergillus nidulans*. *A. nidulans* is an important model organism for studying eukaryotic cell biology. A 2150-fold purification of nuclear RNase P was achieved with a yield of 2.3%, by combining five chromatography steps. The 580 kDa complex contains seven polypeptides (125, 85, 45, 33, 30, 21, 19 kDa) that consistently co-purify with nuclear RNase P activity and its RNA subunit (Han, Lee, and Kang 1998).

Various roles such as substrate binding, localization, RNA binding and catalysis were suggested for protein subunits of human RNase P. Several of them physically interact with precursor tRNA (True and Celander 1998). Although human RNase P does not require ATP or GTP to be active, the subunit Rpp20 is an ATPase (Li and Altman 2001) with a yet unknown role.

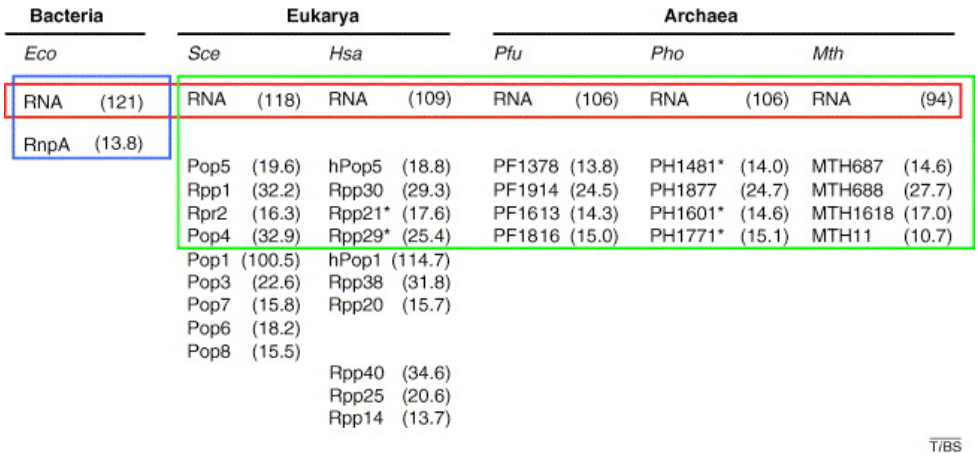

Figure 1-5- Comparison of RNase P holoenzymes in the three domains of life
Homologous components of the RNase P holoenzyme from *E. coli (Eco), S. cerevisiae (Sce), Homo sapiens (Hsa)*, Pyrococcus furiosus (*Pfu), Pyrococcus horikoshii (Pho)* and *Methanothermobacter thermoautotrophicus (Mth*) are aligned horizontally. The molecular mass (in kDa) of each subunit is indicated in parentheses. The green box highlights the minimal holoenzyme composition, shared before the divergence of the Archaea and Eukarya, the RNA subunit and at least four of the protein subunits. Figure taken from (Evans, Marquez, and Pace 2006).

Substrate recognition by eukaryal RNase P

Little is known about substrate recognition for eukaryotic RNase P. In contrast to *E. coli*, the 3' CCA is not encoded in the eukaryotic tRNA genes, but rather is added after pre-tRNA processing. Thus, the 3'-CCA is not likely recognized by eukaryotic RNase P (Chen and Pace 1997). In human, the minimum substrate for RNase P, must contain the T stem and T loop (Yuan and Altman 1995) in addition to acceptor stem and 5' leader, which makes it more specific for substrate cleavage than bacterial RNase P. This explains why several non-tRNA substrates are cleaved by RNase P of Bacteria but not Eukarya (McClain, Guerrier-Takada, and Altman 1987; Forster and Altman 1990).

1.7. Organelle RNase P

Specific RNase P activities were observed in organelles that should make them bacteria-like according to the endosymbiont theory on their origin: α-proteobacterial for mitochondria and cyanobacterial for chloroplasts (Margulis 1970; Gray and Spencer 1996; Gray, Burger, and Lang 1999; Lang, Gray, and Burger 1999). MtRNase P activity has been examined in rat liver (Manam and Van Tuyle 1987), *S. cerevisiae* (Hollingsworth and Martin 1986; Morales et al. 1989; Dang and Martin 1993), *A. nidulans* (Lee et al. 1996), *Trypanosoma brucei* (Salavati, Panigrahi, and Stuart 2001), potato (Marchfelder and Brennicke 1994), *Oenothera berteriana* (Marchfelder, Schuster, and Brennicke 1990) and human (Doersen et al. 1985; Rossmanith and Karwan 1998a; Holzmann et al. 2008). Only little is known about the protein subunits of these various mitochondrial RNase P complexes. Judging from the mtP RNA structures (if present at all) that are highly derived and inactive in the absence of proteins (Miller and Martin 1983; Morales et al. 1989; Morales et al. 1992; Baum, Cordier, and Schon 1996), mtP proteins may be as derived and difficult to recognize.

1.7.1.1. **Chloroplast RNase P**

Chloroplast RNase P is expected to be similar to that of Cyanobacteria. Notwithstanding, a protein-only RNase P has been reported in spinach chloroplast that resists micrococcal nuclease likely because it apparently lacks an RNA component (Wang, Davis, and Gegenheimer 1988; Thomas et al. 1995; Thomas et al. 2000). Spinach chloroplast RNase P contains an unknown number of protein subunit(s) of about 70 +/- 5 kDa (Thomas et al. 2000).

1.7.1.2. Mitochondrial RNase P

Mitochondrial RNase P activity has been found in HeLa cells (Dorsen et al. 1985), rat liver (Manam and Van Tuyle 1987), wheat (Hanic-Joyce and Gray 1990), various yeasts such as *S. cerevisiae* (Hollingsworth and Martin 1986; Morales et al. 1989), *Saccharomycopsis fibuligera*, (Wise and Martin 1991a), *Kluyveromyces lactis* (Wilson, Ragnini, and Fukuhara 1989), *Saccharomyces exiguus*(Wise and Martin 1991b), *Saccharomyces douglasii* (Ragnini et al. 1991), *Saccharomyces chevalieri, Saccharomyces ellipsoideous, Saccharomyces diastaticus*, (Sbisa et al. 1996), *Saccharomyces castellii*, (Petersen et al. 2002), the protist *Reclinomonas americana* (Lang et al. 1997), the prasinophyte green alga *N. olivacea* (Turmel et al. 1999), *Trypanosoma brucei* (Salavati, Panigrahi, and Stuart 2001), potato (Marchfelder and Brennicke 1994), carrot (Franklin, Zwick, and Johnson 1995), *A. nidulans* (Lee et al. 1996) and Human (Holzmann et al. 2008) is different from nuclear RNase. Interestingly mitochondrial RNase P that faithfully cleaves mitochondrial tRNA precursors, does not process *E. coli* pre-tRNA$^{Tyrsu3+}$ (Rossmanith and Karwan 1998b). Moreover, nuclear RNase P, which is capable of cleaving pre-tRNA$^{Tyrsu3+}$, does not cleave mitochondrial pre-tRNA^{Tyr} (Rossmanith and Karwan 1998b). The same principle of difference in substrate specificity was used for distinction of nuclear and mitochondrial RNase P with pre-tRNA^{His} (Lee et al. 1996).

Initially, mtRNase P has been studied in *S. cerevisiae*, subsequent to the finding that its RNA subunit is mtDNA-encoded and that mutation in the respective gene were defective in tRNA processing (Miller and Martin 1983; Underbrink-Lyon et al. 1983). Following

purification of the activity, a large nuclear-encoded 105-kDa P protein subunit (*RPM2*) was identified, which is required for mtRNase P activity (Morales et al. 1992; Dang and Martin 1993). Sequence analysis has further identified *rnpB* genes in mtDNAs of several additional budding yeasts, *Reclinomonas americana* (Lang et al. 1997), the prasinophyte green alga *N. olivacea* (Turmel et al. 1999), *S. pombe* (Paluh and Clayton 1996), and *S. ocotosporus* (Seif et al. 2003). The yeast and fission yeast *rnpB* gene sequences are highly A+T rich and vary drastically in size (423 nt for *S. cerevisiae* (Morales et al. 1989), 227 nt for *Torulopsis glabrata* (Shu et al. 1991)), and as short as 140 nt for *Saccharomycopsis fibuligera* (Wise and Martin 1991b), make it difficult to predict this secondary structure. The gene appears to be absent from mtDNAs of land plants, animals, a great number of protists and non-ascomycete fungi (Lang, Gray, and Burger 1999).

Purification of mtRNase P in *A. nidulans* resulted in a not further characterized 232 kDa ribozyme consisting of at least seven polypeptides and a mtDNA-encoded RNA subunit (Lee et al. 1996). 5' endonuclease activity was also found in wheat (Hanic-Joyce and Gray 1990). In potato an RNA moiety with three proteins are detectable in fractions with RNase P activity. Since their abundances do not closely correlate with activity, it remains unclear whether any of these three proteins are constitutive for RNase P activity.

Studies in plant tRNA processing uncovered an active RNase P in mitochondria. Whether the plant mitochondrial RNase P contains essential RNA and protein components is not yet clear. In mitochondrial RNase P, the cage-shaped pseudoknot (pairings P1–P4) known as the basic structure of P RNA is the only conserved sequence in some cases (Seif et al. 2003). Among all P RNAs the mt-P RNA are the most divergent, as they are vary in both size and secondary structure (Figure 1-6).

