Domain Structure in Yeast tRNA Ligase†

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ABSTRACT: Yeast tRNA ligase is one of two proteins required for the splicing of precursor tRNA molecules containing introns. The 95-kDa tRNA ligase has been purified to homogeneity from a strain of *Escherichia coli* which overexpresses the protein. The ligation reaction requires three enzymatic activities: phosphodiesterase, polynucleotide kinase, and ligase. By partial proteolytic digestion, we have produced fragments of tRNA ligase which contain the constituent activities. These results provide evidence for a model in which the three constituent activities of ligase are located in three distinct domains separated by protease-sensitive regions. We have also located the active adenylylated site in the ligase domains. It is lysine-114. The tRNA ligase sequence in this region has limited homology to the active-site region of T4 RNA ligase.

In yeast, about a tenth of the tRNA genes contain introns (Ogden et al., 1984). These introns are removed from tRNA precursors in a two-step reaction (Peebles et al., 1979) which requires the action of at least two enzymes (Figure 1). In the first step, an endonuclease cleaves the splice junctions and releases the linear intron. The specificity of the endonuclease is such that the exons are left with 5′-OH and 3′-cyclic phosphate termini (Peebles et al., 1983). These are joined together in an ATP-dependent reaction by tRNA ligase. tRNA ligase possesses three separate enzymatic activities, all of which are required for the ligation reaction (Greer et al., 1983a). First, a cyclic phosphodiesterase opens the cyclic phosphate to give a 2′-phosphate. The γ-phosphate of ATP is transferred to the 5′-OH end by a polynucleotide kinase activity. tRNA ligase is adenylylated, and in the ligation reaction, AMP is transferred to the 5′-PO_4 to form an activated 5′–5′-phosphoanhydride bond. The 3′-OH then attacks, AMP leaves, and the 3′–5′-phosphodiesterase bond is formed. The 2′-phosphate is still present and must be removed by a phosphatase (McCraith & Phizicky, 1990). In this mechanism, the phosphate at the splice junction is derived from the γ-phosphate of ATP, and the original phosphate at the 5′ splice is not part of the mature tRNA molecule.

In previous studies, we have purified the tRNA ligase to homogeneity from yeast extracts (Phizicky et al., 1986). The gene for tRNA ligase has been cloned and sequenced (Westaway et al., 1988). tRNA ligase is a single 95-kDa polypeptide chain, and the purified protein contains all of the activities described above.

There is reason to believe that these activities are located in separate domains. Bacteriophage T4 expresses an RNA ligase, and interestingly, the gene for RNA ligase, gene 63, is closely linked to the pseT gene which codes for an enzyme which possesses polynucleotide kinase, phosphatase, and phosphodiesterase activities (Snyder, 1983). Together, these two enzymes can replace yeast tRNA ligase in the in vitro splicing reaction (Greer et al., 1983b). (Because of the phosphatase activity of the pseT protein, the spliced product does not contain a 2′-phosphate at the splice junction.) Thus, T4 carries out with two proteins, a reaction that is catalyzed by one in yeast and in wheat (Konarska et al., 1981; Schwartz et al., 1983). Although the sequence homology between yeast tRNA ligase and the T4 proteins is, at best, weak (discussed further below), the genetic separation of the ligase and kinase functions in T4 suggests that they may constitute separate domains in the yeast enzyme.

In this paper, we have used partial proteolysis to produce fragments of the 95-kDa yeast tRNA ligase. The constituent activities were found to be located in separate fragments confirming the domain hypothesis.

Both T4 RNA ligase and yeast tRNA ligase activate the 5′-phosphate with an AMP (Uhlenbeck & Gumport, 1982; Greer et al., 1983a). As in DNA ligase, AMP is covalently bound to the enzyme. The site of adenylylation has been determined in T4 RNA ligase (Thøgersen et al., 1985). AMP is coupled to the enzyme in a phosphoamide linkage with the γ-amino group of a lysine residue. In this paper, we describe the isolation and characterization of the adenylylated peptide from yeast tRNA ligase and its characterization. We confirm that in this case as well the adenylylation site is a lysine residue.

Materials and Methods

Assays. The tRNA ligase assay was performed as described by Greer et al. (1983a) and Phizicky et al. (1986) except that synthetic pre-$tRNA^{Phe}$ was used as the substrate. The synthesis of pre-$tRNA^{Phe}$ by T7 RNA polymerase has been described by Reyes and Abelson (1987). In these experiments, the precursor was labeled with [α-^{32}P]UTP, and it had a specific activity of $\sim 10^5$ dpm/pmol.

The polynucleotide kinase assay was performed as described by Pick et al. (1986). The preparation of the substrate for the cyclic phosphodiesterase assay, the assay conditions, and the conditions for separating the product and reactant have been described by Schwartz et al. (1983) and Greer et al. (1983a). Incorporation of [α-^{32}P]ATP into tRNA ligase and the analysis of the adenylylated protein by SDS–polyacrylamide gel electrophoresis have been described by Greer et al. (1983a).

