Purification and Characterization of the *Saccharomyces cerevisiae* Mitochondrial Leucyl-tRNA Synthetase*

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We have purified the product of the NAM2 gene, the mitochondrial leucyl-tRNA synthetase, from yeast mitochondria. The purified protein cross-reacts with antibodies raised against the product of a *LacZ/NAM2* gene fusion and antibodies raised against the purified *Escherichia coli* leucyl-tRNA synthetase. The mass as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is about 100 kDa, consistent with the size predicted by the gene sequence (102 kDa). The N-terminal sequence of the protein has been determined and shows that the first nine amino acids predicted by the gene sequence have been removed, probably during transport into the mitochondria.

Pre-mRNA splicing in yeast mitochondria is a complex process that requires proteins encoded by both the nuclear and the mitochondrial genome. The nuclear gene *NAM2* of *Saccharomyces cerevisiae* was originally identified and defined by dominant alleles able to suppress mutations that inactivate the maturase encoded by the fourth intron of the mitochondrial cytochrome b gene (*b14* maturase) (Dujardin *et al.*, 1980). This maturase is required for the excision of two introns, its own intron *b14*, and the fourth intron (*a14*) of the mitochondrial gene encoding subunit I of cytochrome oxidase (*cox1*) (Dhawale *et al.*, 1981; De La Salle *et al.*, 1982; Labouesse and Slonirmski, 1983; Labouesse *et al.*, 1984). These two introns are similar, and both encode proteins. The *a14* protein has an endonuclease activity (Delahodde *et al.*, 1989; Wemlau *et al.*, 1989), but despite considerable homology with the *b14* maturase it does not appear to act as an RNA maturase under normal conditions. However, a single substitution causing a glutamate to be replaced by a lysine activates a "latent" maturase activity in the *a14* protein, which is then able to participate in the excision of both *a14* and *b14* (Dujardin *et al.*, 1982). Furthermore, genetic studies on the NAM2 suppressor alleles have indicated that the protein encoded by the *a14* intron is essential for suppressor activity (Dujardin *et al.*, 1983). This led to the hypothesis that the effect of the suppressor mutations is to enable the NAM2 protein to activate the *a14* maturase by a protein-protein interaction, possibly by changing the affinity of the NAM2 protein for this maturase, and that in the wild-type situation the NAM2 protein would interact with the *b14* maturase (Herbert *et al.*, 1988a). This hypothesis is supported by the observation that when cloned on a multicopy plasmid the wild-type NAM2 gene has a low but measurable suppressor activity (Labouesse *et al.*, 1987).

The NAM2 gene has been cloned and sequenced; analysis of the sequence shows that it could encode a protein of 894 amino acids with a molecular mass of 102 kDa. The deduced protein sequence shows considerable homology with the *Escherichia coli* leucyl-tRNA synthetase (LRS),1 34% identical residues and 20% conservative replacements in 839 amino acids that can be unambiguously aligned, suggesting that the NAM2 gene might encode the mitochondrial leucyl-tRNA synthetase (mLRS). This was confirmed by the observation that the levels of the mLRS in crude mitochondrial extracts varied with the copy number of the NAM2 gene and that no activity was detected in extracts made from a strain in which the gene was deleted. Finally, antibodies raised to the C-terminal part of the NAM2 protein inhibit mLRS activity in crude extracts (Herbert *et al.*, 1988a). Independently, Tzagoloff *et al.* (1988) cloned the *S. cerevisiae* mLRS, and the coding sequence of the gene was identical to the sequence of the NAM2 gene.

To date, two aminoacyl-tRNA synthetases have been shown to be involved in fungal pre-mRNA splicing, the tyrosyl-tRNA synthetase in *Neurospora crassa* (Akins and Lambowitz, 1987) and the mLRS in *S. cerevisiae* (Herbert *et al.*, 1988a). Thus, in addition to their aminoacyl-tRNA synthetase functions it is reasonable to assume that these proteins are able to bind to the pre-mRNA, and in the case of the mLRS, possibly form a complex with a pre-mRNA maturase. To further our studies on the role of the mLRS in mitochondrial pre-mRNA splicing and its interactions with other components of the splicing system we decided to purify the protein. Here we report a procedure for a rapid and simple purification of the mLRS, a preliminary characterization of the enzyme, and the determination of the N-terminal sequence of the purified protein.