Recent studies demonstrated that the human mtRNase P is a protein-only enzyme composed of at least three protein subunits, which are all essential for RNase P activity (Holzmann et al. 2008). MRPP1, an RNA (guanine-9-) methyltransferase, is proposed to provide a tRNA-binding specificity to RNase P enzyme. The role of the MRPP2, a short-

chain dehydrogenase/reductase that binds tightly to MRPP1, is not clear. MRPP3 with putative metallonuclease and RNA-binding domains is not stables associated with the other two proteins, suggested to provide the enzymatic activity. These MRPP proteins have no similarity to any of the known nuclear RNase P protein subunits (Holzmann et al. 2008). This can be considered as another fact on the evolution of RNA-based enzymes to a more complex enzyme containing numerous proteins that are playing the role of catalytic RNA.

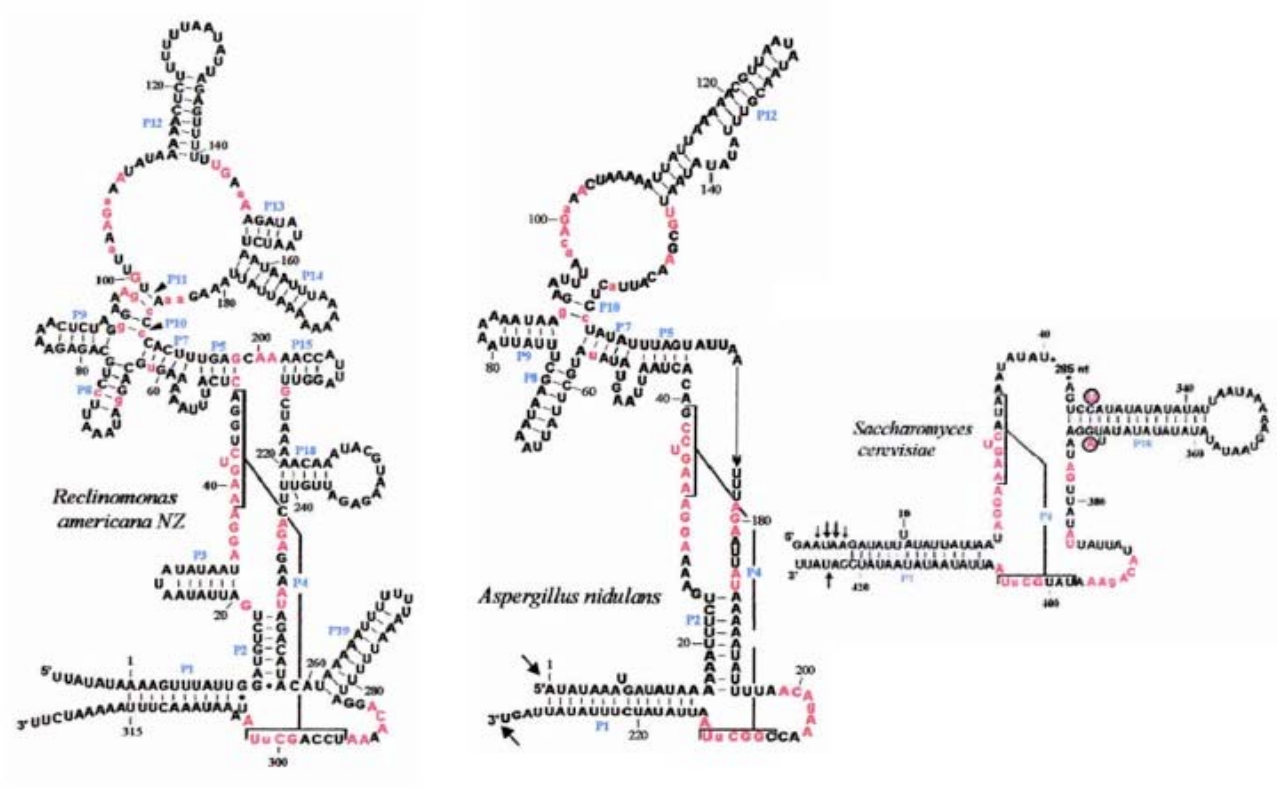

Figure 1-6- Schematic mtP RNA secondary structure models of *Reclinomonas americana*, *A. nidulans and S. cerevisiae.* The sequences in red are conserved in the minimum bacterial consensus structure. Figure taken form (Seif et al 2003).

1.8. Purification Methods for mitochondrial RNase P

Many standard enzymatic purification procedures perform solubilization of cellular components by detergents, dissociation of sulfide bonds by 2BME or DTT, and 'stabilization' by EDTA. These substances may all cause dissociation of protein complexes; in the case of EDTA by "sequestering" metal ions such as Mg^{2+}, $Ca^{2+,}$ and Fe^{3+}. In addition, unphysiologically high salt concentrations are used for precipitation of enzymes and for their elution from chromatographic columns, which may also disrupt protein interactions. The characterization of enzymes that were purified this way may provide valuable biochemical information, including the minimum number of polypeptides that constitute an active enzyme, and its structure. Yet, the applied procedures may introduce structural artifacts, in particular dissociation of large complexes, and change in enzymatic properties.

In the case of mitochondrial RNase P, the available purification protocols also include detergents, various chemicals, heat shock, ammonium sulfate precipitation, and numerous separation steps in the presence of high salt concentrations. For instance, purification of *A. nidulans* mtRNase P from a whole cell lysate (starting from 2.5 kg of *Aspergillus* mycelia) resulted in a more than 4000 fold enriched enzyme, yet at a yield of only 5%. This ribozyme consists of at least seven not further identified polypeptides (~55, 41, 40, 26, 24, 18 and 16 kDa), and numerous small pieces of an mtDNA-encoded 232 nt RNA that fold into a P RNA structure (Lee et al. 1996; Lee, Lee, and Kang 1996). That this preparation is in fact both homogenous and reflecting the structure of the native ribozyme remains questionable. The P RNA which carries the active center of the ribozyme is not only heavily degraded, but the reduction in the number of protein subunits leads to a reduction of the specific activity in some purification steps. This suggests that the ribozyme complex is labile under the given conditions, casting doubts on the 100% reference value that was used for calculation of enzyme yields, and suggesting an even lower yield than just 5%.

Similar purification procedures were used in yeast, ultimately resulting in a fraction containing a prominent 105 kDa protein (yet several minor protein 'contaminants' remain), and small pieces of the mtP RNA subunit (Morales et al. 1992). Most strikingly, the

identified P RNA pieces lack sequences required for the active ribozyme center of the RNA (i.e., the core sequence including the P4 helix). Therefore it remains questionable whether the measured RNase P activity is due to the prominent 105 kDa protein combined with small P RNA pieces or the minor proteins that were interpreted as contaminants. As in *A. nidulans*, the yield of purified enzymatic activity is marginal, and the 100% value for calculating ribozyme yields is as questionable. Taken together, results from these two systems suggest that mtRNase P is part of a relatively large, labile RNA-protein complex – as yet of unknown size.

The case of protein-only RNase P in human mitochondria

Recent studies on human mitochondrial RNase P reveal that it is a protein-only activity, and that its purification to homogeneity will be challenging if not impossible (Holzmann et al 2008). Initial purification attempts were unsuccessful due to the rapid loss of activity and poor recovery. Therefore, the authors used a combinatorial purification/proteomics approach called partial proteome overlap purification (PPOP). PPOP is based on only partial purification of the activity with a variety of purification steps, and the identification of protein subunits in active fractions by mass spectrometry. A comparison of all results led to the identification of common protein subunits. From these, three candidate P proteins were chosen according to their predicted function for in vitro reconstitution studies of RNase P activity. It turns out that human mtRNase P is composed of a minimum of only three proteins (a tRNA methyltransferase, a short-chain dehydrogenase/reductase family member, and a protein of hitherto unknown functional and evolutionary origin, possibly representing the enzyme's metallonuclease moiety), but that these three proteins are integrated into large protein complexes of varying size (Figure 1-8) (Holzmann et al. 2008; Walker and Engelke 2008).

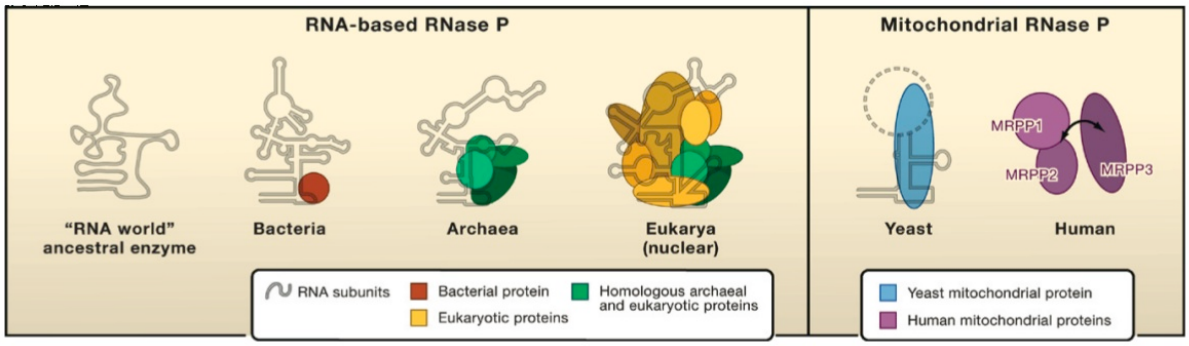

Figure 1-7- Evolution of RNase P

Left, increase in protein subunits of the yet known RNA-based RNase P of Bacteria, Archaea and Eukarya. **Right**, the minimum known protein subunits for an active mtRNase P in *S. cerevisiae* (with the only known protein subunit and a partially identified RNA subunit) and *H. sapiens* (three protein subunits and no RNA). The arrow indicates that MRPP3 binds to the two-protein sub-complex only weakly or dynamically. Figure taken from (Walker and Engelke 2008).