Purification of tRNA Ligase. The purification of tRNA ligase from an *Escherichia coli* strain engineered to overproduce the protein is described in detail in a separate publication (Xu et al., 1990). A brief description of the procedure follows:

The construction of the expression vector for tRNA ligase has previously been described (Phizicky et al., 1986). The

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Table I: Purification of Yeast tRNA Ligase from an *E. coli* Overproducing Strain

step	total volume (mL)	total protein	total act. (KU)	sp act. (units/mg)	purification (*x*-fold)	recovery (%)
crude extract	700	18 g	190	10.5	1	100
polymin P supernatant	620	12.4 g	170	13.7	1.3	89.4
heparin–agarose	360	305 mg	110	360	34.3	58
Blue Trisacryl M	170	64 mg	70	1093	104	37
hydroxyapatite	70	42 mg	60	1428	136	32
Sephadex G-150	100	25 mg	58	2320	220	30

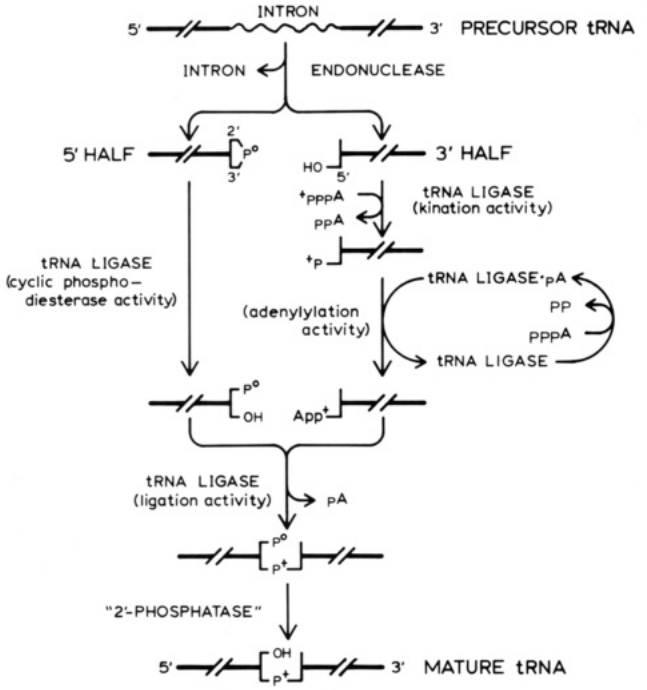

FIGURE 1: Mechanism of yeast tRNA splicing. The bold lines represent the mature domains of the tRNA, and the squiggly line signifies the intron. Endonuclease cleaves the phosphodiester bonds at the 5′ and 3′ splice sites and forms a 5′ half-molecule with a 2′,3′-cyclic phosphate and 3′ half-molecule with a 5′-hydroxyl. tRNA ligase cleaves the 2′,3′-cyclic phosphodiester bond, leaving a 2′-phosphate, and adds a γ-phosphate from an ATP molecule to the 5′-hydroxyl. tRNA ligase then adenylylates the 5′-phosphate with another ATP molecule and ligates the two half-molecules together. Finally, the 2′-phosphate is removed by a 2′-phosphatase (Greer et al., 1983; Phizicky et al., 1986; McCraith & Phizicky, 1990).

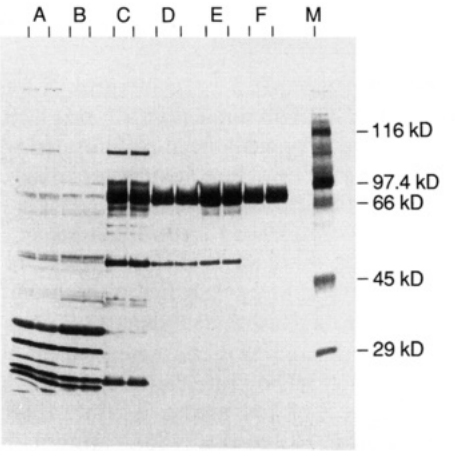

FIGURE 2: Purification of yeast tRNA ligase. The purification of yeast tRNA ligase from a strain of *E. coli* which overproduces the enzyme was performed as described under Materials and Methods and in Table I. This figure shows the resolution of the proteins from various fractions of the purification by electrophoresis on an SDS–polyacrylamide gel visualized by silver staining. The nature and quantity of protein loaded to each pair of lanes are as follows: lane A, 70 μg of crude extract; lane B, 60 μg of polymin P supernatant; lane C, 8.5 μg of the peak fraction from heparin–agarose chromatography; lane D, 3.7 μg of the active fractions from Blue Trisacryl in chromatography; lane E, 6 μg of the peak fractions from hydroxylapatite chromatography; lane F, 2.5 μg of the active fractions from Sephadex G-150 gel filtration; lane M, molecular weight markers.

