Interplay between substrate recognition, 5' end tRNA processing and methylation activity of human mitochondrial RNase P

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ABSTRACT

Human mitochondrial ribonuclease P (mtRNase P) is an essential three-protein complex that catalyzes the 5' end maturation of mitochondrial precursor tRNAs (pre-tRNAs). Mitochondrial RNase P Protein 3 (MRPP3), a protein-only RNase P (PRORP), is the nuclease component of the mtRNase P complex and requires a two-protein *S*-adenosyl-methionine (SAM)-dependent methyltransferase MRPP1/2 subcomplex to function. Dysfunction of mtRNase P is linked to several human mitochondrial diseases, such as mitochondrial myopathies. Despite its central role in mitochondrial RNA processing, little is known about how the protein subunits of mtRNase P function synergistically. Here, we use purified mtRNase P to demonstrate that mtRNase P recognizes, cleaves, and methylates some, but not all, mitochondrial pre-tRNAs *in vitro*. Additionally, mtRNase P does not process all mitochondrial pre-tRNAs uniformly, suggesting the possibility that some pre-tRNAs require additional factors to be cleaved *in vivo*. Consistent with this, we found that addition of the TRMT10C (MRPP1) cofactor SAM enhances the ability of mtRNase P to bind and cleave some mitochondrial pre-tRNAs. Furthermore, the presence of MRPP3 can enhance the methylation activity of MRPP1/2. Taken together, our data demonstrate that the subunits of mtRNase P work together to efficiently recognize, process, and methylate human mitochondrial pre-tRNAs.

Keywords: RNase P; PRORP; MRPP; mitochondria; pre-tRNA methylation

INTRODUCTION

Ribonuclease P (RNase P) catalyzes one of the first steps of tRNA maturation, the removal of the 5' leader sequences from precursor tRNAs (pre-tRNAs). There are two classes of evolutionarily distinct RNase Ps: RNA-based (ribozyme) and protein-based enzymes (Holzmann et al. 2008; Gobert et al. 2010; Taschner et al. 2012). RNase P is essential wherever pre-tRNAs are transcribed and is present in the nucleus, mitochondria, and chloroplasts of cells. The protein-only form of RNase P was first identified in human mitochondria and consists of three subunits: Mitochondrial RNase P Protein 1 (TRMT10C or MRPP1), Mitochondrial RNase P Protein 2 (MRPP2, 17 β -hydroxysteroid dehydrogenase type 1, SDR5C1), and Mitochondrial RNase P Protein 3 (MRPP3 or human PRORP, catalytic nuclease subunit) (Holzmann et al. 2008; Vilardo et al. 2012). Subsequently, Protein-only RNase Ps (PRORPs) have been dis-

covered in some land plants (e.g., *Arabidopsis thaliana*; Gobert et al. 2010; Gutmann et al. 2012) and other unicellular Eukaryotic organisms (e.g., *Trypanosoma brucei*; Taschner et al. 2012). It is noteworthy that in archaea and bacteria, 5' tRNA enzymes containing a similar catalytic domain to PRORPs were also recently identified (HARPs, Homologs of Aquifex RNase P). These enzymes were shown to be the smallest RNase P with independent 5' end pre-tRNA processing endonuclease activity (Nickel et al. 2017). In contrast to human mtRNase P, PRORPs are stand-alone enzymes that do not require accessory proteins. Most studies of protein-based RNase Ps have focused on PRORPs because these enzymes are homologous to MRPP3 and provide a tractable system for study (Gobert et al. 2010, 2013; Gutmann et al. 2012; Howard et al. 2012, 2013, 2016; Karasik et al. 2016; Klemm et al.

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2016; Pinker et al. 2017). As a consequence, little is known about how the components of the metazoan mtRNase P complex function.

In mitochondria, RNAs are transcribed in polycistronic units. In each polycistronic unit, most protein-coding and rRNA transcripts are separated (or “punctuated”) by tRNAs. Therefore, 5′ end and 3′ end tRNA processing is required for the processing of most key mitochondrial RNAs (Ojala et al. 1981). 5′ End cleavage by mtRNase P has been suggested to be the first step in the hierarchical tRNA processing in mitochondria (Rossmann et al. 1995; Lopez Sanchez et al. 2011; Reinhard et al. 2017). More than 50% of mitochondrial disease-causing mutations in the mitochondrial genome are found in tRNA genes (Abbott et al. 2014). Because precursor tRNA processing is likely the first step in the mitochondrial tRNA maturation pathway, disabling this function leads to accumulation of mitochondrial precursor tRNAs along with unprocessed RNA transcripts (Rackham et al. 2016; Sen et al. 2016). Accumulation of unprocessed RNA in the mitochondria affects downstream processes such as mitochondrial ribosome assembly and translation with detrimental effects on mitochondrial function and viability. It is therefore predicted that mitochondrial RNA processing plays a role in mitochondrial diseases. Consistent with this idea, mtRNase P dysfunction is linked to several mitochondrial diseases including maternally inherited essential hypertension (Wang et al. 2011), mitochondrial myopathy (Bindoff et al. 1993; Rossmann and Karwan 1998), MELAS (Li and Guan 2010), and HSD10-disease (Deutschmann et al. 2014; Chatfield et al. 2015; Vilardo and Rossmann 2015; Falk et al. 2016).

All three subunits of mtRNase P are strictly required in vivo; knockout of any component in flies leads to larval lethality (Sen et al. 2016). In addition, knockdown of TRMT10C (MRPP1) and MRPP3 in human cell lines was shown to affect 5′ end mitochondrial pre-tRNA processing and mature mitochondrial tRNA levels (Holzmann et al. 2008; Lopez Sanchez et al. 2011). This further emphasizes that in general, processing activity of MRPP3 is dependent on other components of mtRNase P. Similarly, knockout of the MRPP3 gene in mice arrests embryogenesis at a very early stage (Rackham et al. 2016). Furthermore, mice containing a conditional knockout of MRPP3 develop cardiomyopathy, show signs of mitochondrial dysfunction, and exhibit severe defects in 5′ end RNA processing (Rackham et al. 2016). MRPP3 has also been shown to further influence tRNA modifications—for example, a missense mutation in MRPP3 was identified as an important factor responsible for differences in mitochondrial tRNA methylation patterns (Hodgkinson et al. 2014). Thus, the correct mtRNase P function is likely to be essential for human health.

Little is known about the structure and function of the mtRNase P complex; there are no structures or structural models and limited mechanistic and biochemical informa-

tion available, although structures of truncated MRPP3 have been solved (Howard et al. 2012; Li et al. 2015; Reinhard et al. 2015; Karasik et al. 2016). As such, the role of the interaction between MRPP3 and the MRPP1/2 subcomplex in enhancing substrate binding and catalysis is a key question in the function of human mtRNase P (Holzmann et al. 2008). The MRPP1/2 subcomplex has been predicted to methylate the N1 of adenine or guanine residues at the ninth position of 19 mitochondrial tRNAs (Vilardo et al. 2012; Suzuki and Suzuki 2014). TRMT10C (MRPP1) is the S-adenosyl-methionine (SAM)-dependent methyltransferase subunit in the MRPP1/2 subcomplex, and MRPP2 likely acts as a scaffold protein (Vilardo et al. 2012).

Here, we investigate how TRMT10C (MRPP1), MRPP2, and MRPP3 work together to recognize and cleave pre-tRNA substrates. Our work with recombinant mtRNase P reveals that the complex can specifically bind, process, and methylate several human mitochondrial pre-tRNAs that are not recognized and processed by single-subunit plant PRORPs. Additionally, a subset of human mitochondrial pre-tRNA substrates are not processed efficiently by the recombinant mtRNase P complex, suggesting that additional components or modifications may be required to facilitate cleavage of these substrates. We further demonstrate that the TRMT10C (MRPP1) cofactor SAM enhances the ability of the mtRNase P complex to bind and cleave some substrates. Overall, our studies deepen the insight into how the components of mtRNase P act synergistically to bind, process, and methylate human mitochondrial pre-tRNAs.