1.9. Rational, Working Hypotheses and Objectives of the Project

The published literature on the analysis and purification of mitochondrial RNase P (Morales et al. 1989; Marchfelder and Brennicke 1994; Marchfelder 1995; Lee et al. 1996) does not address two questions of central importance: (i) if its activity is a soluble constituent of the mitochondrial matrix that may be extracted without the use of detergents (as its ancestral, bacterial form (Stark et al. 1978)); or if it is instead tightly associated with mitochondrial membranes (a view more consistent with experiments in human (Holzmann et al. 2008)), or if it may be either soluble or membrane-bound, depending on the species; (ii) related to this question, in species that have a demonstrated mtP RNA subunit (yeast, *A. nidulans*), its quantification in membranes *versus* inner mitosol has not been reported, although quantitative tracing of P RNA would be straightforward. In fact, the quantity of RNAs in the cellular starting material (i.e., the 100% value for tracing) is not only a valid quantitative marker, but easily determined in the presence of potent RNA inhibitors. In contrast, quantification of the activity may only start at most advanced stages of purification, when the bulk of unspecific RNase activities are removed. It is therefore entirely possible that the reported purified ribozymes in yeast and *A. nidulans* are not at all representative of the native level of activity, a view that is consistent with the reported extremely low yields. For instance in *A. nidulans*, 2.5 kg wet weight cells from 150-l culture had to be used to purify sufficient mtP RNA for identification of its P RNA subunit by gel electrophoresis; indeed, the yield was not only low but most of the RNA turned out to be degraded (Lee et al. 1996). The situation is similar in yeast, where the P RNA is not only heavily degraded, but a region constituting part of is active center was also not recovered from the purified fraction (Morales et al. 1989).

To address these questions, we have undertaken experiments to purify the *A. nidulans* mtRNase P, based on mild procedures with and without the use of detergents, and to quantitatively trace its RNA subunit by RT PCR experiments – either in soluble or membrane fractions.

CHAPTER 2: MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Media and solutions

Complete medium (for one liter): 2% salt solution, 1% vitamin solution, 1.5% casamino acid (Sigma), 1% glucose (BDH Chemical), 2% peptone (DIFCO Laboratories), 1% yeast extract (Becton, Dickinson and Company), 1.2% agar (Roch).

Salt solution (for one liter): potassium chloride (26 g) (Fisher Scientific), magnesium sulphate 7 H_2O (26 g) (American Chemicals (A&C)), potassium dihydrogen phosphate (76 g)(Biopharm), trace-elements solution (50 ml).

Trace element solution (for one liter): sodium borate 10 H_2O (40 mg) (AnalaR), copper sulphate 5 H_2O (400 mg) (International Biotechnologies Inc. (IBI)), ferric phosphate 2 H_2O (800 mg) (J.T. Baker Chemical), manganese sulphate 2 H_2O (800 mg) (A&C), sodium molybdate 2 H_2O (800 mg) (Fisher Scientific), zinc sulphate (Biopharm) (8 mg).

Vitamin solution (for one liter): thiamin (50 mg), biotin (10 mg), nicotinic acid (100 mg), calcium D-pantothenate (200 mg), pyridoxine HCl (50 mg), riboflavin (100 mg), all were obtained from Sigma Chemical Company.

Standard growth medium (liquid): 0.5% yeast extract, pH adjusted to 5.8 with KH_2PO_4(Fisher), 3% glycerol (Bioshop) and 0.5% ethanol (Bioshop).

Mitochondrial matrix extraction buffer (buffer A): 50 mM bis-tris/HCl (Bioshop) pH: 7.5, 100 mM NH_4Cl (Biopharm), 10 mM $MgCl_2$ (Bioshop), 10% glycerol.

RNase P activity reaction buffer (PA buffer): 10 mM tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM NH_4Cl. To stop RNase P reactions before loading them on gels.

Stop buffer is added (95% formamide (sealed under N2) (Fisher), 0.05% bromophenol blue (IBI), 0.05% xylene cyanole (IBI).

Polyacrylamide gel for RNase P assays: 7% of Acrylamide/bis-acrylamide 29:1, 40% (Bioshop), 7 M urea (Sigma), 3.9% TBE 20x, 0.8% ammonium persulfate (AnalaR), 0.05% TEMED (Invitrogen).

BN-PAGE and BNC gel: Roche acrylamide ultra-pure and BioRad bis-N,N-methylene bis-acrylamide (as a weight-to-weight ratio of 32:1) were dissolved in water, to obtain a 49.5% stock solution.

BN-PAGE buffers:

- Cathode buffer, pH: 7.0 at 4 °C, 500 mM tricine (Bioshop), 150 mM bis-tris, with or without 0.2% Coomassie Blue G-250
- Anode buffer, pH: 7.0 at 4 °C, 500 mM bis-tris
- 3X Gel buffer, pH: 7.0 at 4 °C, 1.5 M aminocaproic acid (ACA) 750 mM (Sigma), 150 mM bis-tris
- Coomassie Blue G-250 5%, 750 mM, ACA 750 mM solution

Gel staining buffers:

- Fixation buffer: 10% ethanol (Bioshop), 5% acetic acid (Bioshop)
- Destaining buffer: 40% methanol (Fluka), 10%, acetic acid
- Coomassie Blue G-250 coloration buffer: 50% acetic acid, 50% methanol, 500 mg Coomassie Blue G-250.

2.2. Cell culture, mitochondrial isolation, and extraction of soluble matrix proteins

A. nidulans, FGSC 4, Glasgow wild type, was grown on complete medium for 72 hours by shaking (100 rpm) at room temperature. The mycelia were disrupted with an equal mixture of 20-30 and 50-70 mesh size white sand (Sigma) using a mortar and pestle, and the fragmented cell material was extracted from the sand by repeated washing with 0.6 M D-sorbitol

(Bioshop). The combined solutions were loaded on sucrose step gradients (60% (7ml) -32% (7 ml) -23% (5 ml), 15% (5 ml)) and centrifuged for 90 min at 25 000 rpm at 4 °C (mitochondria will move to the layer between 60-32% (Figure 2-1)). The mitochondrial band was collected, mixed with about 4 times its volume 80% sucrose (~5ml), layered below a second step gradient (60% (7ml), 32% (7ml), 15% (4ml) sucrose), and centrifuged for a second round. Intact mitochondria will float to the interface between 32 and 60% sucrose. After dilution of the mitochondrial fraction with 5% sucrose solution and centrifugation at 15000 rpm for 15 min, the mitochondrial pellet was kept frozen at -80^0 C until use.

Soluble matrix proteins are extracted from the highly purified mitochondria by mechanical disruption with an equal ratio mixture of 125-212 micron and 425-600 micron glass beads (Sigma) in matrix extraction buffer (buffer A), with and without the use of detergents (1% Triton X-100 or 0.3 M digitonin). The soluble fraction (after centrifugation at 4000 rpm for 30 min) was concentrated with an Amicon Ultra centrifuge filter (50k MWCO) (Millipore), and the protein concentration of the matrix extract was determined with a BioRad protein assay kit.

2.3. RNase P activity assays

The RNase P substrate (pre-tRNA proline) was produced from the respective mitochondrial *R. americana* gene plus 15 nt of its upstream leader sequence. The gene was cloned in the vector pFBS/EcoRV (2.9 kb), from which it was PCR-amplified using primers 5'-GAAATTAATACGACTCACTATAGGGCTAGTA-3' and 5'-TCACTAAAGGGAACAAAAGCTGGGT-3'. The amplified DNA was end-repaired with 4 u/µl Klenow (Roche) and T7 DNA polymerase (Invitrogen) for 30 min at 12°C, and the reaction was stopped with EDTA (5 mM final concentration) by incubating for 10 min at 65°C. The DNA was then purified from 1.2% low-melting agarose gels (after a run for one hour at 80 V), by cutting out bands of interest, freezing them at -20°C for 30 min, and centrifuging twice (14000 rpm) for 15 min at room temperature. The DNA was recovered from the liquid supernatants, checked on an agarose gel, and stored in frozen aliquots for *in*

vitro transcription of the RNA. *In vitro* transcription was performed in the presence of [α^{32} P] ATP (10 mCi/ml)(PerkinElmers),with 2 μg of DNA and Invitrogen T7 RNA polymerase (2 u/μl), following the supplied protocol. The labeled 117 nt pre-tRNA was separated by agarose electrophoresis, and the band cut out and incubated in 1% SDS at 37 °C overnight. After phenol/chloroform extraction and ethanol precipitation, the pre-tRNA was ready for mtRNase P assays, in PA buffer with 100 mM added $MgCl_2$ for 15 min. RNase P will cleave the labeled pre-tRNA (117 nt) into a mature tRNA (78 nt) and a 5' leader sequence (39 nt). Reactions were stopped with stop-buffer and loaded on 7% polyacrylamide gels, and run for 4 hours at 200V at room temperature. The BioRad PROTEIN II 1-D electrophoresis system was cooled by water circulation. The 7% electrophoresis gels were then exposed to a Kodak film overnight.