**MATERIALS AND METHODS**

*Yeast Strains and Growth Medium.*—The rho*,mit* wild-type strain CW04 has the nuclear genotype α·his3-11,15,leu2-3,112,ade2-1,ura3-1,try1-1,can1-100,NAM2* (Banroques *et al.*, 1986). HM200/1 is a rho*,mit* strain carrying the plasmid YCPGMC068 and has the same nuclear genotype as CW04 except that it is nam2::LEU2. Construc-

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1 The abbreviations used are: LRS, leucyl tRNA synthetase; mLRS, mitochondrial LRS; mMRS, mitochondrial methionyl tRNA synthetase; SDS, sodium dodecyl sulfate.
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Purification of the NAM2-encoded mLRS was followed by assaying the formation of the tRNA charged with radioactive -leucine. This reaction was carried out at 10 mM ATP, 10 mM MgCl₂ with methionine as a substrate. Initial velocity conditions were used for the determination of the kinetic constants and 10-min reactions for following the enzyme fractionation and optimizing the conditions. After incubation, 20 µl (10 µl for initial velocity measurements) of the aminoacylation mixture was applied to a column of heparin-Ultrogel (to 0.1%), concentrated separately 10-12 times with Centricon 10-300 (Amicon) ultrafilters, and then frozen in liquid N₂ before storage at -90 °C. The specific activity of the homogeneous fraction was 350 units/mg of protein. The enzyme peak eluted from the heparin-Ultrogel column represented 57% of the total activity detected in the lysate, and the homogeneous fractions represented 28% of the peak activity. One unit is defined as the amount of enzyme that catalyzes the formation of 1 nmol of tRNA-Leu 20 pµl (10 pl for initial velocity measurements) of the aminoacylation reaction was carried out at 36 mM ATP, 25 mM MgCl₂ for leucine incorporation and at 10 mM ATP, 10 mM MgCl₂ with methionine as a substrate. Initial velocity conditions were used for the determination of the kinetic constants and 10-min reactions for following the enzyme fractionation and optimizing the conditions. After incubation, 20 µl (10 µl for initial velocity measurements) of the aminoacylation mixture was applied to a GF/B filter, and the cold trichloroacetic acid-insoluble radioactivity was counted in a liquid scintillation counter.

**RESULTS AND DISCUSSION**

**Aminoacyl-tRNA Synthetase Activities in Crude Extracts**

Under normal conditions mitochondrial aminoacyl-tRNA synthetases are low abundance proteins. To facilitate our attempts to purify the *S. cerevisiae* mLRS we decided to use a vector that overexpresses the NAM2 gene. HM200/1 harbors a UASGAL10/NAM2 gene fusion cloned on a centromeric plasmid (Herbert et al., 1988a). In order to determine if this would be a suitable starting material for the purification we decided to assay the mLRS in whole cell and mitochondrial lysates of galactose-grown HM200/1 and the wild-type equivalent CW04. As an internal control the level of the mitochondrial methionyl-tRNA synthetase (mMRS) was also measured. Both enzymes are able to charge *E. coli* tRNA so this was used as a readily available substrate (Schneller al., 1988b). The membranes were incubated for 2 h at 37 °C with one of the antisera described above followed by incubation with a second antibody (anti-rabbit IgG) linked to alkaline phosphatase. Addition of nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt caused the immunolabeled proteins to develop a purple colour.

**N-terminal Microsequencing**—Fractions 74 and 75 from the heparin-Ultrogel column were pooled and trichloroacetic acid precipitated (final concentration, 5%) in the presence of 0.15% sodium deoxycholate. The pellet was resuspended in Laemmli sample buffer (Laemmli 1970) and the pH adjusted to 7 with the 1 M Tris/HCl base. Appropriately purified mLRS was obtained by Dr. Leberman, EMBL, Grenoble) or against a chimeric protein resulting from the fusion of the 5’ region of *Laez* gene (280 N-terminal amino acids) and a 3’ fragment of the NAM2 gene (363 C-terminal amino acids). Construction of fusion protein and preparation of antisera have been described elsewhere (Herbert et al., 1988).

After polyclonality of the mLRS gel electrophoresis immunoblotting was performed as described by Towbin et al. (1979). The membranes were incubated for 2 h at 37 °C with one of the antisera described above followed by incubation with a second antibody (anti-rabbit IgG) linked to alkaline phosphatase. Addition of nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt caused the immunolabeled proteins to develop a purple colour.