RESULTS

mtRNase P binds mitochondrial precursor-tRNAs nonuniformly

Although the human mtRNase P complex was discovered almost a decade ago, it is largely unknown how it recognizes and binds its mitochondrial substrates (Holzmann et al. 2008). The pool of native mtRNase P substrates is diverse; in human mitochondria, there are four subtypes of tRNAs (Type 0–III). The (mt)tRNAs belonging to types I, II, and III all differ from the canonical cloverleaf secondary structure of nuclear tRNAs, whereas type 0 (mt)tRNAs resemble the features of nuclear ones (Helm et al. 2000; Suzuki et al. 2011). We selected six mitochondrial pre-tRNA [(mt)pre-tRNAs] substrates to study representatives of all four classes including a substrate [(mt)pre-tRNA^{Ser(AGY)}] that human mtRNase P is not expected to process in vivo (Fig. 1; Rossmann 1997). The exact lengths of 5′ and 3′ flanking ends of mtRNase P substrates have not yet been established; therefore, we used our knowledge of *A. thaliana* single-subunit PRORP enzymes that are homologous to MRPP3 to design the 5′ and 3′ ends of our (mt)pre-tRNA substrates. PRORPs preferentially bind substrates with

FIGURE 1. Proposed secondary structures of different types of human mitochondrial pre-tRNAs used in this study. Black arrows indicate the 5' cleavage site, and circles mark the m1A9/m1G9 methylation sites for MRPP1/2.

short (<5–8-nt) 5' leaders and do not bind the 3' trailer of pre-tRNAs (Brillante et al. 2016; Howard et al. 2016; Karasik et al. 2016). Thus, the (mt)pre-tRNA substrates studied here possess 6–7-nt long 5' leader sequences and lack mature 3' CCA ends (Fig. 1).

To determine how each component of the mtRNase P complex interacts with (mt)pre-tRNAs, we measured the binding constant (K_D or $K_{D,app}$) for the mtRNase P complex components (MRPP1/2 subcomplex and MRPP3) and six (mt)pre-tRNAs of interest using a fluorescence anisotropy binding assay. We find that the isolated mtRNase P components generally do not associate tightly with the selected substrates (Table 1; Fig. 2A,B). MRPP3 alone binds all of the (mt)pre-tRNAs substrates weakly with K_D values > 4 μ M (Table 1; Fig. 2A). The MRPP1/2 subcomplex similarly binds most substrates weakly ($K_{D,app}$ > 2 μ M) with the exception of (mt)pre-tRNA^{Ile} and (mt)pre-tRNA^{Ser(UCN)}, which it binds more tightly ($K_{D,app}$ values of 104 ± 24 nM and 730 ± 196 nM, respectively) (Table 1; Fig. 2B).

We next sought to establish how the fully reconstituted mtRNase P complex (MRPP1/2:3) interacts with (mt)pre-tRNA substrates. For these assays, first, we incubated pre-folded (mt)pre-tRNAs with 150 nM MRPP1/2. After MRPP1/2 and pre-tRNA binding reached equilibrium (5 min), we added varying concentrations of MRPP3 (0–10 μ M, Fig. 2C,D) and measured additional changes in fluorescence anisotropy. In general, we find that the presence of the MRPP1/2 subcomplex enhances (mt)pre-tRNA bind-

ing by 30- to 330-fold over binding to MRPP3 alone (Table 1). However, these enhancements are not uniform. The substrates fall into two categories: those that bind the complex somewhat tighter than MRPP3 alone [three- to fivefold: (mt)pre-tRNA^{Val}, (mt)pre-tRNA^{Ser(UCN)}, (mt)pre-tRNA^{Ser(AGY)}] and those that bind the complex significantly tighter (30- to 330-fold: (mt)pre-tRNA^{Leu(UUR)}, (mt)pre-tRNA^{Ile}, (mt)pre-tRNA^{Met}). As anticipated the substrate that binds the weakest is (mt)pre-tRNA^{Ser(AGY)}. Notably, the mtRNase P complex exhibits the tightest $K_{D,app}$ (13.5 ± 2.3 nM) for (mt)pre-tRNA^{Ile}. These results indicate that although a subset of human (mt)pre-tRNAs are recognized with high affinity by mtRNase P ($K_{D,app}$ values are in the nanomolar range), there are other (mt)pre-tRNAs that have a weaker affinity ($K_{D,app}$ values in the micromolar range).

To compare substrate selectivity of human mtRNase P complex with single-component PRORP1 and 2 enzymes we tested the ability of *A. thaliana* PRORP1 and PRORP2 to bind our six human (mt)pre-tRNA substrates (Table 1; Supplemental Table 2). We find that only (mt)pre-tRNA^{Ser(UCN)} binds to PRORP1 and 2 with a comparable K_D value (60–120 nM) to that of their native *A. thaliana* substrates. All other (mt)pre-tRNAs were bound at least sevenfold less tightly (K_D = 880 nM to >10 μ M, Supplemental Table 2). Thus, mtRNase P uses strategies or interactions for recognizing its substrates that are different from the single-component PRORP1 and 2 enzymes.

TABLE 1. Human mtRNase P binds and processes a subset of mitochondrial pre-tRNA substrates efficiently

Human (mt) pre-tRNA	K_D , MRPP3 (nM)	$K_{D,app}$, MRPP1/MRPP2 (nM)	$K_{D,app}$, mtRNase P ^a (nM)	K_D , PRORP1 (nM)
(mt)pre-tRNA ^{Leu(UUR)}	9200 ± 1600	>2000	50.6 ± 5.3	883 ± 174
(mt)pre-tRNA ^{Ile}	4400 ± 600	104 ± 24	13.5 ± 2.3	4105 ± 1400
(mt)pre-tRNA ^{Val}	21,700 ± 2600	>2000	4124 ± 560	>>10,000
(mt)pre-tRNA ^{Met}	19,300 ± 5500	>2000	707 ± 168	>5000
(mt)pre-tRNA ^{Ser(UCN)}	12,200 ± 2200	730 ± 196	3556 ± 540	123 ± 23
(mt)pre-tRNA ^{Ser(AGY)}	43,900 ± 7900	>2000	12,660 ± 1312	>>10,000
Human (mt) pre-tRNA	$K_{1/2}$ ^{MRPP3} (nM)	k_{obs} or $k_{max,cleav}$ mtRNase P (min ⁻¹) ^b	$k_{obs,PRORP1}$ (min ⁻¹)	
(mt)pre-tRNA ^{Leu(UUR)}	423 ± 107	0.41 ± 0.04	0.06 ± 0.002	
(mt)pre-tRNA ^{Ile}	389 ± 85	0.80 ± 0.06	0.05 ± 0.003	
(mt)pre-tRNA ^{Val}	-	0.03 ± 0.01	0.08 ± 0.005	
(mt)pre-tRNA ^{Met}	933 ± 299	0.45 ± 0.07	0.09 ± 0.017	
(mt)pre-tRNA ^{Ser(UCN)}	-	0.06 ± 0.01	0.69 ± 0.020	
(mt)pre-tRNA ^{Ser(AGY)}	-	0.06 ± 0.01	0.09 ± 0.005	

All individual binding and kinetic experiments were performed in parallel for the different substrates to ensure comparability of data. All fluorescence binding measurements were carried out in 30 mM MOPS pH = 7.8, 150 mM NaCl, 1 mM DTT, and 20 nM Fl pre-tRNAs. K_D values were calculated from a fit of a binding isotherm to the concentration dependence of the anisotropy data.

^aMeasurements for mtRNase P were performed as follows: First, folded pre-tRNA was incubated with 150 nM MRPP1/2 for 5 min, and then varying amounts of MRPP3 were added. Fluorescence anisotropy values were read after 5 min further incubation.