The M1 RNA for the positive control was produced by amplification of the *E. coli rnpB* gene with primers 5'GAAATTAATACGACTCACTATAGGGAAGCTGACCAGACAGTCGC 3' and 5'AGGTGAAACTGACCGATAAGCC 3'. The PCR product was purified, transcribed overnight at 37 °C *in vitro* with T7 RNA polymerase (Invitrogen), and purified with a G-50 spin colum

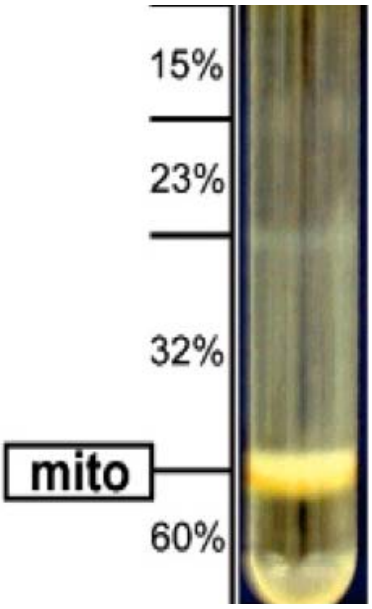

Figure 2-1– Isolation of mitochondria by sucrose gradient centrifugation.
The sucrose step gradient is formed by layering four sucrose solution of different density (15% (5ml), 22% (5ml), 32% (7ml) and 60 % (7ml)) in Beckman ultracentrifuge tubes (25 x 89 mm). The disrupted mycelia is loaded on top of the gradient, and after centrifugation for 90 min at 25 000 rpm at 4 °C, the mitochondrial fraction can be found on top of the 60% layer at which its density matches with surrounding sucrose. The mitochondrial fraction was harvested from the first gradient, and then brought up to higher sucrose concentration for the second flotation gradient.

2.4. Blue Native PAGE separation

Gradient Blue Native PolyAcrylamide Gel Electrophoresis (4-13%) was performed as previously published (Schagger and von Jagow 1991; Schagger 1995; Wittig, Braun, and Schagger 2006; Wittig and Schagger 2007). 6% Coomassie Blue G-250 was added to the samples (matrix extracts) immediately before loading them on the gel, which was run at 60 V, overnight, in a cold room. Gels were run in Hoefer (18 x 16 cm) electrophoresis chambers, or in BioRad Econo-Glass Columns (0.7 x 10 cm).

After electrophoresis, gels were either stained with Coomassie Blue G-250 and/or silver nitrate, depending on the expected intensity of proteins bands (silver staining is most sensitive in protein staining). The silver staining procedure followed instructions provided by the BioRad silver stain kit. For Coomassie Blue G-250 staining (which binds preferentially to basic and hydrophobic residues of proteins), the gel is incubated first in fixation buffer (30 min.), and then for four hours in Coomassie Blue G-250 staining buffer under slow agitation (~50 rpm). The gel was destained with destaining buffer until the desired band resolution was obtained.

To test RNase P activities of protein bands in gels, the respective zones (0.5 cm wide) were cut out and electro-eluted in GeBAflex- midi tubes in electrophoresis tank extraction buffer, for an hour (90 V) at room temperature.

2.5. Preparative Blue Native Column Electrophoresis (BNC)

Blue native column separation was developed in our lab to permit large scale purification of protein complexes. It is based on the regular BN-PAGE protocol, using a cylindrical running chamber filled with polyacrylamide gel (e.g., 7%; (Figure 2-2)). The mitochondrial extracts (~500 µg) mixed with 6% of Coomassie Blue G-250 were separated at constant voltage (120 V), using the same electrophoresis buffers as in regular BN-PAGE. Samples were collected every hour from a 200 µl dialysis-cup placed at the outlet of the column, and kept frozen at -80° C for RNase P activity tests and protein identification by mass spectrometry.

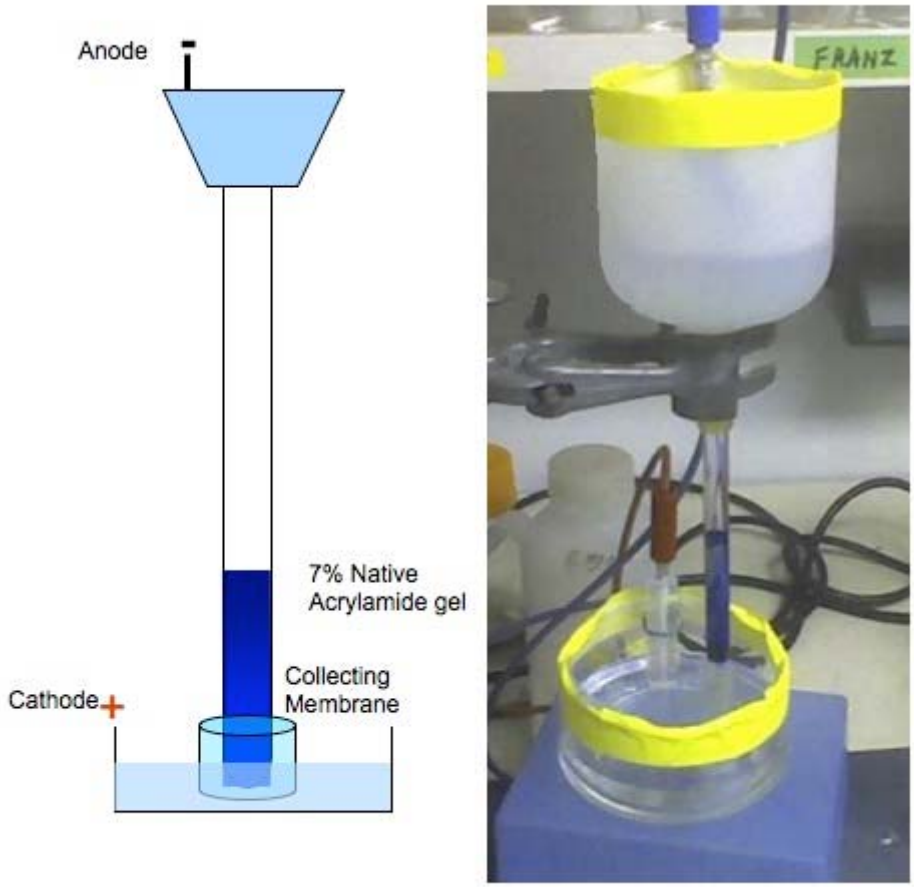

Figure 2-2- Preparative Blue Native Column: BNC is a purification method developed in our lab. The polyacrylamide gel (desired percentage and height) is polymerized inside the column. The tank at the top of the column (anode) contains cathode buffer and the bottom tank anode buffer. The electrodes are connected to a power system at 120V. The sample containing 6% Coomassie Blue G-250 is loaded with a Pasteur pipette and samples are collected from the dialysis cup at regular intervals.

2.6. Identification of mtP RNA by RT-PCR

RNA samples were purified using the RNeasy Plus kit by QIAGEN and RT-PCR assays were performed on 10 ng total RNA with AMV reverse transcriptase (cDNA synthesis), followed by PCR amplification with the Expand High Fidelity PCR system containing Taq polymerase and Tgo polymerase (Roch). The PCR primer sequences of the *A. nidulans* mtRNase P were 5' GGAAAGTCCGACACTAATTAAG 3' and 5' GTCTTTGGCCGAATTAATATAG 3', with annealing temperature of 55 °C for 35 cycles, which amplifies the P2, P4, P5 and P7 regions of the P RNA. The expected PCR product had a length of 117 nt.

2.7. Protein identification by mass spectrometry

To identify protein subunits of given fractions, Liquid Chromatography / tandem Mass Spectrometry (LC-MS/MS) (tandem MS to derive the sequence of individual peptides) was performed by a service at the Institute for Research in Immunology and Cancer (IRIC), including functional annotation by Mascot (Perkins et al. 1999). For sample preparation, proteins were TCA-precipitated and re-dissolved in ammonium bicarbonate plus TCEP, and digested with trypsin overnight at 37 °C. The samples were then dried in a SpeedVac and re-dissolved in ACN / FA. Twenty-five µl of each samples were injected into a C18 pre-column (0.3 mm i.d. x 5 mm) and separated on a C18 analytical column (150 µm i.d. x 100 mm). Each full MS spectrum (generated by -Orbitrap mass spectrometer (Thermo Fisher)) was followed by three MS/MS spectra (four scan events), where the three most abundant multiply charged ions were selected for MS/MS sequencing. The data were analyzed using the Mascot 2.1 (Matrix Science) search engine.

Functionally unidentified (hypothetical) proteins were verified with PSI-BLAST for matches to known proteins in reference databases. PSI-BLAST is a tool that finds distant relatives of a protein from a multiple alignment of the top-scoring BLAST results. The iterative PSI-BLASTing was continued until the best E-value was achieved.