sequence at the N-terminal methionine codon was modified and linked to the p-tac promoter in the expression vector pKK223-3 to give the plasmid pKK223-3-RLGX. This systems provides good overexpression of the 95-kDa ligase protein, but we observed some instability of the protein when it was expressed in a normal *E. coli* strain (Phizicky et al., 1986). Consequently, we transferred the plasmid to the protease-deficient strain SW1064 (Δlon, htp R, lacZ am, trp am, pho am, Sup C^{ts} mal rpsL phe rel [F′laci^{Q}lacZ::Tn5]). This strain is designated SW1068. The protein is considerably more stable in this strain, and fewer low molecular weight forms of tRNA ligase can be detected by rabbit anti-tRNA ligase IgG in Western blots. Strain SW1068 was grown in LB broth and tRNA ligase expression induced by addition of IPTG at a final concentration of 0.2 mM. The cells were resuspended in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10% glycerol. Before sonication to produce the cell extract, the following mixture of protease inhibitors was added: 0.2 mM EGTA, 0.2 mM benzamidine, 0.4 μg/mL aprotinin, 20 μg/mL bacitracin, 0.4 μg/mL pepstatin A, and 0.04 mM PMSF. The PMSF was freshly prepared as a 200 mM stock solution in isopropyl alcohol. In the early steps of the purification, protease inhibitors were added to all the buffers at half the concentrations given above. In most steps, the buffer was buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 10% glycerol, and 1 mM 2-mercaptoethanol).

The purification table (Table I) is for a preparation which started with 200 g of *E. coli* SW1068 cell paste. Figure 2 shows the results of an electrophoretic separation of the proteins at each stage of the purification. The major band which contaminates the 95-kDa tRNA ligase in the hydroxylapatite and Blue-Trisacryl-M fractions is a fragment of tRNA ligase, and it is not removed until the final gel filtration step.

The peak fractions from the final gel filtration step were concentrated by using an Amicon stirred ultrafiltration cell with a Ym 10 filter. tRNA ligase was stored at −20 °C at a concentration of ~10 mg/mL in buffer A containing 0.2 M NaCl and 50% glycerol.

Partial Proteolysis of tRNA Ligase to Produce Fragments. Two methods were used to produce fragments of tRNA ligase: Pure tRNA ligase was partially digested by incubation with trypsin at 4 °C in buffer A and 0.2 M NaCl. The ligase to trypsin ratio was 10:1. Stoichiometric quantities of bovine trypsin inhibitor were added to terminate the reaction. The time course of digestion was followed by electrophoretic separation of aliquots of the reaction mixture by SDS–polyacrylamide gel electrophoresis.

We observed that chromatography of pure tRNA ligase on a heptylagarose column resulted in the degradation of the protein into quite specific fragments, which corresponded in molecular weight to the fragments produced by partial trypsin

proteolyses. The degradation did not occur when tRNA ligase was mixed separately with either ammonium sulfate or the column resin. We do not understand the reason, but the same results were obtained several times with several different preparations of heptylagarose that were prepared by the method of Shaltiel (1974) using Sepharose CL-6B activated by the method of March et al. (1974). The fragments which resulted from heptylagarose seemed to be more specific (although of the same general size) than those obtained by partial trypsin digestion. Therefore, most of the work we describe was done with fragments derived from heptylagarose chromatography.

Five milligrams of purified ligase in buffer A was adjusted to 1.5 M $(NH_4)_2SO_4$ and applied to a 5-mL heptylagarose column which had previously been equilibrated with 1.5 M $(NH_4)_2SO_4$ and buffer A. The column was eluted with 15 mL of buffer A, and the fragmentation of ligase was monitored by resolving the peptides by electrophoresis on an SDS–polyacrylamide gel. Fragments of tRNA ligase, produced as described above, were further purified by rechromatography on either heparin–agarose or heptylagarose.

Each of the fragments was characterized by N-terminal Edman degradation. The proteins were electrophoretically blotted onto a sheet of activated glass fiber filter paper as described by Aebersold et al. (1986). Automated Edman degradations and on-line PTH-amino acid analyses were performed using an Applied Biosystems Model 477A/120 system, essentially as suggested by the manufacturer. Protein sequence analysis was done by manual comparison of HPLC chromatograms.