^bThese k_{max} values were derived from k_{obs} versus MRPP3 concentration plots for (mt)pre-tRNA^{Ile}, (mt)pre-tRNA^{Leu(UUR)}, and (mt)pre-tRNA^{Met}. For all other (mt) pre-tRNAs, the data represent the k_{obs} values using 500 nM MRPP1/2 and 1.5 μ M MRPP3.

mtRNase P exhibits 5' end processing of tightly bound (mt)pre-tRNAs

To further understand mtRNase P substrate selectivity we measured single-turnover cleavage rate constants for the six (mt)pre-tRNAs discussed above (Fig. 1). Previously, we used fluorescence polarization-based multiple-turnover assays for monitoring pre-tRNA 5' end cleavage by RNA-based and single-component protein-only RNase Ps (*A. thaliana* PRORPs) (Liu et al. 2014; Howard et al. 2016). Here, we adapted this method to monitor the cleavage of 5'-fluorescein (5-FAM)-labeled (mt)pre-tRNAs by the fully reconstituted human mtRNase P complex in single-turnover cleavage assays. We measured the single-turnover rate constants (k_{obs}) and $K_{1/2}$ ^{MRPP3} values for mtRNase P using limiting substrate (20 nM fluorescent pre-tRNA) and a range (100 nM–2 μ M) of MRPP3 concentrations at 28°C while MRPP1/2 concentration was maintained constant at near saturating levels (500 nM) (Fig. 3A,B; Supplemental Fig. 3B).

All of the (mt)pre-tRNAs that bind with relatively high affinity to the mtRNase P complex [(mt)pre-tRNA^{Leu(UUR)}, (mt)pre-tRNA^{Ile}, (mt)pre-tRNA^{Met}] have $K_{1/2}$ ^{MRPP3} values, ranging from ~390 to 930 nM, greater than the measured binding affinities ($K_{D,app}$ values in CaCl₂), suggesting that $K_{1/2}$ ^{MRPP3} does not reflect a thermodynamic binding equilibrium under single-turnover conditions (Table 1). The $K_{D,app}$ values that we report were measured in CaCl₂ under conditions that permit binding but do not promote sub-

strate cleavage (Karasik et al. 2016). Although unlikely, we cannot rule out the possibility these conditions do not entirely reflect binding of substrates to mtRNase P in MgCl₂, when cleavage can occur. The k_{obs} values for these substrates range between 0.4 and 0.8 min⁻¹ (Table 1; Fig. 3C) and correlate well with rate constants previously measured for single-subunit PRORPs cleaving their native substrates (Brillante et al. 2016; Howard et al. 2016; Karasik et al. 2016). In contrast, (mt)pre-tRNAs that have low affinities for mtRNase P [(mt)pre-tRNA^{Val}, (mt)pre-tRNA^{Ser(UCN)}, (mt)pre-tRNA^{Ser(AGY)}] exhibit ~100-fold lower single-turnover activity (k_{obs} ~0.06 min⁻¹) (Table 1; Fig. 3C); the cleavage of these substrates was so slow that we were unable to accurately measure $K_{1/2}$ ^{MRPP3} values. We also considered the possibility that the length of the pre-tRNA 5' leader or 3' trailer sequences impacts cleavage by mtRNase P (Supplemental Fig. 4). To test this possibility, we measured the end-point cleavage activity of mtRNase P of substrates with long length 5' leaders and 3' trailers. We found that the length of 3' and 5' sequences does not significantly alter the ability of mtRNase P to cleave (mt)pre-tRNA^{Ser(UCN)} (cleavage is undetectable) and (mt)pre-tRNA^{Ile} (able to cleave). Our findings that the same substrates that bind poorly are also cleaved inefficiently by mtRNase P in single-turnover assays (in which binding is not the rate-limiting step) suggest that these may not be oriented properly in the bound complex.

As mentioned earlier, we find that most human mitochondrial pre-tRNAs are not bound tightly by *A. thaliana*

FIGURE 2. Presence of MRPP1/2 is required for efficient recognition of mitochondrial pre-tRNAs. (A) Representative figure of pre-tRNA binding to MRPP3 measured by changes in fluorescence polarization. Twenty nanomolars of FI-(mt)pre-tRNA^{Leu(UUR)} was titrated against increasing concentrations of MRPP3 (0–50 μ M) using standard binding conditions. (B) Representative figure of pre-tRNA binding to MRPP1/2 measured by changes in fluorescence polarization. Twenty nanomolars of FI-(mt)pre-tRNA^{Leu(UUR)} was titrated against increasing concentrations of MRPP1/2 (0–1 μ M) using standard binding conditions. (C) Representative figure of pre-tRNA binding to MRPP3 in the presence of 150 nM MRPP1/2 measured by changes in fluorescence polarization. Twenty nanomolars of FI-pre-(mt)tRNA^{Leu(UUR)} was titrated against the increasing amount of MRPP3 (0–1 μ M) using standard binding conditions. (A.U.) arbitrary unit. (D) Average dissociation constants for the mtRNase P complex and investigated pre-tRNAs, measured as described in C.

PRORP1 and PRORP2; therefore, we sought to address whether cleavage catalyzed by PRORP1 and 2, as measured by single-turnover reaction rates, is also slow for these substrates. In good agreement with our binding data, PRORP1 and PRORP2 processed most human (mt)pre-tRNAs with single-turnover rates more than 10-fold slower than that of mtRNase P, except for (mt)pre-tRNA^{Ser(UCN)} (Fig. 3D; Supplemental Table 2). (Mt)pre-tRNA^{Ser(UCN)} is not processed efficiently by the mtRNase P but is cleaved by PRORP1 and 2 with single-turnover rate constants comparable to that of their native substrates (~ 0.3 – 0.7 min^{−1}) (Fig. 3D; Supplemental Table 2). Together with our binding data, this suggests that human mitochondrial pre-tRNAs are recognized differently by *A. thaliana* PRORPs and human mtRNase P.

MRPP3 can enhance the methylation activity of the MRPP1/2 subcomplex

The MRPP1/2 subcomplex was previously shown to methylate in vitro transcribed human (mt)pre-tRNAs (Vilardo

et al. 2012). Here, we measured the single-turnover rate constants for MRPP1/2 catalyzing methylation of (mt)pre-tRNAs containing potential m1A9/m1G9 modification sites [(mt)pre-tRNA^{Ile}, (mt)pre-tRNA^{Leu(UUR)}, and (mt)pre-tRNA^{Val}] (Figs. 1 and 4A). To determine the rate of methylation, we developed a fluorescence primer extension assay for the mtRNase P complex (described in Materials and Methods; Fig. 4B). Additionally, we also assessed if the presence of MRPP3 modulates MRPP1/2 methylation activity. Our data demonstrate that the single-turnover rate constants ($k_{\text{obs, meth}}$) for methylation of (mt)pre-tRNA^{Ile} are 0.13 ± 0.02 min^{−1} catalyzed by near-saturating MRPP1/2 concentrations and that the addition of MRPP3 does not alter the $k_{\text{obs, meth}}$ value for this substrate (Fig. 4C,F). In comparison, methylation rates for (mt)pre-tRNA^{Leu(UUR)} are a modest twofold higher when MRPP3 is present (Fig. 4D,F) (0.08 ± 0.01 min^{−1} in the absence and 0.15 ± 0.03 min^{−1} in the presence of MRPP3). MRPP1/2 also catalyzed methylation of (mt)pre-tRNA^{Val}, however, the rates are significantly slower ($k_{\text{obs, meth}} < 0.005$ min^{−1}) compared to the other two (mt)pre-tRNA substrates, suggesting that there are differences in how

MRPP1/2 binds and methylates each (mt)pre-tRNA substrate (Fig. 4E).

SAM is required for mtRNase P to efficiently bind and process some human mitochondrial pre-tRNAs

As discussed above, we found that some human mitochondrial pre-tRNAs are not efficient substrates for the reconstituted mtRNase P complex in vitro. However, in vivo RNA sequencing experiments suggest that most mitochondrial tRNAs are processed by the mtRNase P complex, including (mt)pre-tRNA^{Val} and (mt)pre-tRNA^{Ser(UCN)} (Rackham et al. 2016). This led us to consider whether some substrates, such as (mt)pre-tRNA^{Val} and (mt)pre-tRNA^{Ser(UCN)}, might require either additional modification steps prior to mtRNase P cleavage, or additional cofactors, such as a protein or small molecule.