CHAPTER 3: RESULTS

3. RESULTS

In this section, we will present results for the localization of mitochondrial active RNase P in soluble *versus* membrane fractions, and the enrichment of the ribozyme with different procedures. To select an effective purification protocol for *A. nidulans* RNase P that uses a minimum number of steps, and to avoid as many chemicals and conditions known to dissociate protein complexes as possible, a variety of methods was initially tested. These include kinetic centrifugation (sucrose or glycerol gradients), RNA affinity, Mono Q, and Mono S columns, and separations by BN-PAGE. Following initial results, the most promising means of purification is Blue Native preparative polyacrylamide gel Column electrophoresis (BNC) (Figure 2-2). BNC is based on a similar principle as the BN-PAGE procedure published by others (Wittig and Schagger 2008), with the difference that a large preparative column with a continuous polyacrylamide gel is used for separation, and that fractions are continuously recuperated in a dialysis cup as they leave the column (see Material and Methods, Chapter 2). These fractions are then monitored for RNase P activity (cleavage of a radioactively labeled tRNA precursor), and active fractions are analyzed for their protein subunit composition by mass spectrometry.

3.1. RNase P purification from *A. nidulans* mitochondria, without use of detergents

Contrary to results in yeast (R. Daoud, unpublished results), the yield of soluble mitochondrial matrix proteins is extremely low in *A. nidulans* (~3.5 mg protein from ~0.8 g wet mitochondria, typically isolated from ~100 g wet weight mycelia). This indicates that mitochondrial proteins of *A. nidulans* are in general more tightly integrated with mitochondrial membranes. To investigate the extent to which this also applies to mtRNase P, soluble mitochondrial extracts were separated on 7 % BNC, 200V, and 500 µl fractions were collected every hour during a period of 12 hours (for details, see Material and Methods). RNase P assays clearly indicate the presence of activity in fractions 7 to 10 (Figure 3-1). Yet, non-specific RNases co-migrating with RNase P degrade the substrate and cleavage products to an extent that the amount of mature tRNA produced by RNase P is difficult to

estimate. Unexpectedly, the A+T rich 5' leader sequence is more resistant to this unspecific degradation than the tRNA itself, a property that might be used for its identification. To identify proteins in the active fractions (7-10) they were pooled, and analyzed by mass spectrometry. Based on LC/MS/MS, twenty proteins were identified (Table S-1). Surprisingly, seventeen of these proteins do not have a reported function and are annotated in GenBank as 'hypothetical protein', and the three identified proteins have no evident function related to RNase P activity. These include (i) citrate synthase, an enzyme that condensates AcCoA with OAA to form citrate and CoA, the first step of the citric acid cycle; and (ii) the soluble alpha and (iii) beta chains of mitochondrial ATP synthase. Taking previously published results into account, only three proteins were found (hypothetical proteins of 28 and 39 kDa, and the 55 kDa isocitrate dehydrogenase) that have a molecular mass close to those previously reported for mtRNase P (55, 41, 40, 26, 24, 18 and 16 kDa) (Lee et al. 1996). However, Lee et al. estimated the molecular size of their polypeptides according to a standard molecular mass marker in the gel, which might slightly deviate from theoretical calculations based on sequence data (which is reported in the mass spectrometry analyses). It is therefore possible that more proteins are identical in the published *versus* our RNase P purifications.

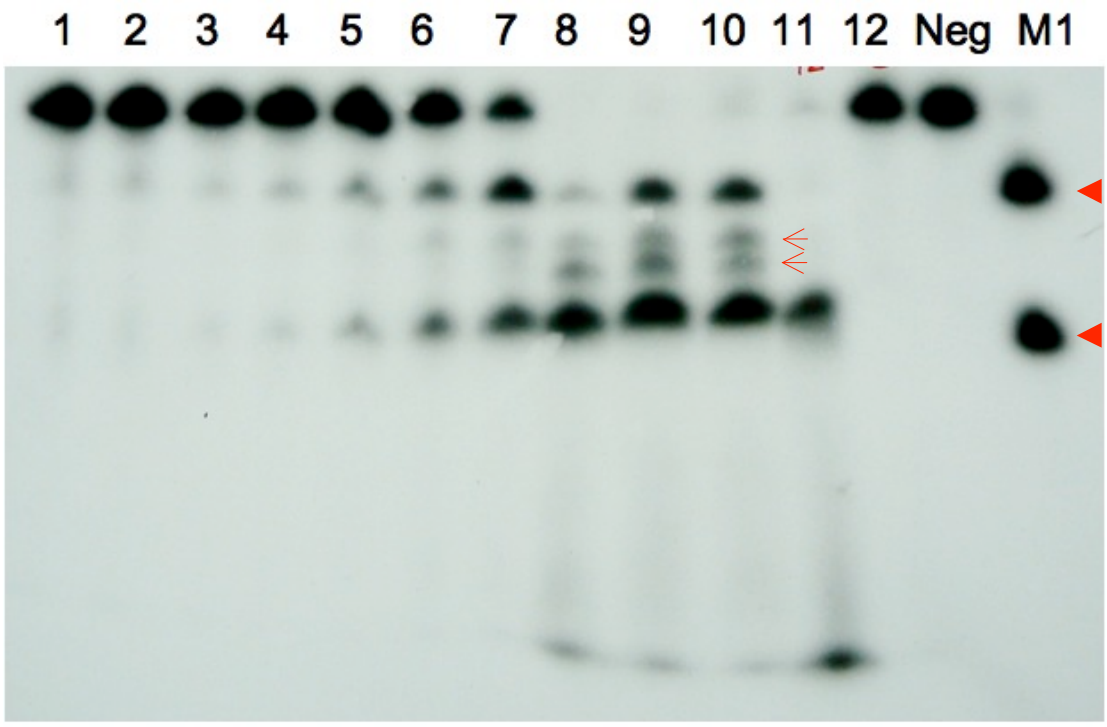

Figure 3-1- RNase P activity of BNC-separated soluble mitochondrial fractions (without detergent). Purified mitochondria were disrupted in buffer A by mixing with glass beads, and the matrix extract (after removing membranes by centrifugation) was subjected to 7% continuous BNC (20x7.5mm; 200 V). Fractions (1-12) were collected on an hourly basis, and RNase P activity was determined with radioactively end-labeled tRNA precursor RNA. The negative control (Neg) is without addition of enzyme and the positive control a cleavage with *in-vitro* synthesized P RNA of *E. coli* (M1). The bands corresponding to pre-tRNA (117 nt); mature tRNA (78 nt) and the removed 5' leader sequence (39 nt) are indicated with thick arrows. Most RNase P activity was found in fractions 6 thought 10, together with some "non-specific" nuclease activities that co-migrate with RNase P under the chosen conditions and that are strongest in fractions 8 and 11 (i.e., overlapping the RNase P peak and potentially separable from RNase P by further purification steps). Unspecific bands are labeled with thin arrows. The RNA bands in the M1 lane are slightly more distant due to a migration artifact caused by the higher purity (absence of proteins) of the M1 sample.

3.2. RNase P purification from *A. nidulans* mitochondria with detergents

We used two types of non-ionic detergents for solubilization of RNase P from mitochondria: the very mild digitonin and Triton X 100. The addition of 0.3 M digitonin to extraction buffer A doubled the amount of solubilized proteins (see Table S-2), and treatment with 1% Triton of the remaining membrane fraction, after digitonin treatment, recovered even more material (three times that of digitonin; ~ 20.8 mg) (Table S-3). The soluble fraction was loaded on 7% BNC and the hourly collected fractions were tested for enzyme activity. Non-specific RNA degradation increased with both detergents, though somewhat less with digitonin than Triton (Figures 3-2 and 3-3). Enzyme activity was spread out over most BNC fractions when using detergents, i.e., there was no homogenous fraction as in previous experiments without detergent. We selected the most active fractions 1, 2, 6, and 8 for identification of proteins by mass spectrometry, and for identification of a common core of proteins related to mtRNase P activity (Table S-2). Twenty-five of these proteins were initially annotated as hypothetical proteins, but we were able to assign potential functions by PSI-Blast in 21 instances (Table-S2). Three of the most active fractions isolated from Triton-treated extracts contained seventy-five proteins, of which again most (64) were without functional annotation. Sixteen of them remained unknown even after PSI-Blast re-annotation. Ten proteins are common among all active fractions (Table 3-1).