Purification of the Adenylylated Peptide. One milligram of pure tRNA ligase was incubated in a 0.2-mL reaction mixture containing 100 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 3 mM dithiothreitol, and 10 μCi of [α-^{32}P]ATP for 5 min at 30 °C. Then 16 μL of unlabeled 1 mM ATP was added, and the incubation was continued for another 10 min at 30 °C. For complete digestion of ligase by chymotrypsin, 1-chloro-3-(tosylamino)-7-aminoheptan-2-one-treated chymotrypsin (TLCK-chymotrypsin, Sigma) was added to 1 mg of adenylylated ligase at a substrate to protease ratio of 10:1 (w/w). The digestion was carried out at 37 °C for 30 min. For partial digestion of ligase, 1 mg of the adenylylated protein was made 5 M in urea, and TLCK-chymotrypsin was added at a substrate to protease ratio of 10:1 (w/w). Incubation was for 30 min at 37 °C. After digestion, the reaction mixtures were heated for 3 min at 100 °C and centrifuged for 1 min in an Eppendorf centrifuge.

A two-step procedure was used to purify the complete chymotrypsin adenylylated peptide. The hydrolysate was first fractionated by HPLC on a Vydec TP546 RP-C4 column (4.6 × 250 mm). Elution was achieved with gradient system 2 (see below). The major radioactivity peak was rechromatographed on the same C_4 column using gradient system 3.

Partial chymotryptic digests were fractionated as above using gradient system 2. The major radioactivity peaks were collected and dried in a Speed-Vac (Savant) vacuum concentrator. Each peptide peak was redissolved in 0.2 mL of 20 mM NH_4HCO_3 (pH 8.3) and separately digested with 15 μg of protease V8 (sequence grade) at 25 °C for 20 h. The digest was then loaded onto the RPC4 column and developed with gradient system 1.

The solvent and gradient systems for HPLC were as follows: solvent A, 0.1% trifluoroacetic acid (sequanal grade) in water (double glass distilled); solvent B, 0.1% trifluoroacetic acid in acetonitrile/H_2O (60:40) v/v; gradient system 1, 100% A to 40% B in 30 min and 40% B to 100% B in 60 min; gradient system 2, 100% A to 50% B in 30 min and 50% B to 100% B in 90 min; gradient system 3, 100% A to 50% B in 30 min, 50% B to 60% B in 60 min, and 60% B to 100% B in 30 min. All fractionations were carried out at ambient temperature, with a flow rate of 0.7 mL/min. The peaks were detected by monitoring the absorbance at 214 nm.

Sequence Analysis of Peptides. Peptide sequence analysis was performed as described above for the tRNA ligase fragments, except that the HPLC fractions were spotted directly onto the TFA-activated, polyprene-coated, glass fiber disks (prepared essentially as suggested by Applied Biosystems, Inc.).

Mass Spectrometry Analysis. Samples collected from reverse-phase HPLC in polypropylene microcentrifuge tubes were concentrated to dryness by using a vacuum centrifuge. The sample was redissolved in a few microliters of 5% (v/v) aqueous acetic acid. Approximately 2 μL of the sample solution was added to 1 μL of the sample matrix on a 1.5 × 6 mm stainless-steel sample stage. The sample matrix was a mixture of dithioerythritol (5:1) (Witten et al., 1984) and camphorsulfonic acid (6 mM) (DePauw et al., 1984). Positive-ion, fast atom bombardment (FAB) mass spectra were obtained by using a JEOL HX-100HF high-resolution, double-focusing, magnetic-sector mass spectrometer operating at 5-kV accelerating potential and a nominal resolution of 3000. Sample ionization was accomplished using a 6-keV Xe atom beam. A JEOL DA5000 data system was used to control instrument parameters and collect the spectral data.

Results

Purification of tRNA Ligase. We have previously described a construct in which tRNA ligase is overproduced in *E. coli* (Westaway et al., 1988). Although we observed enhanced synthesis of the 95-kDa protein and also of ligase activity in extracts from this strain, we also observed many smaller fragments which cross-reacted with antisera to the intact protein. Apparently, yeast tRNA ligase is unstable in *E. coli*. We largely overcame that problem by transferring the expression plasmid into a protease-deficient strain of *E. coli* (lon$^-$, htp R$^-$). In addition, a mixture of protease inhibitors was added to buffers used in the early purification steps. The purification procedure is briefly described under Materials and Methods and in more detail in a separate paper (Xu et al., 1990). As can be seen in Table I and in Figure 2, we can now obtain milligram quantities of the pure 95-kDa protein. There is still fragmentation of the protein, and only in the final gel filtration step is the intact protein separated from a partial fragment, which is the main contaminant at later stages in the purification.