As a first step, we investigated if the presence of the methyl donor, SAM, affects the binding affinity of the six (mt)pre-tRNAs used in this study (Fig. 1.) We added a high concentration of SAM (25 μ M) to our fluorescence

FIGURE 3. Some human mitochondrial pre-tRNAs are efficiently cleaved by the mtRNase P complex in vitro. (A) Representative figure of single-turnover cleavage of 20 nM (mt)pre-tRNA^{Leu(UUR)} measured using changes in fluorescence polarization in the presence of the mtRNase P complex (closed triangles), MRPP3 only (open circles), or MRPP1/2 only (open diamonds). Control reaction containing only FI-(mt)pre-tRNA^{Leu(UUR)} is marked by closed circles. (B) Dependence of single-turnover rate constant, k_{obs} , for cleavage of (mt)pre-tRNA^{Leu(UUR)} on the concentration of MRPP3 in the presence of 0.5 μ M MRPP1/2. (Black square) k_{obs} measured in the presence of 0.5 μ M MRPP1/2. (C) Average single-turnover cleavage rates at saturating MRPP3 in the presence of 0.4 μ M MRPP1/2 for investigated pre-tRNAs. (D) Average observed single-turnover cleavage rates catalyzed by saturating concentrations of PRORP1 and 2 for investigated human pre-tRNAs.

anisotropy binding assays and measured (mt)pre-tRNA binding affinities for the MRPP1/2 and mtRNase P complexes (Supplemental Tables 3 and 4). We find that the presence of SAM enhances the binding affinity of (mt)pre-tRNA^{Val} for both the MRPP1/2 and the mtRNase P complexes ≥ 15 -fold (Fig. 5A,B; Supplemental Table 3). Next, we determined if this effect is due to the presence of SAM or methylation of the m1A9 site of (mt)pre-tRNA^{Val}. We first measured the (mt)pre-tRNA^{Val} binding affinities for MRPP1/2 and mtRNase P in the presence of the cofactor product of methyl transfer, SAH, demonstrating an at least approximately threefold but up to sixfold decrease in $K_{D,app}$ (Fig. 5A,B; Supplemental Table 3). To further examine whether methylation is required for the increased binding affinity, we measured the $K_{D,app}$ of MRPP1/2 and mtRNase P for a methylation site mutant of (mt)pre-

tRNA^{Val} [(mt)pre-tRNA^{Val/C9}]. The addition of either SAM or SAH enhances the binding affinity of the (mt)pre-tRNA^{Val/C9} mutant to the same extent as for the wild-type substrate (Fig. 5A,B; Supplemental Table 3). This suggests that the presence of SAM, but not methylation, is important for (mt)pre-tRNA^{Val} substrate recognition.

In addition, we find that (mt)pre-tRNA^{Leu(UUR)} binds the MRPP1/2 complex approximately four- to fivefold tighter in the presence of SAM and SAH (Fig. 5A), similar to (mt)pre-tRNA^{Val}. However, binding affinities for mtRNase P or MRPP1/2 subcomplex for the remaining four substrates are not significantly altered in the presence of SAM (Supplemental Table 4).

We next investigated if the presence of SAM and SAH impact the ability of mtRNase P to cleave the 5' end of (mt)pre-tRNAs. We find that the single-turnover rate constants for the 5' end cleavage of (mt)pre-tRNA^{Val} by mtRNase P is increased by three- to sevenfold in the presence of SAH or SAM, respectively (Fig. 5C; Supplemental Table 3). Moreover, the single-turnover cleavage rates for (mt)pre-tRNA^{Val/C9} catalyzed by mtRNase P exhibit a similar pattern (Fig. 5C; Supplemental Table 3). Despite this rate enhancement, it is noteworthy that the rate constant for the processing of (mt)pre-tRNA^{Val} in the presence of SAM is approximately three times slower than for any of the other investigated (mt)pre-tRNAs without the presence of SAM (Table 1; Supplemental Table 3). Addition of SAM or SAH did not enhance the single-turnover rate constants for any of the other (mt)pre-tRNAs that we investigated (Supplemental Table 4). Our results demonstrate that SAM increases the binding efficiency and 5' end processing catalyzed by mtRNase P for a subset of (mt)pre-tRNAs.

Some mitochondrial pre-tRNAs exhibit low melting temperatures and structural instability

We have previously observed that not all investigated in vitro transcribed human mitochondrial pre-tRNAs were bound and processed efficiently by mtRNase P. This prompted us to study the structural integrity of in vitro transcribed pre-tRNAs by UV melting experiments. In general,

FIGURE 4. MRPP1/2 methylates human mitochondrial pre-tRNAs. (A) Schematic representation of m1A9/m1G9 methylation catalyzed by MRPP1/2 in the presence of SAM. (SAH) S-adenosyl homocysteine. (B) Scheme for primer extension methylation assays. 5-FAM labeled primers (black arrow) were designed to base pair with the anticodon loop. In the absence of m1A9/m1G9 methylation, reverse transcriptase (RT) reads all the way to the 5' end of pre-tRNA (dashed line). When pre-tRNA is methylated at m1A9 or m1G9 position, RT stops at this modification site. (C–E) Representative gels for methylation of (mt)pre-tRNA^{Ile}, (mt)pre-tRNA^{Leu(UUR)}, and (mt)pre-tRNA^{Val} in the presence (left panel) and absence (middle panel) of MRPP3. Substitution of SAH for SAM was used as a negative control for methylation (right panel). Black arrows indicate the full-length and methylation stop primer extension products. Asterisks indicate nonspecific bands (SAM-independent bands appear in the presence of both SAM and SAH, whereas SAM-specific bands are only present when assay contains SAM). (F) Example quantification of the gel-based primer extension methylation assays for (mt)pre-tRNA^{Ile} (open symbols) and (mt) pre-tRNA^{Leu(UUR)} (closed symbols) in the presence (circles) and absence (squares) of MRPP3.

FIGURE 5. Presence of SAM enhances recognition and 5' end processing of some mitochondrial pre-tRNAs by mtRNase P. (A) Average dissociation constants for (mt)pre-tRNA^{Val} and (mt)pre-tRNA^{Leu(UUR)} for MRPP1/2 in the absence or presence of 25 μM SAM or SAH. (B) Average dissociation constants for mtRNase P and (mt)pre-tRNA^{Val} in the absence or presence of 25 μM SAM or SAH. (C) Average observed single-turnover cleavage rates for the mtRNase P complex and (mt)pre-tRNA^{Val}. The reaction was measured by gel-based cleavage assay in the presence of 625 nM MRPP1/2, 1875 nM MRPP3, and 80 nM labeled pre-tRNA.

tRNAs exhibit several structural transitions while unfolding as a function of increasing temperature, manifesting as hyperchromicity that can be monitored at 260 nm (Mustoe et al. 2015). The first major transition is traditionally assigned to the melting of the L-shaped tertiary structure (Mustoe et al. 2015). Therefore, we focused on dissecting the first transition in the UV melting curve of mitochondrial pre-tRNAs (Fig. 6A).