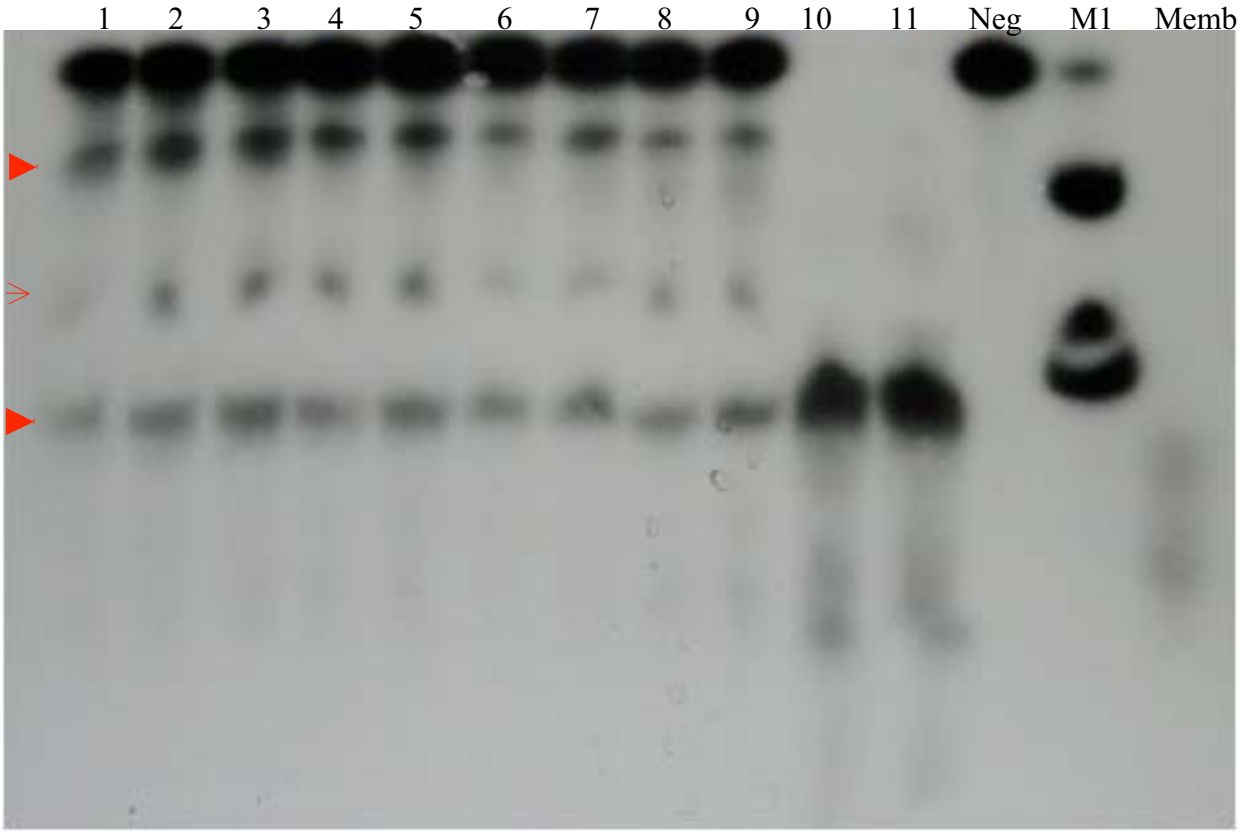

Figure 3-2- RNase P activity of BNC-separated soluble mitochondrial fractions (0.3 M digitonin). Separation conditions were as described for the detergent-free extract (Fig. 2-1). M1 is the positive control with the *E. coli* M1 P RNA. Bands corresponding to pre-tRNA (117 nt), mature tRNA (78 nt) and the 5' leader sequence (39 nt) are indicated with thick arrows. The negative control (Neg) contains no enzyme; unspecific bands are labeled with thin arrows. The last lane (Memb) shows the activity of the solubilized membrane fraction (in 3 and 5% Triton), which is strongly enriched in non-specific RNase.

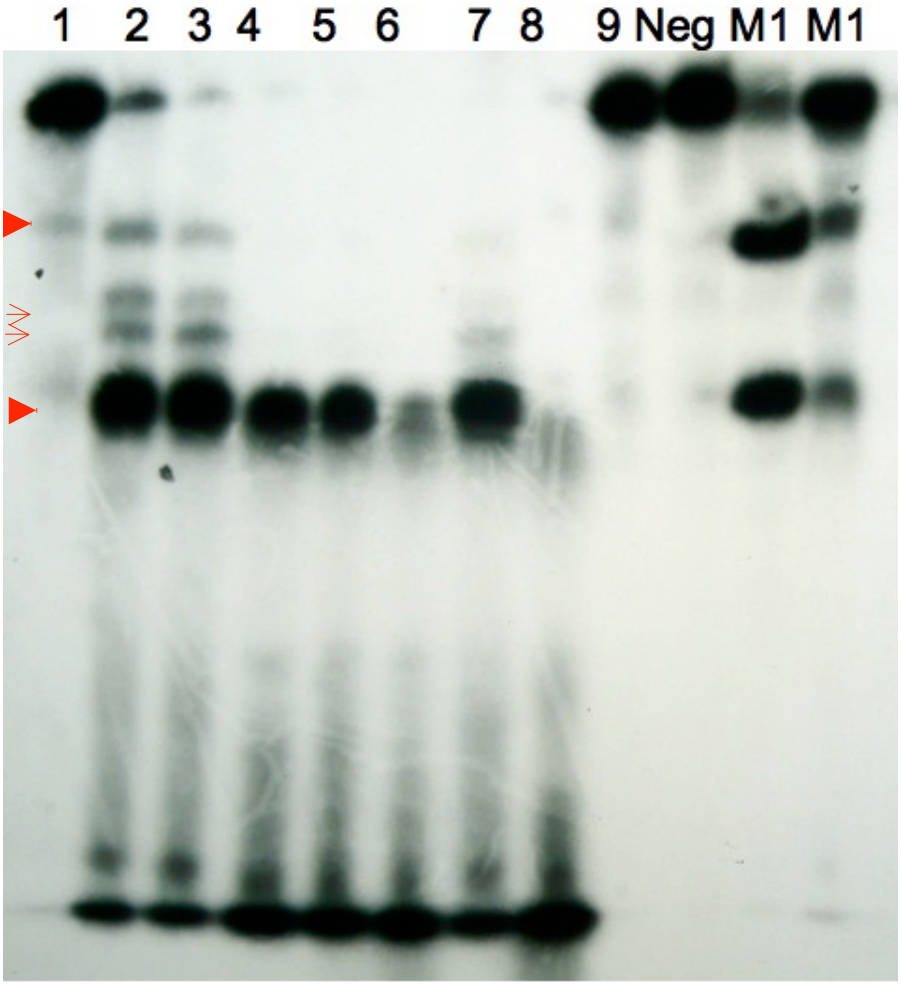

Figure 3-3- RNase P activity of BNC-separated soluble mitochondrial fractions (1% Triton). The membrane and mitochondrial debris removed by centrifugation (14 000 rpm, 15 min.) from digitonin treated mitochondria, were treated with 1% Triton and loaded on 7% BNC (20x7.5mm; 200 V). Fractions (1-9) were collected at hourly intervals. The fractions were tested for RNase P activity. The positive control is with *E. coli* P RNA, M1, and a negative control (Neg.) is sample mixture without enzyme. Thick arrows indicate the bands corresponding to pre-tRNA (117 nt), mature tRNA (78 nt), and the removed 5' leader sequence (39 nt). Thin arrows show unspecific bands. It seems that Triton causes substantially more degradation of the substrate, tRNA and 5' leader sequences than digitonin.

Table 3-1: Common proteins among active RNase P fractions from several purification experiments (with and without detergent).

	Protein ID	Protein Description	Protein Mass
1*	|XP_659127.1|	ATPA_NEUCR ATP synthase alpha chain; mitochondrial precursor [*A. nidulans* FGSC A4]	61899
2*	|XP_659919.1|	ATPB_NEUCR ATP synthase beta chain; mitochondrial precursor [*A. nidulans* FGSC A4]	54818
3*	|XP_664321.1|	hypothetical protein AN6717.2 [*A. nidulans* FGSC A4] (malate dehydrogenase, mitochondrial)	37724
4*	|XP_658036.1|	hypothetical protein AN0432.2 [*A.* nidulans FGSC A4] (NADH-cytochrome b5 reductase, putative)	36050
5*	**|XP_658766.1|**	**hypothetical protein AN1162.2 [*A. nidulans* FGSC A4] (Unknown)**	**24889**
6*	|XP_659910.1|	hypothetical protein AN2306.2 [*A. nidulans* FGSC A4] (ubiquinol-cytochrome c reductase iron-sulfur)	26291
7*	**|XP_661447.1|**	**hypothetical protein AN3843.2 [*A. nidulans* FGSC A4](Unknown)**	**68592**
8	|XP_663859.1|	hypothetical protein AN6255.2 [*A. nidulans* FGSC A4](cytochrome c oxidase polypeptide VIb)	11021
9	|XP_664235.1|	hypothetical protein AN6631.2 [*A. nidulans* FGSC A4] (ATPase activity)	19482
10*	**|XP_680705.1|**	**hypothetical protein AN7436.2 [*A. nidulans* FGSC A4] (Unknown)**	**55807**

Table 3-1: Common proteins among active RNase P fractions from several purification experiments. (BNC 7% without detergent, with digitonin, and with Triton). Proteins that also occur in partially purified human mtRNase P carry asterisks. The functions in square brackets are predicted by PSI-BLAST, and unknown proteins are in bold. The two underlined protein masses are proteins with molecular mass similar to those previously published (Lee et al. 1996).

3.3. Searching for P RNA

To confirm that all active fractions also contained mtRNase P as expected, these were analyzed for the presence of the *A. nidulans* mtP RNA by RT-PCR (starting from 300 ng total RNA per assay, see also Material and Methods). The PCR product of expected size (191 bp) is equally amplified from all active fractions, with various alternative primer pairs (Figure 3-4). A second weaker band of different size is due to a duplicated and expressed *rnpB* pseudogene in mtDNA of *A. nidulans* (L. Forget, unpublished results).