Partial Proteolytic Digestion of tRNA Ligase. Two methods were used to produce distinct fragments of tRNA ligase. First, tRNA ligase was partially digested with trypsin. Figure 3 shows the time course of fragmentation under conditions of partial digestion. Three major products were separated by SDS gel electrophoresis with molecular weights of 65K, 55K, and 40K. Lesser amounts of a 25-kDa fragment could also be seen. It is clear that there are sites in the protein which are particularly sensitive to trypsin. The second method used to produce fragments is less conventional. We had originally used a heptylagarose column to purify tRNA ligase (Greer et al., 1983a; Phizicky et al., 1986). However, when this column was used to purify tRNA ligase produced in *E. coli*, Western blot analysis of fractions eluting from the column using anti-tRNA ligase antibody showed that specific fragmentation of the protein was occurring. The major products

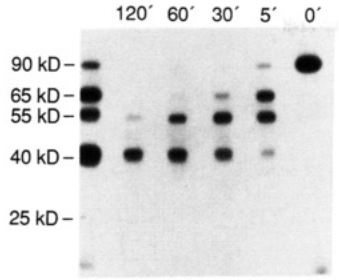

FIGURE 3: Time course of the partial digestion of tRNA ligase with trypsin. Pure adenylylated tRNA ligase, labeled with [^{32}P]AMP, was digested with trypsin at 0 °C. The ratio of tRNA ligase to trypsin in the reaction mixture was 10:1. This is an autoradiogram of an SDS–polyacrylamide gel in which the polypeptides in the reaction mix were separated by electrophoresis.

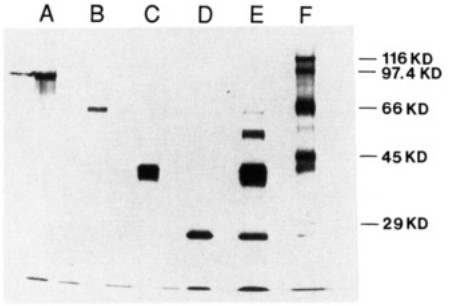

FIGURE 4: Purified tRNA ligase fragments. tRNA ligase fragments, produced as described under Materials and Methods, were purified by further chromatographic steps. The purity of each of the fragments used in further experiments was characterized by SDS–polyacrylamide gel electrophoresis. Lane A, 0.32 μg of tRNA ligase, 95 kDa; lane B, 0.2 μg of the 65-kDa fragment; lane C, 0.6 μg of the 40-kDa fragment; lane D, 0.54 μg of the 25-kDa fragment; lane E, partial trypsin digestion of tRNA ligase as described in Figure 3; lane F, molecular weight markers.

were similar in molecular weight to those generated by partial trypsin digestion.

We do not understand why fragmentation of the protein was occurring on heptylagarose columns. It could be that the column was contaminated with a protease or that an unknown chemical reaction is cleaving the protein. In any case, this method proved to be useful in the isolation of fragments of tRNA ligase, since the distribution of molecular weights of the fragments was considerably sharper than in the partial tryptic digests.

Purification and Characterization of Three Fragments of tRNA Ligase. Three of the principle fragments of tRNA ligase, those with molecular weights of 65K, 40K, and 25K, were purified further by rechromatography. Aliquots of the pure fractions are displayed on an SDS–polyacrylamide gel (Figure 4). These fragments all cross-react with antisera to the 95-kDa protein.

The fragments were further characterized by N-terminal sequencing. The 65- and 40-kDa fragments both have the same N-terminal sequence as the intact 95-kDa protein. The 25-kDa protein is from the C-terminus. Its N-terminal sequence begins at residue 615: Gly-Asn-Asn-Gln-Gln-Lys-Thr-Pro... [see Westaway et al. (1988)].

We do not known the C-terminal sequence of this fragment, but if the C-terminus is intact, the peptide is 213 amino acids long with a predicted molecular weight of 24.3K.

Enzymatic Activity of tRNA Ligase Fragments. Each of the fragments were tested for tRNA ligase activity in the splicing of a synthetic pre-tRNA^{Phe} precursor. In these reactions, endonuclease was added to cleave the precursor and provide the substrate for ligase action. None of the fragments,

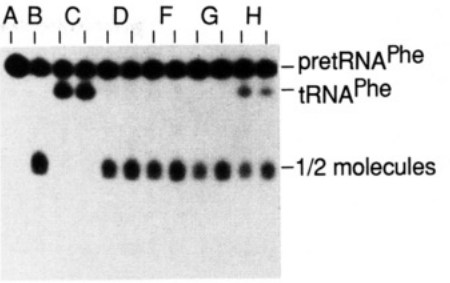

FIGURE 5: Activity of tRNA ligase fragments as measured in the splicing assay. Splicing reactions, as described under Materials and Methods, were carried out by using tRNA ligase or fragments of tRNA ligase as indicated and a partially purified endonuclease. This is an autoradiogram of a polyacrylamide gel in which [^{32}P]pre-tRNA^{Phe} and the product of splicing were separated by electrophoresis. Reactions shown in lanes B–H contained endonuclease. A, pre-tRNA^{Phe}, control; B, endonuclease alone; C, 95-kDa ligase; D, 65-kDa fragment; F, 40-kDa fragment; G, 25-kDa fragment; H, 65- and 25-kDa fragments. (Note that lanes C, D, F, G., and H are duplicates.)