Analyzing the UV melting temperature of (mt)pre-tRNA^{Ile}, (mt)pre-tRNA^{Val}, (mt)pre-tRNA^{Leu(UUR)}, (mt)pre-tRNA^{Met}, and (mt)pre-tRNA^{Ser(AGY)}, we found that they exhibited two to four major transitions similarly to previous findings for other tRNAs, such as human mitochondrial tRNA^{Ser(UCN)} (Mustoe et al. 2015). First transitions of (mt)pre-tRNA^{Ile}, (mt)pre-tRNA^{Val}, and (mt)pre-tRNA^{Ser(AGY)} manifested above 42°C (Fig. 6A; Supplemental Table 5) suggesting proper folding. On the other hand, for (mt)pre-tRNA^{Leu(UUR)} and (mt)pre-tRNA^{Met} we observed first transitions with lower melting temperature ($28 \pm 0.2^\circ\text{C}$ and $32.6 \pm 1.1^\circ\text{C}$, respectively) than the physiological temperature (37°C) (Fig. 6A; Supplemental Table 5). This indicates that at physiological temperature these two mitochondrial tRNAs (as transcribed and refolded with our standard protocol and without any modifications) may not be fully folded or may exhibit a difference from the typical and expected for tRNAs structure. Because most reported 5' end pre-tRNA cleavage reactions by mtRNase P have been performed at 28°C , we sought to address the effect

of an increased temperature closer to physiological levels on mtRNase P nuclease activity. At 37°C we observed no 5' end cleavage of (mt)pre-tRNA^{Leu(UUR)} and (mt)pre-tRNA^{Met} by mtRNase P (Fig. 6B). Notably, melting curves for (mt)pre-tRNA^{Ser(UCN)} did not show the expected pattern of a fully folded tRNA suggesting that this (mt)pre-tRNA may have multiple overlapping transitions or it does not adopt the L-shaped tertiary structure.

DISCUSSION

Given the essential nature of human mtRNase P and its direct link to disease, it is important to understand how this protein complex functions. Despite its importance, it is

FIGURE 6. Structural integrity of (mt)pre-tRNAs is important for pre-tRNA recognition. (A) Representative figures for UV melting experiment for five investigated (mt)pre-tRNAs: (mt)pre-tRNA^{Leu(UUR)} (squares), (mt)pre-tRNA^{Ile} (diamond), (mt)pre-tRNA^{Val} (triangles), (mt)pre-tRNA^{Met} (circles), (mt)pre-tRNA^{Ser(AGY)} (upside-down triangles). (B) The mtRNase P-complex processes the 5' end of (mt)pre-tRNA^{Leu(UUR)} and (mt)pre-tRNA^{Met} at 28°C but not at 37°C .

mostly unclear how mtRNase P binds and processes its (mt) pre-tRNA substrates. Significantly, how the three components of the mtRNase P interact remains a major question. Here we directly address these critical knowledge gaps by investigating how human mtRNase P binds, cleaves, and methylates representative human mitochondrial pre-tRNAs. Our data demonstrate that there is extensive interplay between the mtRNase P components (MRPP1/2/3 and the TRMT10C [MRPP1] cofactor SAM) required to recognize, process, and methylate human mitochondrial pre-tRNAs. We found that mtRNase P exhibits distinct modes of substrate recognition for individual human mitochondrial pre-tRNAs, in contrast to the homologous single-protein component PRORPs that bind and cleave their native substrates more uniformly (Howard et al. 2016).

The mitochondrial genome encodes 22 tRNAs that fall into four different types (0–III) based on their predicted secondary structures. The secondary structures of type 0 (mt)tRNAs are similar to those of canonical nuclear tRNAs, whereas types I–III vary distinctly. Type I–III (mt) tRNAs differ in the length of their anticodon stems, D- and T-loops, or lack the D-loop entirely [(mt)pre-tRNA^{Ser(AGY)}; Watanabe et al. 2014; Rackham et al. 2016]. In our study, we investigated the processing of pre-tRNAs representative of all four categories of mitochondrial pre-tRNAs: (mt)pre-tRNA^{Leu(UUR)} (type 0), (mt)pre-tRNA^{Ser(UCN)} (type I), (mt)pre-tRNA^{Ile} (type II), (mt)pre-tRNA^{Met} (type II), (mt)pre-tRNA^{Val} (type II), and (mt)pre-tRNA^{Ser(AGY)} (type III). These pre-tRNAs are of significant interest because all are hot spots for mutations responsible for a plethora of human mitochondrial diseases (Rossmanith and Karwan 1998; Yan et al. 2006; Wang et al. 2011; Wortmann et al. 2012). Additionally, (mt)pre-tRNA^{Val} is of note because it is transcribed between the two mitochondrial rRNAs and is proposed to play an unusual and noncanonical structural role as part of the human mitochondrial ribosome (Amunts et al. 2015; Greber et al. 2015).

Using our selected substrates, we determined how both individual components of the mtRNase P (MRPP3 and MRPP1/2) and the mtRNase P complex bind type 0–III (mt)pre-tRNAs. We find that MRPP3 and MRPP1/2 individually bind most (mt)pre-tRNAs with low affinity ($K_{D,app}$ values in μ M range). However, for (mt)pre-tRNA^{Leu(UUR)}, (mt)pre-tRNA^{Ile}, and (mt)pre-tRNA^{Met}, binding affinities are significantly ($K_{D,app}$ values in nM range) enhanced when the entire complex is present. This suggests that the different components in the mtRNase complex depend on each other to recognize some substrates. The substrates that bind weakly to mtRNase P include a variety of types [type I (mt)pre-tRNA^{Ser(UCN)}, type II (mt)pre-tRNA^{Val}, and type III (mt)pre-tRNA^{Ser(AGY)}]. The inability of the complex to bind (mt)pre-tRNA^{Ser(AGY)} was not surprising because this substrate lacks the entire D-loop and stem (Fig. 1) and is not a substrate of mtRNase P in vivo (Rossmanith 1997). However, the weaker binding of (mt)pre-tRNA^{Val}

and (mt)pre-tRNA^{Ser(UCN)} was unexpected because there are no obvious structural or sequence differences that explain the low affinity with mtRNase P.

The efficiency of mtRNase P catalyzing the 5' end cleavage of particular (mt)pre-tRNAs follows the same pattern as substrate binding; (mt)pre-tRNAs that associate tightly are processed eight to 20 times faster (single-turnover $k_{obs} = 0.4$ – 0.8 min^{-1}) than substrates that bind weakly ($k_{obs} \sim 0.06 \text{ min}^{-1}$). We note that the estimated multiple-turnover constants ($k_{cat} \sim 0.4$ – 0.7 min^{-1}) are similar to these observed kinetic constants in single-turnover assays, suggesting that product release is not a rate-limiting step in the catalytic cycle of mtRNase P, similarly to the plant homologs (Supplemental Table 6; Supplemental Fig. 5; Howard et al. 2016). The correlation between weak binding and slow cleavage rates at saturating enzyme concentrations suggests that these pre-tRNA substrates do not bind optimally for cleavage. Notably, all (mt)pre-tRNAs that are recognized and processed efficiently by human mtRNase P are type 0 or II substrates. However, not all type II pre-tRNAs are efficiently processed by mtRNase P, such as (mt)pre-tRNA^{Val}, suggesting that the “type” of (mt)pre-tRNA structure is insufficient to predict how mtRNase P will recognize any given substrate. Our data indicate that the recombinant mtRNase P does not recognize all substrates equally, leading us to posit that additional factors beyond MRPP1/2 and MRPP3 may be required to ensure that all (mt)pre-tRNAs are efficiently processed in cells.