The low yield of mtRNase P in purification experiments without detergents prompted us to estimate the proportions of soluble *versus* membrane-attached mtRNase P. Yet, testing of activities in membrane fractions is not possible, as detergents that are required to dissolve membranes and release RNase P also activate unspecific RNase, leading to degradation of the RNA substrate and its products (Figure 3-2 and 3-3). Attempts to inhibit this non-specific nuclease activity by the addition of total yeast RNA (for competitive substrate inhibition) were unsuccessful. Therefore, to quantify mtRNase P, we traced mtP RNA by RT-PCR. From these data it is evident that the majority of P RNA is in the membrane fraction, even after digitonin treatment (Figure 3-5) Triton releases an additional proportion (~ 30%) into solution, but this RNA is less than expected based on addition of total amounts (Figure 3-5). This was most likely due to RNA degradation after addition of the detergent.

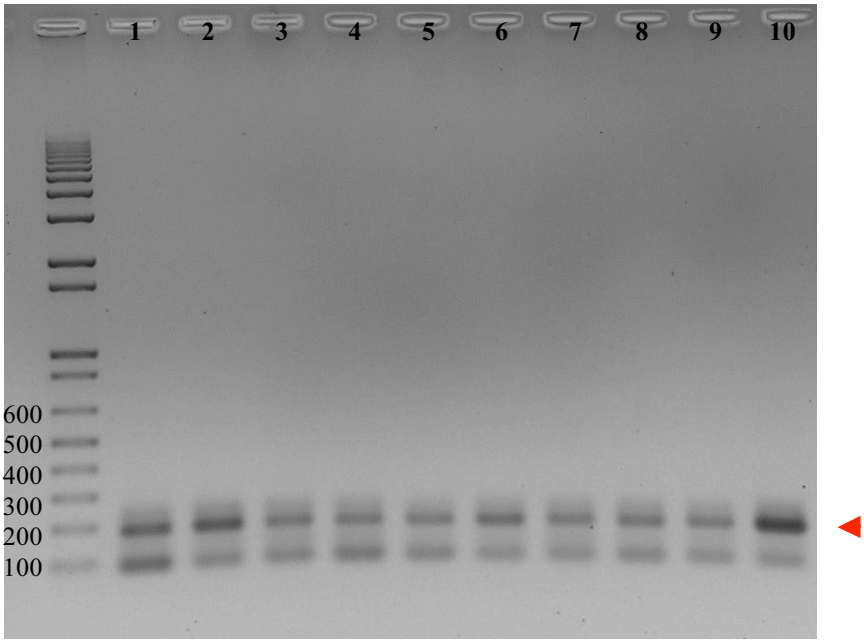

Figure 3-4- Presence of P RNA in purified active fractions with RT-PCR .

P RNA in active fractions collected from 7% BNC (treated with digitonin and Triton). 10 ng of the RNA reverse transcribed DNA that was present in active fractions, was amplified by PCR (55 °C annealing temperature for 35 cycles) (see also material and methods). 1 µl of the PCR products was loaded on 0.8% agarose gel .The expected RT-PCR product (191 nt) appeared in all the active fractions. Lane 1-3, Triton purification; lane 4- 6, digitonin purification; lane 7-9, are the non detergent enriched fractions. 10 is a positive control with total mitochondrial RNA.

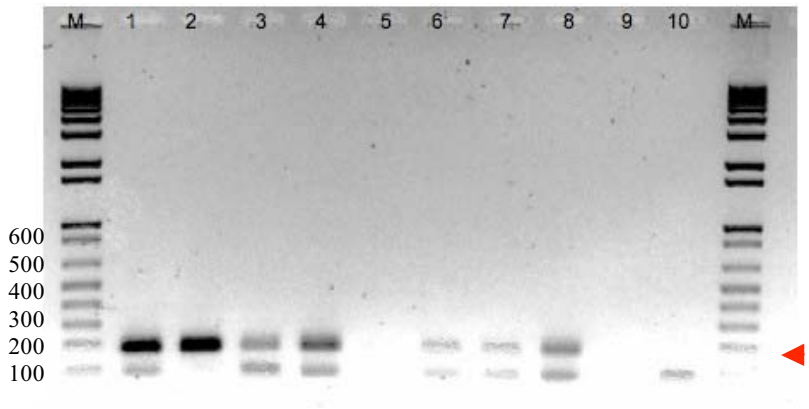

Figure 3-5- Presence of P RNA in soluble and membrane fractions in the presence of digitonin and Triton. RT-PCR was applied as outlined in material and methods. 1 μl of PCR products was loaded on a 0.8% agarose gel; the expected size of the RT-PCR product is 191 nt. Comparison of amplification products: intact mitochondria (1-2), digitonin treated membranes (3-4), digitonin treated soluble matrix (5-7), and 1% Triton treatment of the remaining membrane fraction after 0.3 M digitonin treatment: insoluble membrane (8) and soluble fraction (10). Lane 9 is negative control.

CHAPTER 4: DISCUSSION AND PERSPECTIVES

4. DISCUSSION AND CONCLUSION

This project reveals that the major part of the *A. nidulans* mitochondrial RNase P is in the membrane fraction. We have succeeded in developing enrichment procedures for the enzyme both with and without detergents. According to our results, the RNase P holoenzyme is much larger than previously published (Lee et al. 1996), and as in human mitochondria (Holzmann et al. 2008) it seems that a homogenous mitochondrial RNase P complex may indeed not exist. When purifying RNase P from a detergent-treated membrane fraction, a spectrum of large complexes of different size and composition is obtained. Among ten common protein lists (Table 3-1) we suggest three unknown proteins (AN1162.2, AN3843.2, AN7436.2) as potentially essential subunits of *A. nidulans* mtP RNase P.

4.1. Advantages of BN PAGE and BNC for RNase P purification

Published purification methods for mtRNase P are complex multi-step procedures, applying detergents in combination with non-physiological conditions (such as high salt, EDTA, heat shock etc; Lee et al. 1996; Marchfelder, A 1994; Manam, S , 1987, Morales 1989), likely resulting in the dissociation of enzyme complexes. The aim of these procedures is purification of an activity with a minimum number of subunits, rather than characterization of its native conformation. According to our initial idea, elimination of detergents from the purification procedure would help to preserve a native state of the enzyme, which may then also be more easily purified as a homogenous complex. Yet according to our results, the major fraction of mtRNase P in *A. nidulans* appears to be membrane-bound, as it is in humans, which eliminates the option of a detergent-free purification (except for a minor, soluble fraction of mtRNase P that we consider non-representative for the cellular activity). We show here that the comparison of BNC-purified detergent-free and -treated fractions allows pinpointing of proteins that are linked to the RNase P activity. BNC, a preparative variant of BN PAGE, combines its advantages with the option to separate about an order of magnitude more material. Although the separation of complexes by BNC is reduced due to a

larger electrophoresis chamber and the application of much more material, eluted fractions from a first separation can be easily re-purified in a second run.

Our results open the possibility of future experiments, following to a similar strategy as in human, i.e., identification of its essential subunits by identifying common proteins in all fractions with RNase P activity, and its *in vitro* reconstitution from the individual components (Holzmann et al. 2008).

4.2. The effects of Detergents: to use or not to use?

Our main criticism of previously published mtRNase P purification methods have been the use of detergents (Triton and cholate salts), leading to low yield of activity and strongly degrading mtP RNA (if at all present). Yet, our results with *A. nidulans* mtRNase P show that the major fraction of mtRNase P is not a soluble, homogenous ribonucleo-protein complex, and that detergents are in fact required to isolate it from the membrane fraction. The idea of a soluble RNase P comes from bacterial systems and from eukaryotes (their nucleus-encoded form), where detergents are not required for purification. In fact, we also show that a minute fraction of the *A. nidulans* mitochondrial ribozyme is soluble without detergents, and when purified, contains 20 proteins. Yet, we do not consider this fraction as representative for the cellular activity. In the other words, this soluble complex is one of the several representative forms of RNase P.

A very mild detergent like digitonin increases both the yield and the number of proteins in the isolated *A. nidulans* complex by about 1.6 times. One percent Triton is even more efficient in solubilizing the activity, but increases the number of proteins in the purified fraction more than twice. Results from purification of human mtRNase P suggest a similar situation, in which the non-ionic detergent Tween 20 solubilizes the enzyme into a wide size range of complexes of varying protein composition. Based on these findings, we suspect that also the *A. nidulans* detergent-purified mtRNase P fraction is non-homogenous, but rather a continuum of complexes that contain mtRNase P activity. This proposal is consistent with the absence of distinct bands in BN-PAGE, when separating *A. nidulans* extracts obtained with detergents. It is unclear whether this also applies to the minor soluble fraction of the

ribozyme, as the amount of isolated material has been too small to test it for homogeneity in BN PAGE.

Whereas human mtRNase P has been characterized by comparing the protein composition of all active complexes, this method is so far restricted to protein-only enzymes. In case of mtRNase P which depends on intact P RNA for activity, all of the tested detergents activate non-specific nucleases, leading to substantial degradation of the P RNA, as documented previously for yeast, *Aspergillus* and plant mitochondria (Marchfelder and Brennicke 1994; Lee et al. 1996; R.Daoud unpublished data). We interpret this nuclease activation as the result of membrane dissociation, liberating otherwise secluded enzymes. In early stages of purification (i.e. crude detergent-treated lysates), identification and quantification of RNase P activity is therefore virtually impossible, leading to imprecise estimates of enzyme yields during purification. This may also explain the enormous amount of *Aspergillus* hyphae (2.5 kg) that was required for a published mtRNase P purification with Triton (Lee et al. 1996): most of the activity and the integrity of the ribozyme was likely lost in early stages of the procedure and only a minor more stable sub-fraction has served for further purification.