**Polyacrylamide Gel Electrophoresis and Immunoblotting**—10% Polyacrylamide-SDS gel electrophoresis was performed according to Laemmli (1970). When needed, appropriate portions from column fractions were concentrated before electrophoresis by precipitation with 80% acetone at -20 °C. Immunoblotting was performed with 10% polyacrylamide minigel (383 C-terminal amino acids).

The membranes were incubated for 2 h at 37 °C with one of the antisera described above followed by incubation with a second antibody (anti-rabbit IgG) linked to alkaline phosphatase. Addition of nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt caused the immunolabeled proteins to develop a purple colour.

**Separation of the tRNA-Leu isoacceptors by RPC-5 Chromatography**—A standard incubation mixture (90 µl) containing 16 µg/ml *E. coli* total tRNA (Sigma type XXI) and appropriate concentrations of ATP and MgCl₂ were incubated at 20 °C for 10 min with either *E. coli* LRS or purified mitochondrial mLRS. Labeling with *E. coli* LRS was done with [3H]leucine (16.3 mCi/mmol) and with mLRS with [3H]leucine (16.3 mCi/mmol). After incubation, aminoacylated mixtures were deproteinized with phenol-chloroform extraction; nucleic acids were precipitated with 78% cold ethanol and dried under vacuum. The tRNA pellets from each assay were dissolved in water. Aliquots of the 3H- and 14C-labeled products were mixed together and diluted with RPC-5 column buffer. These mixtures (500-600 µl) were then separated on an RPC-5 column as described by Roe et al. (1973).

**RESULTS AND DISCUSSION**

**Aminoacyl-tRNA Synthetase Activities in Crude Extracts**—Under normal conditions mitochondrial aminoacyl-tRNA synthetases are low abundance proteins. To facilitate our attempts to purify the *S. cerevisiae* mLRS we decided to use a vector that overexpresses the NAM2 gene. HM200/1 harbors a UASGAL10/NAM2 gene fusion cloned on a centromeric plasmid (Herbert et al., 1988a). In order to determine if this would be a suitable starting material for the purification we decided to assay the mLRS in whole cell and mitochondrial lysates of galactose-grown HM200/1 and the wild-type equivalent CW04. As an internal control the level of the mitochondrial methionyl-tRNA synthetase (mMRS) was also measured. Both enzymes are able to charge *E. coli* tRNA so this was used as a readily available substrate (Schneller et al., 1976). The results in Table I show that the level of the mMRS is essentially the same in mitochondrial lysates of both HM200/1 and CW04. However, the level of the mLRS was approximately 45 times higher in mitochondrial lysates of HM200/1 compared with CW04. This indicated that galactose-grown HM200/1 would be a suitable starting material for attempts to purify the mLRS. It should be remembered that...
as neither synthetase was measured with its cognate mitochondrial tRNA the activities of the mLRS and mMRS measured in this experiment are not comparable.

Purification of the mLRS—Using antibodies raised against the product of a LacZ/NAM2 gene fusion, we were able to show that no NAM2 gene product was detectable in postmitochondrial supernatants of HM200/1 (data not shown). This indicated that essentially all of the overproduced mLRS is transported into the mitochondria. Thus, as mitochondrial proteins represent approximately 10% of total cellular protein, the isolation of mitochondria gave a significant first purification step and removed the possible problem of contaminating cytoplasmic LRS in the final preparation. The next step was an ammonium sulfate precipitation, most of the enzyme was found to precipitate from a cleared mitochondrial lysate at 40–85% saturation ammonium sulfate. This yielded a further 2-fold purification and concentrated the enzyme. After this the protein was redissolved and dialyzed to remove the ammonium sulfate and to change the buffer for the final stage of the purification. This was performed by column chromatography on heparin-Ultrogel using KC1 gradients. Upon chromatography the enzyme migrated as a single peak independent of the slope of the gradient. Steep gradients (35–450 mM KC1) permitted the rapid isolation of a rather concentrated fraction (with ~50% purity); flat gradients (150–300 mM KC1) led to the isolation of homogeneous enzyme with a specific activity of 350 units/mg of protein. The enzyme peak eluted from the column represented approximately 57% of the total activity detected in the lysate, the homogeneous fractions representing 28% of the peak activity. The activity measured with total E. coli tRNA co-migrated with the activity charging E. coli tRNA-Leu and the protein detected by the antibodies raised against the LacZ/NAM2 fusion protein (Fig. 1). Fractions eluted with ~260 mM KC1 contained a single protein, whose molecular mass was assessed by SDS-polyacrylamide gel electrophoresis and Coomassie staining to be 100 kDa (Fig. 1), consistent with the size predicted from the NAM2 gene sequence (Labouesse et al., 1987). This protein cross-reacts with antibodies raised against the LacZ/NAM2 fusion protein and with antibodies raised against the purified E. coli LRS (Fig. 2), thus confirming the considerable similarity between the proteins predicted by a comparison of their primary sequences (Herbert et al., 1988a). This purification protocol is simple and rapid; the ability to construct NAM2 mutants in appropriate expression vectors and purify the corresponding proteins will facilitate the understanding of the role of the mLRS in mitochondrial pre-mRNA splicing.