To gain further insight into the interplay between 5' end pre-tRNA processing and methylation activities of mtRNase P, we measured the single-turnover methylation rates of MRPP1/2 in the presence and absence of MRPP3. These assays were performed using the (mt)pre-tRNAs that possess m1A9/m1G9 methylation sites [(mt)pre-tRNA^{Ile}, (mt)pre-tRNA^{Leu(UUR)}, (mt)pre-tRNA^{Val}]. We find that single-turnover rates for methylation were at least five times slower than 5' end processing [Table 1; Fig. 4, e.g., $k_{obs,cleav} \sim 0.8 \text{ min}^{-1}$ and $k_{obs,meth} \sim 0.13 \text{ min}^{-1}$ for (mt)pre-tRNA^{Ile}], suggesting that cleavage may occur prior to methylation. This finding agrees with previous studies that assessed (mt)pre-tRNA^{Ile} methylation catalyzed by mtRNase P (Vilardo and Rossmanith 2015). We note that measured single-turnover methyltransferase activities with our (mt)pre-tRNAs were significantly lower (~ 10 -fold) than previously measured for TRMT10C (MRPP1) and its paralog ($k_{obs} = \sim 1 \text{ min}^{-1}$), TRMT10A with 3' end mature substrates (Vilardo and Rossmanith 2015; Falk et al. 2016; Krishnamohan and Jackman 2017). This suggests that the methyltransferase activity of TRMT10C (MRPP1) may be facilitated by tRNA 3' end maturation and further indicates that methyl transfer is not the first step of tRNA maturation pathway in the mitochondria. Recent work indicated that 5' end processing and methylation of (mt)pre-tRNA^{Ile} catalyzed by mtRNase P are carried out by the

components of mtRNase P independently, and do not affect one another, further suggesting the structural, but not catalytical role of MRPP1/2 in 5' end processing (Vilardo et al. 2012; Vilardo and Rossmanith 2015). Although our results with (mt)pre-tRNA^{Ile} and (mt)pre-tRNA^{Val} corroborate this suggestion, we find that the presence of MRPP3 modestly increases the rate constant for methylation of (mt)pre-tRNA^{Leu(UUR)} by MRPP1/2. This implies that the two functions of mtRNase P are not entirely decoupled, and that there may be cooperation between the 5' end tRNA processing and methyltransferase functions of mtRNase P for some substrates.

Based on in vivo experiments (Holzmann et al. 2008; Rackham et al. 2016), (mt)pre-tRNA^{Val} is predicted to be a substrate of mtRNase P. However, we show that this substrate is not recognized and processed efficiently by recombinant mtRNase P in vitro. It has been previously demonstrated that (mt)tRNA^{Lys} requires methylation at the ninth base in order to fully adopt the cloverleaf secondary structure (Helm et al. 1998; Sissler et al. 2004; Motorin and Helm 2010). This result raised the possibility that methylation of (mt)pre-tRNA^{Val} at the ninth position is necessary for this substrate to fold (and subsequently be processed by mtRNase P), akin to (mt)tRNA^{Lys}. Consistent with this model, we observed that the binding and cleavage of (mt)pre-tRNA^{Val} by mtRNase P is significantly enhanced by the addition of SAM (Fig. 5). However, similar effects are observed upon either addition of SAH, a SAM analog that does not function as a cofactor for methylation catalyzed by MRPP1/2, or using the A to C9 methylation site mutant of (mt)pre-tRNA^{Val} (Fig. 5). These results suggest that the SAM-mediated effect on (mt)pre-tRNA^{Leu(UUR)} and (mt)pre-tRNA^{Val} binding affinity and processing by mtRNase P is linked to cofactor binding to MRPP1/2 rather than pre-tRNA methylation. There are several examples in the literature in which SAM acts as an effector molecule affecting protein function and activity; SAM is an allosteric regulator of human cystathione-β-synthase and histidine trimethylase EgtD from *Mycobacterium smegmatis* (Lin et al. 2012; Jeong et al. 2014; McCorvie et al. 2014). Furthermore, in the presence of SAM, a bifunctional restriction enzyme harboring a methyltransferase domain, RM.BpuSI, had increased processing activity without significant methylation activity (Sarrade-Loucheur et al. 2013), similar to mtRNase P acting on (mt)pre-tRNA^{Val}. Additionally, a recent study showed an increase in melting temperature for TRMT10C (MRPP1) in the presence of SAM indicating a stabilizing effect of the cofactor (Oerum et al. 2018). Based on our data, we propose that the presence of SAM bound to TRMT10C (MRPP1) enhances the ability of TRMT10C (MRPP1) to bind some pre-tRNA substrates. Our finding also agrees well with the previous observation (Vilardo and Rossmanith 2015) that MRPP1/2 mainly provides structural support for MRPP3.

Although we observe that SAM is a key factor for recognition of (mt)pre-tRNA^{Val} by MRPP1/2 and subsequently MRPP3, (mt)pre-tRNA^{Ser(UCN)} is bound to MRPP1/2 efficiently (Table 1) but to mtRNase P poorly. Therefore, one possibility is that the recognition of the MRPP1/2-(mt)pre-tRNA^{Ser(UCN)} complex by MRPP3 requires an unidentified mitochondrial factor; however, this needs further testing. (mt)pre-tRNA^{Ser(UCN)} was selected for study because it possesses several unusual secondary structure features (shorter D-, variable loop and connector, longer anticodon stem; Fig. 1). Because it is possible that these unique deviations from canonical tRNA sequence and structural features may impact recognition by mtRNase P, we performed domain/element-swapping studies. We systematically modified the shorter D-, variable loop and connector, longer anticodon stem regions of (mt)pre-tRNA^{Ser(UCN)} to introduce canonical secondary structural elements [using (mt)pre-tRNA^{Ile} as a template]. These modifications in pre-tRNA^{Ser(UCN)}, which were introduced one at a time, did not enhance binding or 5' end processing by mtRNase P (data not shown). It is possible that several of these modifications in pre-tRNA^{Ser(UCN)} may have to be introduced in parallel in order for it to become a better in vitro substrate for mtRNase P. These results further suggest that pre-tRNA^{Ser(UCN)} recognition by mtRNase P in human mitochondria may require additional factors, such as interactions with chaperones that have yet to be discovered.

In nonmetazoan eukaryotes, the MRPP3-homologous PRORP enzymes are stand-alone proteins able to cleave a wide variety of substrates from different organisms (Gobert et al. 2010; Howard et al. 2015; Karasik et al. 2016). To gain insight into potential differences as to how single-protein PRORPs and complex-forming MRPP3 enzymes select and cleave their substrates, we assessed the ability of *A. thaliana* PRORP1 and PRORP2 to recognize and process human (mt)pre-tRNAs. Our results suggest that complex-forming protein-only RNase Ps recognize their substrates differently than single-enzyme PRORPs. Because *A. thaliana* PRORPs recognize the L-shaped tertiary structure of pre-tRNAs (Gobert et al. 2013; Imai et al. 2014; Klemm et al. 2017; Pinker et al. 2017), we propose that mtRNase P may interact with pre-tRNAs by recognizing local structural elements or specific bases within the (mt)pre-tRNAs. Some human mitochondrial tRNAs are known to adopt alternate secondary structures (Lorenz et al. 2017) and mtRNase P may have conformed to be able to recognize these noncanonical structures.

To further dissect the uniqueness of mitochondrial pre-tRNAs used in this study, we investigated their structural integrity by UV melting analysis. We found that some pre-tRNAs [(mt)pre-tRNA^{Leu(UUR)} and (mt)pre-tRNA^{Met}] unfold at lower than physiological temperatures suggesting that these precursors may not be properly structured in vivo. It

is possible that these in vitro transcribed pre-tRNAs need to be stabilized by other proteins in the mitochondria directly or through tRNA modifications. Our preliminary UV melting data combined with our kinetic data indicate that (mt)pre-tRNAs as transcribed may not fold into the expected structures. It is clear that the systematic and rigorous characterization of (mt)pre-tRNAs structure is required that includes structural probing and crystallographic studies. Because MRPP1/2 act as a platform for both 3' and 5' end pre-tRNA processing and subsequent events (Reinhard et al. 2017), it is conceivable that some of these pre-tRNAs may require the presence of an additional partner, such as a tRNA processing or modification enzyme. The additional partner(s) would bind to this MRPP1/2 platform so as to properly fold problematic tRNAs and prime them for 5' end cleavage; however, this hypothesis needs further testing. Our data indicate that at physiological temperatures some pre-tRNAs may be lacking the proper tertiary structure—for example, the integrity of the elbow region may be compromised. We posit that tRNA canonical structural features would be required for their in vitro binding to and processing by mtRNase P. We have excluded the possibility that mtRNase P is out of its optimal temperature range at 37°C, because it is able to efficiently bind and cleave fully folded *Bacillus subtilis* pre-tRNA^{Asp} at this temperature (Liu et al. 2019). Our findings suggest that some in vitro transcribed human mitochondrial pre-tRNAs at physiological temperatures have reduced thermostability and that their in vivo processing by mtRNase P may require either additional factors or modifications. Although the conditions (such as Mg²⁺ concentration and pH) for the UV melting experiments were selected to match those found in mitochondria, these in vitro conditions may not fully recapitulate the actual in vivo environment in mitochondria.