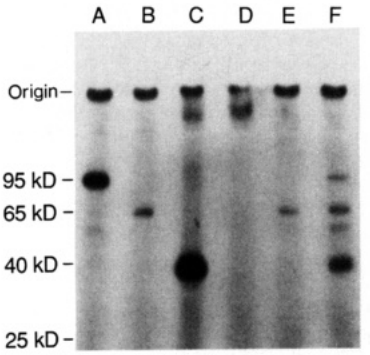

FIGURE 6: Adenylylation activity of tRNA ligase fragments. The adenylylation assay in which [α-^{32}P]ATP is mixed with tRNA ligase is described under Materials and Methods. The proteins are separated by electrophoresis on an SDS–polyacrylamide gel. This is an autoradiogram of that gel. A, 95-kDa tRNA ligase; B, 65-kDa fragment; C, 40-kDa fragment; D, 25-kDa fragment; E, 65- and 25-kDa fragments; F, adenylylated fragments in a partial trypsin digest of tRNA ligase (as in Figure 3).

Table II

	enzyme concn (pmol/μL)	kinase act. (units/μL)	sp act. (units/pmol)
95 kDa	0.35	0.5	1.4
65 kDa	0.3	0.7	2.3
40 kDa	1.5	0	0
25 kDa	2	0.12	0.06
65 kDa + 25 kDa	0.3	0.056	0.19

acting alone, could catalyze this reaction (Figure 5). However, it is interesting that addition of the 65-kDa and the 25-kDa fragment together partially restored the activity. These two fragments, the N-terminal 65-kDa fragment and the 25-kDa C-terminal fragment, presumably constitute most of the protein (although since we do not know the C-terminal sequences of either, some internal residues could be missing). We do not know whether the two fragments act independently to carry out this reaction or whether they fold together, reconstituting the intact protein. In any case, the complete ligation reaction requires virtually the entire protein.

Each of the fragments was incubated with [α-^{32}P]ATP and analyzed by SDS–polyacrylamide gel electrophoresis (Figure 6). Both the 65- and 40-kDa fragments were adenylylated. The 25-kDa fragment was not. Addition of the 25-kDa fragment to the 65-kDa fragment did not increase the adenylylation of the 65-kDa fragment. From this result, we infer

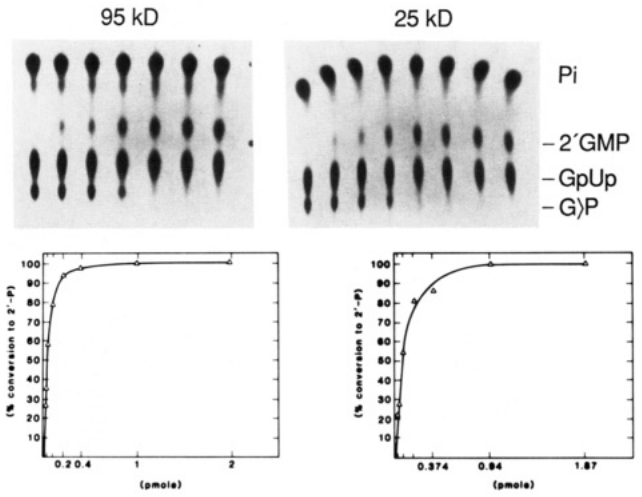

FIGURE 7: Phosphodiesterase activity of fragments of tRNA ligase. The phosphodiesterase activity of each of the tRNA ligase fragments was compared to that of the intact 95-kDa enzyme. Only the 25-kDa fragment had significant activity. The top panels show the raw data of the assay. In this assay, a polynucleotide, $(U)_nGP^*$, is treated with phosphodiesterase (Greer et al., 1983a). Pancreatic ribonuclease is then added to the reaction mix. The nucleotides are then separated by chromatography on poly(ethylenamine) thin-layer plates. The 2′-GMP product is quantified, and the results are shown in the bottom panels.

Table III

	E-AMP	ligase act.	kinase act.	cyclic phosphodiesterase act.
95 kDa	+	+	+	+
65 kDa	+	−	+	−
40 kDa	+	−	−	−
25 kDa	−	−	<5%	+
65 kDa + 25 kDa	+65 kDa	+	+reduced	+

that the adenylylation site is in the N-terminal 40-kDa fragment and that this segment of the protein is sufficient for adenylylation.

The fragments were assayed for polynucleotide kinase activity (Table II). The specific activity of the 65-kDa fragment in this assay was somewhat greater than that of the intact protein. The 40-kDa fragment had no activity, and the 25-kDa fragment had a small activity (~5% that of the intact enzyme). Surprisingly, when added to the 65-kDa fragment, the 25-kDa fragment had an inhibitory effect. We assume that the low level of activity of the 25-kDa fragment was due to a contaminating fragment of about the same size which was derived from the C-terminal portion of the 65-kDa fragment or the center of the molecule but this assumption remains to be proved.