4.3. *Aspergillus* mtRNase P, a membrane-attached enzyme

Several unsuccessful attempts were made to extract and separate soluble *Aspergillus* mitochondrial protein extracts using BN-PAGE. In all instances, the yield of extracts was negligible, and the material did not separate into a clear band pattern. In contrast, in other species (e.g., *S. cerevisiae, Arabidopsis thaliana, E. coli*), only small amounts of mitochondria or cells are required to produce soluble extracts (that may represent close to half of the starting material), which separate into clearly visible bands in BN PAGE (R. Daoud, unpublished results) (see also Figure 4-1). Evidently, the organization of otherwise soluble protein complexes is different in *A. nidulans*, apparently tightly integrated with membranes. Out results imply that a similar situation occurs in the case of mtRNase P.

The hypothesis that the *Aspergillus* mtRNase P is membrane-bound is strengthened by experiments that trace mtP RNA in various mitochondrial fractions by RT PCR. Our results show that the use of detergents increases the amount of P RNA in solution and decreases it in the membrane fraction (Figure 3-5). Mild dissociation with 0.3 M digitonin releases a small fraction of P RNA into a low molecular weight fraction. When treating the remaining membrane fraction (pellet) from the 0.3 M digitonin treatment (which still contains most P RNA) with 1% Triton, it results in further solubilization of P RNA, but only up to about 30%, and leads to an increasing loss of RNA by degradation. This reveals that RNase P is either tightly integrated with the membrane, or that it is part of a very large complex that sediments with the membrane fraction, and that this conformation protects the RNA subunit from the attack of unspecific nucleases.

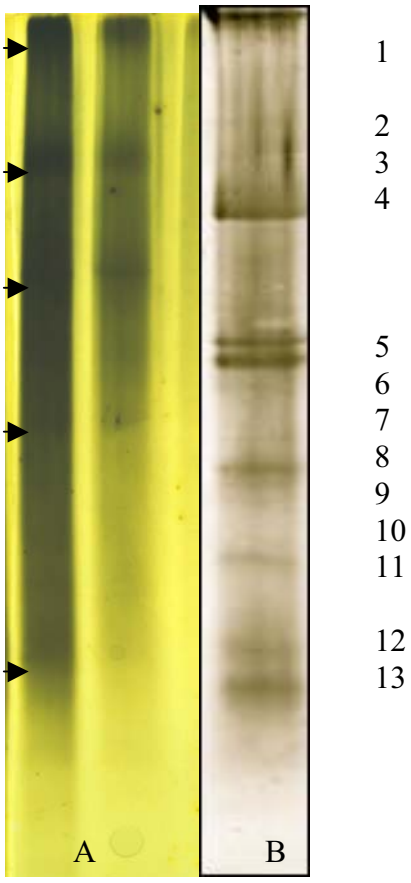

Figure 4-1- Visualization of stained mitochondrial protein complexes separated on a 4-13% gradient BN-PAGE. To increase the intensity of bands, the gels were silver-stained. A) *A. nidulans* mitochondrial matrix after Triton treatment. B) Mitochondrial protein complexes in *S. cerevisiae* (Figure taken from R. Daoud). About thirteen distinct complexes are found in *S. cerevisiae*, whereas less than five weak bands with a background smear appear in *A. nidulans* under the same conditions. The yellow color is due to prolonged development by silver staining.

4.4. In search of common protein subunits in complexes with mtRNase P activity

The comparison of proteins among all fractions with RNase P activity is expected to reveal the components that are essential for activity, and in fact our comparison reveals only ten common protein candidates. The predicted functions of these proteins (Gene Bank or re-annotation of hypothetical proteins by PSI-Blast) do not show potential functional relationship with P RNase activity in seven instances. However three functionally unidentified proteins can be considered as P protein candidates, and may be further studied. Two of these proteins have molecular masses (24 and 55 kDa) close to those that were previously reported as components of an *A. nidulans* mtRNase P reported by Lee et al. 1996.

Remarkably, a comparison of the list of identified proteins that are found together with RNase P activity in *A. nidulans* with that of human (Holzmann et al. 2008) reveals an 80% match. These include the alpha and beta subunits of ATP synthase, cytochrome c oxidase polypeptide VIb, malate dehydrogenase, NADH-cytochrom b5 reductase, and ubiquinol cytochrom c reductase iron-sulfur protein. Although these protein functions are evidently unrelated to RNase P activity, it is reasonable to assume that they are organized in a large super-complex together with RNase P, combining a variety of mitochondrial functions, and evidently in a similar way in human and *A. nidulans*. The hypothesis that RNase P is part of a large complex is not that far-fetched, as human nuclear RNase P functions as a transcription factor for RNA Pol III, in transcription of tRNAs and other small non-coding RNAs (Reiner et al. 2006). Furthermore human nuclear RNase P subunits are also associated with Pol I and its transcription initiation factors (Reiner et al. 2008).

4.5. PERSPECTIVES

This project raises the important question of mtRNase P localization in mitochondria of *A. nidulans*, and its minimum subunit composition in comparison with other eukaryotes. To obtain more precise answers, it will be essential to find detergents and conditions that will allow RNase P to dissolve into a more homogenous fraction. Moreover, the issue of activating unspecific endonucleases due to detergent treatment has to be resolved. Our preliminary experiments have not succeeded to suppress these activities by addition of carrier RNAs, or with commercially available RNase inhibitors. We therefore propose the use of *A. nidulans* mutants in genes that express candidate mitochondrial P proteins. This seems feasible as the complete genome sequence is known, and as gene knockout is possible in this species.

Finally, as an alternative to BN-PAGE purification of large complexes, one might consider the use of tags for pull-down purification (Swaffield and Johnston 2001). This technique has the advantage of not relying on the homogeneity of complexes, to be ultimately used if the search for conditions that dissolve *A. nidulans* mtRNase P into a homogenous fraction is unsuccessful – as is the case in human.

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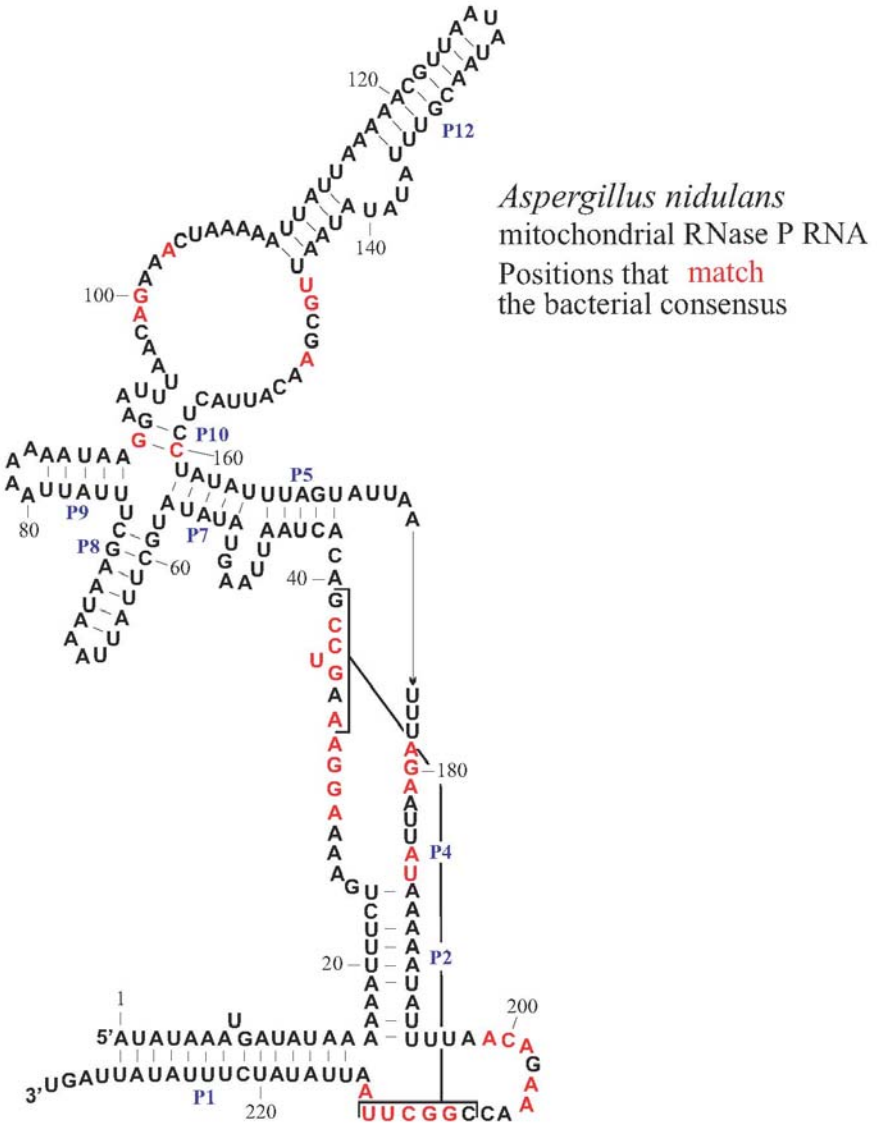
Appendices:

I. A. nidulans P RNA secondary structure



Sequence and partial model published by Lee et.al., Eur J Biochem 234: 297-303; accession # X93307; this model produced by the FMGP: http://megasun.bch.umontreal.ca/People/lang/species/asp/asp.html.