Taken together, our in vitro studies demonstrate that different human mitochondrial pre-tRNAs interact distinctly with mtRNase P, and this recognition mode is potentially an important determinant for 5' tRNA processing. Additionally, we found that some substrates are not efficiently bound and processed by mtRNase P, suggesting a role of other factors in mitochondrial 5' end tRNA processing. In line with this, we found that SAM, the cofactor of TRMT10C (MRPP1), can enhance substrate recognition and 5' processing by mtRNase P. Our data reveal that single-subunit PRORPs and human mitochondrial RNase P have distinct modes of pre-tRNA recognition. In addition, our data provide the first evidence that there is an interplay between the 5' end (mt)pre-tRNA processing and methylation functions of mtRNase P. Together, this work builds on the understanding of the role of MRPP1/2 developed previously (Vilardo et al. 2012) and provides an expanded framework for how the different components of mtRNase P work together to bind, process, and methylate mitochondrial pre-tRNAs.

MATERIALS AND METHODS

In vitro transcription and 5' end labeling of pre-tRNAs

Pre-tRNAs were prepared as previously described (Howard et al. 2012; Karasik et al. 2016). Briefly, to label the 5' end of tRNA with fluorescein, we incubated ~200 μ L of 5' GMPS (guanosine-5-monophosphorothioate) labeled in vitro transcribed pre-tRNA overnight (in Tris-EDTA buffer of pH 7.2) with 20 μ L 45 mM fluorescein (final concentration ~4 mM) at 37°C, and the reaction was purified on a 12% urea-polyacrylamide gel. After gel purification, pre-tRNAs were ethanol-precipitated and the resulting pellet was resuspended in RNase free H₂O. The concentrations of total and labeled pre-tRNA were measured from absorbance readings at 260 nm using a Nanodrop (Thermo Scientific) spectrophotometer using the following extinction coefficients: 909,100 cm⁻¹ mol⁻¹ for (mt)pre-tRNA^{Ile7:0}, 927,300 cm⁻¹ mol⁻¹ for (mt)pre-tRNA^{Leu(UUR)6:0}, 848,600 cm⁻¹ mol⁻¹ for (mt)pre-tRNA^{Val7:0}, 838,200 cm⁻¹ mol⁻¹ for (mt)pre-tRNA^{Met6:0}, 848,600 cm⁻¹ mol⁻¹ for (mt)pre-tRNA^{Ser(UCN)7:0}, and 737,900 cm⁻¹ mol⁻¹ for (mt)pre-tRNA^{Ser(AGY)7:0}.

Protein expression and purification

His₆- Δ 95 MRPP3 was cloned into a pMCSG7 vector and transformed into *Escherichia coli* BL21(DE3) cells. Transformed bacteria were grown overnight (5 mL LB media, 100 μ g/mL ampicillin, 37°C [220 rpm]), and used to inoculate 500 mL of TB media with 50 μ g/mL ampicillin. Cultures were grown at 37°C to an OD₆₀₀ of ~0.8–1.0, and then shifted for 1 h to 18°C. In total, 200 μ M final concentration of isopropyl β -D-1 thiogalactopyranoside (IPTG) was added to induce protein expression, and the culture was grown overnight (~16 h) at 18°C, 220 rpm. Bacterial cells were collected by centrifugation (10,000g, 15 min) and resuspended in lysis buffer (20 mM MOPS pH 7.8, 1 M NaCl, 1 mM TCEP, 0.2% [v/v] Tween, 0.1 mg/mL lysozyme and 0.1 mg/mL phenylmethylsulfonyl fluoride). Cells were lysed by sonication, and the resulting suspension was centrifuged at 34,000g for 1 h at 4°C. The supernatant was loaded onto a HisTrap (GE Healthcare) nickel column and protein was eluted with 500 mM imidazole, 20 mM MOPS pH 7.8, 100 mM NaCl, and 1 mM TCEP. The purified Δ 95 MRPP3 was dialyzed against 20 mM MOPS pH 7.8, 150 mM NaCl, 1 mM TCEP, and 15% glycerol overnight at 4°C and incubated with TEV protease (1:20 protein:TEV protease ratio) to remove the His-tag. His₆-TEV protease was removed by running the sample through a HisTrap nickel column. Δ 95 MRPP3 was then dialyzed against 20 mM MOPS pH 7.8, 1 mM TCEP, 150 mM NaCl, and 2 mM EDTA overnight at 4°C to remove any possible metal contamination, followed by buffer exchange with 20 mM MOPS pH 7.8, 1 mM TCEP, and 150 mM NaCl using a desalting column (BioRad). Protein concentration was measured using a Nano spectrophotometer (Thermo scientific) with molecular weight and extinction coefficient values of 56.4 kDa and 82,500 cm⁻¹ mol⁻¹, respectively.

Full-length MRPP2 and His₆- Δ 39 TRMT10C (MRPP1) were cloned and coexpressed as described (Liu 2013). The TRMT10C (MRPP1) amino-terminal deletion corresponds to the predicted mitochondrial targeting sequence. Initial steps for protein expression and purification, including nickel column purification,

histidine tag, and TEV removal, are identical to those detailed above for $\Delta 95$ MRPP3, with the following exceptions: The antibiotic used was streptomycin (50 $\mu\text{g}/\text{mL}$) and 10% glycerol was added to each purification buffer. The resulting $\Delta 39$ MRPP1/MRPP2 subcomplex was further purified by size exclusion chromatography in 20 mM MOPS pH 7.8, 1 mM TCEP, 100 mM NaCl, and 10% glycerol. Final protein samples were concentrated to 2–4 mg/mL. The ratio of the TRMT10C (MRPP1) and MRPP2 proteins in the subcomplex was determined to be 1:4 based on analytical ultracentrifugation experiments. Therefore, protein concentration was measured based on molecular weight and extinction coefficient values of 149 kDa and 79,520 $\text{cm}^{-1} \text{mol}^{-1}$. *Arabidopsis thaliana* PRORP1 and PRORP2 were expressed and purified from *E. coli* as previously described (Howard et al. 2012; Karasik et al. 2016).

Single-turnover cleavage assays

(mt)pre-tRNA substrates were refolded prior to each cleavage reaction. Pre-tRNAs samples were first heated for 3 min at 95°C and then cooled down to room temperature (~10–15 min). Cleavage reaction buffer (final: 30 mM MOPS pH 7.8, 150 mM NaCl, 1 mM MgCl_2 , 1 mM DTT) was added, and (mt)pre-tRNAs were further incubated for 30 min at room temperature. All single-turnover reactions were performed in the cleavage reaction buffer at 28°C. To initiate a reaction, a mixture of 500 nM $\Delta 39$ MRPP1/MRPP2 complex and variable concentrations (100 nM–2 μM) of $\Delta 95$ MRPP3 in cleavage reaction buffer were added to 20 nM fluorescently labeled pre-tRNA. Changes in fluorescence polarization were followed at 28°C using a ClarioStar plate reader (BMG LabTech). In these assays, we found that the changes in anisotropy observed in the presence of the MRPP1/2 subcomplex and MRPP3 in isolation are small (<1%–10%) relative to the significant changes in anisotropy due to cleavage catalyzed by the mtRNase P complex and as such do not impact the rate constants that we measure (Fig. 3A). The results of our fluorescence polarization kinetic assays were verified using traditional gel-based cleavage assays (Supplemental Fig. 1). For gel-based assays, various time points were obtained by quenching the reaction with the addition of 0.05% Bromophenol blue, 0.05% Xylene cyanol dye, 50% m/v urea, and 0.1 M EDTA. The resulting samples were fractionated on a polyacrylamide–urea gel (12%–20%) and visualized by direct scanning on a Storm 860 imager. Single-turnover assays for (mt) pre-tRNAval and its mutant version were also carried out using gel-based assays (Supplemental Table 3), which is more suitable to measure very low activities ($k_{\text{obs}} < 0.05 \text{ min}^{-1}$) because of reduced background. Densitometry was performed with ImageQuant. Data from at least three independent experiments (unless otherwise indicated) were analyzed using Kaleidagraph 4.1.3. Equation 1 was fit to the data to calculate the observed rate constant (k) and the standard error from the fitting where A is the endpoint, B is the amplitude, and t is the time. In addition, to obtain the $K_{1/2}$ value and maximal rate constant (k_{max}), Equation 2 was fit to the observed rate constants (k) determined at different MRPP3 concentrations (while MRPP1/2 kept at a constant 500 nM). The standard error presented is the error of fitting Equation 2. Furthermore, to ensure that amino-terminal truncation of $\Delta 95$ MRPP3 did not affect the single-turnover activity of mtRNase P, we also reconstituted mtRNase P with $\Delta 45$ MRPP3 lacking only the mitochondrial targeting sequence. We found

nearly identical single-turnover observed cleavage rates for both complexes (Supplemental Table 1).