Each of the fragments was assayed for cyclic phosphodiesterase activity by using the assay described by Greer et al. (1983a; Figure 7). Only the 25-kDa fragment had activity, and it had a similar specific activity to that of the intact protein. Addition of the 65-kDa fragment did not affect the phosphodiesterase activity of the 25-kDa fragment.

The results of all of the assays are summarized in Table III.

Identification of the Adenylylation Site. In the experiment described above, the adenylylation site was localized to the N-terminal 40-kDa fragment. We wished to pinpoint the site of adenylylation precisely. Several approaches were taken to isolate the adenylylated peptide. First, 1 mg of adenylylated tRNA ligase labeled with [α-^{32}P]AMP was completely digested with chymotrypsin, and the peptides were separated by HPLC on a C4 column (Figure 8). The fractions were assayed for radioactivity. There was a single radioactive peak, suggesting that there is a single adenylylation site in the enzyme. The radioactive peak was further purified by a second HPLC run on the same column using a different gradient. The radioactive peak was sequenced by automated sequential Edman degradation. The sequence of the major peptide was Asp-Val-Thr-Ile-Lys-Ala-Asn-Gly-Cys-Ile-Ile-Phe. This peptide is derived from the N-terminal portion of the molecule. It starts at Asp-110 and terminates at Phe-121.

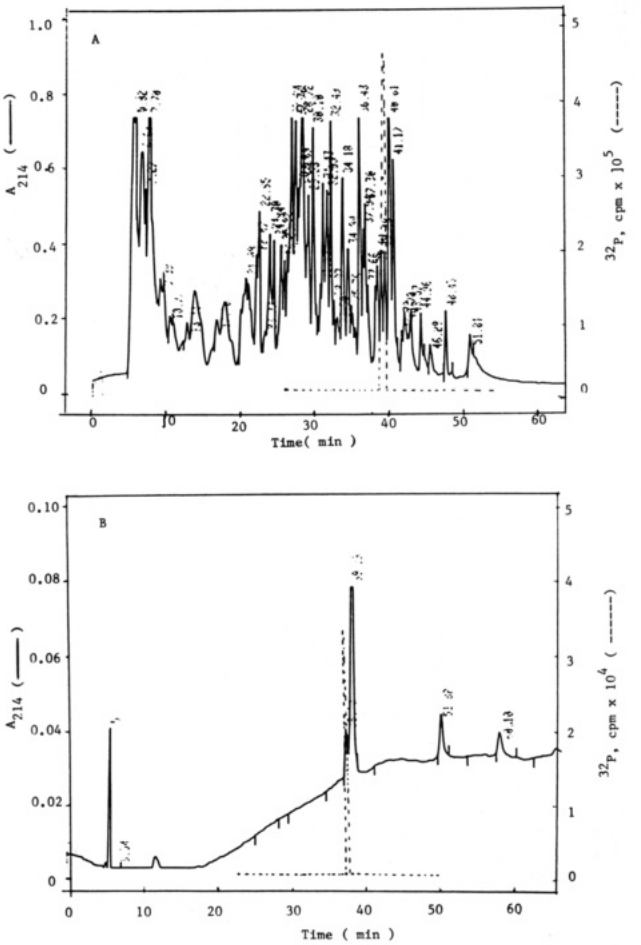

FIGURE 8: Isolation of the adenylylated chymotryptic peptide. One milligram of pure, adenylylated tRNA ligase was digested with chymotrypsin. The digest was chromatographed on a C4 HPLC column loaded and run as described under Materials and Methods. The peak of radioactivity (dotted line) was collected and rechromatographed. The radioactive peak was collected and sequenced.

In a second approach, adenylylated tRNA ligase was partially digested with chymotrypsin in 5 M urea, and the mixture was fractionated by HPLC on a C4 column. The rather broad peak of radioactivity was collected in three tubes (Figure 9). Each tube was lyophilized, and the peptides were separately digested to completion with V8 protease. Each of the digests was further fractionated by HPLC on a C4 column, and the sequence of the radioactive peptide was determined. In each case, the same sequence was observed: Asn-Cys-Thr-Gly-Pro-Tyr-Asp-Val-Thr-Ile-Lys-Ala-Asn-Gly-Cys-Ile-Ile-Phe-Ile-Ser-Gly-Leu-Glu. This sequence includes the previous peptide. It begins at Asn-104 and terminates at Glu-126. The yield of amino acids in early cycles of the sequence determination was consistent with the quantity of adenylylated peptide as determined by quantification of the radioactivity. This suggested that the adenylylated peptide was not a contaminant in these fractions but rather was the major peptide. Since labeled AMP was found in two overlapping peptides with