N-terminal Sequence of the mLRS—The purified mLRS was prepared for sequencing, transferred to Immobilon-P, and the N-terminal sequence was determined as described by Le Caer and Rossier (1988). When the N-terminal sequence of 22 amino acids determined from the protein LSTKRGPPGAVKKLIAIGEKWK was compared with the sequence deduced from the gene it appeared that the first 9 amino acids had been removed. Many proteins of the mitochondrial matrix have a short leader sequence which is necessary for targeting to the mitochondria and is removed during the transport process (reviewed by Hartl et al., 1989). The matrix protease does not have a strict recognition sequence, but most sites fit the consensus sequence of basic, basic or hydrophobic/hydrophobic. This is consistent with the site of proteolysis in the mLRS (MLSRPSSRF/LST), thus it is reasonable to infer that a 9 amino acid leader peptide is removed from the mLRS during or after transport into the mitochondria.

Characteristics of the Purified mLRS—The pH optimum of the purified mLRS is between pH 8.0 and 9.5. The activity is stimulated by KC1, with an optimum of 60–70 mM KC1 in the presence of 5 mM Mg2+. When the Mg2+ concentration is raised to 25 mM the KC1 optimum shifts to 75–175 mM (data not shown). The optimum temperature was found to be 37 °C; however, for practical reasons all incubations were carried out at 20 °C. At 4 mM Mg2+ and using E. coli tRNA-Leu as substrate, the optimal ATP concentration was around 10 mM. When purified mitochondrial tRNA-Leu was used at either 4 or 25 mM Mg2+, the ATP optimum was 10–15 mM. However, when total E. coli tRNA was used as the substrate and the determinations were done at 4 and 25 mM Mg2+, a higher ATP concentration was found to be optimal at 25 mM Mg2+. This led us to examine more carefully the relationship between the optimal ATP concentration and the Mg2+ concentration. We observed that at 5 mM Mg2+ ATP influences the enzyme activity in a typical fashion with a well defined optimum at 7 mM ATP. At 25 mM Mg2+, the enzyme activity reaches a plateau at low ATP concentrations (4–12 mM); higher concentrations of ATP result in additional charging with a broad optimum around 30–35 mM ATP (Fig. 3A). If the Mg2+ concentration is increased still further to 40 mM, the biphasic shape of the curve is maintained, but the ATP optimum is shifted to a higher value (data not shown). In contrast to the mLRS the purified E. coli LRS is inhibited by high ATP concentrations at both 5 and 25 mM Mg2+ (Fig. 3B). At this point it should be remembered that in E. coli there are five tRNA-Leu isoacceptors (Holmes et al., 1977), but in yeast mitochondria there is only one (Berlani et al., 1980). In addition, there is a change in the genetic code between E. coli and yeast mitochondria; in E. coli there are six leucine codons, but in yeast mitochondria there are only two, the CUN family being threonine codons (Li and Tzagoloff, 1979). The E. coli isoacceptors that are equivalent to the yeast mitochondrial leucine tRNA are the tRNA-Leu1/6 (corresponding to the codons UUA UUG). Thus, it is reasonable that the yeast mLRS would discriminate against the other E. coli isoacceptors and that the “extra” charging and second ATP optimum seen at high Mg2+ concentration could correspond to the charging of the other E. coli isoacceptors. To investigate this total E. coli tRNA was charged by the purified mLRS at 5 and 25 mM Mg2+. The charged tRNAs were separated on an RCP-5 column, and the results are shown in

Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Met-tRNA synthase units/mg protein</th>
<th>Leu-tRNA synthase units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial lysate of HM200/1</td>
<td>0.218</td>
<td>0.860</td>
</tr>
<tr>
<td>Mitochondrial lysate of CW04</td>
<td>0.201</td>
<td>0.018</td>
</tr>
<tr>
<td>Total cell lysate of HM200/1</td>
<td>0.037</td>
<td>0.090</td>
</tr>
<tr>
<td>Total cell lysate of CW04</td>
<td>0.027</td>
<td>0.003</td>
</tr>
</tbody>
</table>

The purified mLRS was used to assay the mMRS, and E. coli tRNA-Leu was used to assay the mLRS.
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Fig. 1. Elution profile of the mLRS from the heparin-Utrogel column. Panel A, enzyme activity was followed by aminoacylation of total E. coli tRNA (O—O) or tRNA-Leu (▲—▲) as described under “Materials and Methods,” using 3 µl of the column fractions. Panel B; polyacrylamide gel electrophoresis of 1 µg of protein from fractions 73-82. The position of the molecular mass markers is shown on the left.

Fig. 2. Western blot analysis of purified mLRS. A: Lane 1, E. coli LRS (10 ng); Lane 2, S. cerevisiae mLRS (20 ng). The blot was visualized with antiserum raised against a chimeric β-galactosidase/NAM2 protein. B: Lane 1, S. cerevisiae mLRS (20 ng); lane 2, E. coli LRS (10 ng). The blot was visualized with antiserum raised against the purified E. coli LRS. C: Lane 1, E. coli LRS (1.5 µg); lane 2, molecular mass markers; lane 3, S. cerevisiae mLRS (0.5 µg). Polyacrylamide gel electrophoresis stained with Coomassie blue; the size of the molecular mass markers is indicated on the right of the figure.

Fig. 3. Effect of ATP concentration on the leucine charging of total E. coli tRNA at high and low Mg2+ concentrations. A, S. cerevisiae mLRS; B, E. coli LRS.

Fig. 4. Separation of aminoacylated tRNAs by RCP-5 chromatography. Total E. coli tRNA was aminoacylated using the E. coli LRS in the presence of [3H]leucine (O—O), or the S. cerevisiae mLRS in the presence of [14C]leucine (▲—▲). The products were then mixed and separated by chromatography on RCP-5. The products of the aminoacylation reaction performed with the E. coli enzyme act as markers for the different tRNA-Leu isoacceptors. A, aminoacylation with both enzymes performed at 5 mM Mg2+ and 7 mM ATP. B, aminoacylation with the E. coli LRS performed at 5 mM Mg2+ and 7 mM ATP; aminoacylation with the S. cerevisiae mLRS performed at 25 mM Mg2+ and 32 mM ATP.

Fig. 4. From these results we can see that there is no substantial difference between the tRNAs charged at low and at high Mg2+ concentrations. In particular, the same isoacceptors are charged under both conditions, notably the isoacceptor Leu (which comprises ~90% of the total tRNA-Leu) is not more charged at high Mg2+ concentrations. Thus the biphasic curve obtained when ATP is varied at high Mg2+ remains unexplained. However, it should be remembered that it is only seen when the mitochondrial enzyme is used to charge total E. coli tRNA.

Kinetic Parameters—These data were calculated with total E. coli tRNA under two different conditions. At 5 mM Mg2+ and 7 mM ATP the K_m values for leucine and tRNA were 7.1 and 1.92 µM, respectively. At 25 mM Mg2+ and 32 mM ATP
the $K_m$ values for leucine and tRNA were 7.6 and 2.0 $\mu$M, respectively.

Conclusions—We have presented here a simple and rapid purification scheme for the mLRS of S. cerevisiae which is encoded by the NAM2 gene. This protocol could be modified easily to allow the purification and characterization of mutant proteins originally described in genetic experiments. The purification of the mitochondrial leucyl-tRNA synthetase is of particular interest for two reasons. First, the mitochondrial genetic code differs from the universal code, with the CUN family encoding threonine instead of leucine. Thus, this is the first reported purification of a synthetase that charges a nonuniversal set of tRNAs, and structural studies on the purified mLRS should give us considerable insight into the process of discrimination in tRNA selection. Second, the mLRS is involved in the excision of at least two mitochondrial introns, a14 and b14, as well as in tRNA aminoacylation. Genetic experiments have shown that the maturases encoded by these introns are also needed for splicing; however, it is not known if the mLRS interacts with the intron RNA, the maturases, or both. The availability of the purified enzyme for in vitro studies should help us to begin to answer these questions.

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REFERENCES