$$\text{Fraction cleaved} = A - B^*e^{-kt}, \quad (1)$$

$$k = \frac{k_{\text{max}} [\text{MRPP3}]}{K_{1/2} \text{MRPP3} + [\text{MRPP3}]} \quad (2)$$

Multiple-turnover cleavage assays

Multiple-turnover cleavage assays were performed in 96-well plate format fluorescent polarization assays similarly to single-turnover assays. In these assays, we used 40 nM MRPP3 to achieve consistent $v_0/[E]$ values at a given concentration and excess amount (1 μM) MRPP1/2 to ensure mtRNase P complex formation. The enzyme mix was incubated with 100 nM fluorescently labeled pre-tRNA and varying amounts of unlabeled pre-tRNA in cleavage reaction buffer (final: 30 mM MOPS pH 7.8, 150 mM NaCl, 1 mM MgCl_2 , 1 mM DTT). Initial rates were calculated from the linear decrease of anisotropy. Multiple-turnover kinetic parameters were estimated by fitting Equation 3 (by using the software Prism) to the concentration-dependence of the initial rates:

$$\frac{v_0}{[E]} = \frac{k_{\text{cat}}[S]}{K_M[S]} \quad (3)$$

Fluorescence polarization binding assays

Binding assays were performed in 30 mM MOPS pH 7.8, 150 mM NaCl, 6 mM CaCl_2 , 1 mM DTT at 28°C and 20 nM of 5-FAM labeled and folded (mt)pre-tRNA. The (mt)pre-tRNA substrates were incubated with increasing concentrations (0–50 μM) of $\Delta 95$ MRPP3 or MRPP1/2 (0–2 μM) for 5 min before changes in anisotropy were measured with ClarioStar plate reader (BMG LabTech). We found that equilibrium binding constants after 5 min of incubation were not changed significantly. Twenty nanomolars of fluorescent pre-tRNA (FI-pre-tRNA) was titrated by adding increasing concentrations (0–50 μM) of $\Delta 95$ MRPP3 in the presence of 150 nM $\Delta 39$ MRPP1/MRPP2. In some experiments, 25 μM SAM was added to $\Delta 39$ MRPP1/MRPP2 and FI-(mt)pre-tRNA and incubated for 5 min before the addition of MRPP3. Baseline anisotropy values measured in the absence of the protein but the presence of the FI-pre-tRNA were extracted from measured values. The dissociation constant (K_D) for PRORPs, the apparent dissociation constant ($K_{D,\text{app}}$) for MRPP1/MRPP2 and mtRNase P, and the standard errors were calculated by fitting a binding isotherm (Equation 4) to the fluorescence polarization data from at least three independent experiments (unless otherwise indicated) using Kaleidagraph 4.1.3 software. In Equation 4, A is the observed anisotropy, A_0 is the initial anisotropy, ΔA is the total change in anisotropy, $[P]$ is the concentration of MRPP3, and $K_{D,\text{app}}$ is the dissociation constant:

$$A = A_0 + \frac{\Delta A[P]}{[P] + [K_{D,\text{app}}]} \quad (4)$$

Primer extension methylation assays

Pre-tRNA was folded as described above before each experiment. Methylation reactions were initiated by mixing the unlabeled pre-tRNA and $\Delta 39$ MRPP1/MRPP2 to final concentrations

of 800 nM of and 5 μ M, respectively, in 30 mM MOPS pH 7.8, 150 mM NaCl, 1 mM CaCl_2 , and 1 mM DTT at 28°C. The observed single-turnover methylation rates were measured at varied MRPP1/2 concentrations (2–6 μ M) for (mt)pre-tRNA^{Ile} and (mt) tRNA^{Leu(UUR)}, demonstrating little dependence on the concentration and confirming that 5 μ M MRPP1/2 corresponds to a near-saturating enzyme concentration (Supplemental Fig. 2). To terminate the methylation reaction, 2 μ L of enzyme-pre-tRNA reaction mixture was removed, incubated for 30 sec at 95°C, and then placed on ice. Annealing reactions for primer extensions were set up as follows for each quenched time point: A final volume of a 5 μ L annealing reaction contained 1 μ L of a quenched methylation reaction and 2 μ M of fluorescently labeled primers in 50 mM Tris-HCl (pH 8.1), 30 mM NaCl, and 10 mM DTT. Annealing primer sequences were designed against the anticodon loop for (mt) pre-tRNA^{Ile}, (mt)pre-tRNA^{Leu(UUR)}, (mt)pre-tRNA^{Val}, and (mt)pre-tRNA^{Met} (6-FAM-CTATTATTTACTCTATC; 6-FAM-CCTCTGACT GTAAAG; 6-FAM-CCTAAGTGTAAGTTGGG; and 6-FAM-CGG GGTATGGGCCCG, respectively). Next, annealing reaction mixtures were incubated for 3 min at 95°C followed by cooldown for 10–15 min to 37°C. As a control, previously folded pre-tRNA was used that was not incubated with the enzymes. Two microliters of annealed primer/(mt)pre-tRNA mixture was added to a final volume of 5 μ L RT reaction mixture containing 0.7 U of AMV-RT (Promega), 1 $\times$ AMV-RT reaction buffer (Promega), and 0.5 mM dNTP. The reverse transcription reactions were incubated for 1 h at 37°C and were subsequently quenched with equal amounts of 0.05% Bromophenol blue, 0.05% Xylene cyanol dye, 50% m/v urea, and 0.1 M EDTA and run on 12% polyacrylamide gel. The gels were scanned in a Storm 860 imager and results were evaluated by densitometry (ImageQuant). As a negative control, we substituted SAH for SAM. SAH mimics SAM but lacks the methyl group that is required for the methylation reaction carried out by MRPP1/2. As expected, we did not observe any stops due to methylation in the presence of SAH (Fig. 4C–E). Data from at least three independent experiments were analyzed using Kaleidagraph 4.1.3. Equation 1 was fit to the data to calculate the single-turnover rate constants ($k_{\text{obs, meth}}$) and the standard error.

UV melting experiments

Pre-tRNAs were folded as described above before each experiment using a “UV melting buffer” of the final concentration of 10 mM PIPES pH=7, 150 mM NaCl, 1 mM MgCl_2 , and 1 μ M pre-tRNA. Thermodynamic curves were obtained by heating up the samples 1°C/min from 15°C to 95°C while absorbance at 260 nm was monitored at every 0.5°C or 1°C using a Cary 300 spectrophotometer. Data were corrected with data from blank measurements and analyzed with fitUVData.py and Global Melt Fit (Draper et al. 2000; Mustoe et al. 2015). Most pre-tRNA showed three or four transitions in agreement with previous findings (Mustoe et al. 2015). Average T_m s and standard errors were calculated from at least three independent experiments, and bootstrapping analysis was performed to ensure the robustness of the data where it was applicable.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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