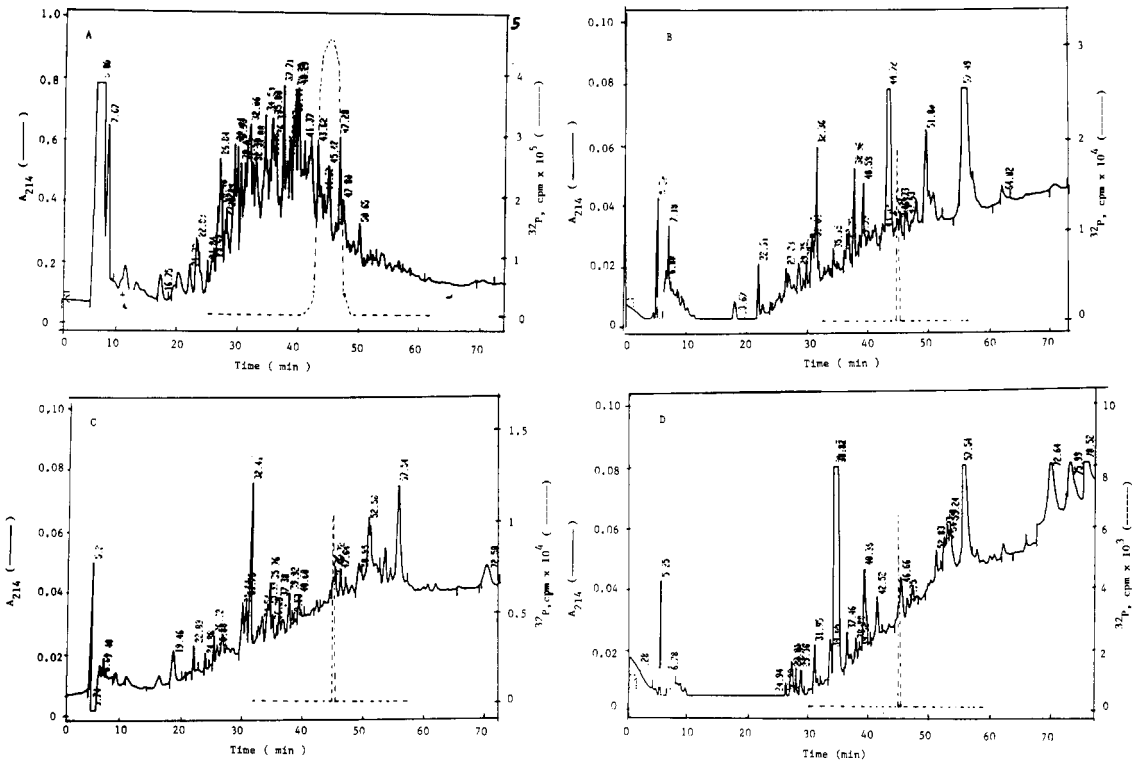

FIGURE 9: Isolation of the adenylylated protease V8 peptide. One milligram of pure adenylylated tRNA ligase was partially hydrolyzed with chymotrypsin. The lysate was chromatographed on a C4 column. The radioactivity eluted in a relatively broad peak (panel A). Three peaks tubes eluting at 43, 45, and 47 min were collected, dried, and redissolved in 200 μL of 20 mM NH_4HCO_3, pH 83, and completely digested with V8 protease. Each digest was rechromatographed: panel B, 43-min peak; panel C, 45-min peak; panel D, 47-min peak. The peak of radioactivity from each run was collected and sequenced. The peptides in all three peaks had the same sequence.

different purification properties, it seems certain that the adenylylation site falls between residues 110 and 121.

The AMP linkage to the protein was hydrolyzed during Edman degradation so it was not possible to determine the precise site of adenylylation by this method. Mass spectrometry was used to prove that the peptide was adenylated. A complete chymotryptic digest was fractionated by HPLC on a C4 column, and the peak of radioactivity was rechromatographed on an RP18 column (see Materials and Methods). Sequence analysis confirmed the result obtained previously, and the mass spectrum gave a value of 1622.59 for the monoisotopic protonated molecular ion (Figure 10). This value corresponds to the peptide Asp-Val-Thr-Ile-Lys-Ala-Asn-Gly-Cys-Ile-Ile-Phe with the added adenyl group (calculated value = 1622.74). A second component in the spectrum was observed at m/z 1293.69 corresponding to the peptide without the adenyl group (calculated value = 1293.68). The presence of this second component in the spectrum was expected given the labile nature of the phosphoramide bond. A third component in the spectrum (m/z 1316.5) was attributed to a peptide impurity in the sample. Although these results confirm which peptide was adenylated, they do not establish which residue is adenylylated. We assume that it is lysine-114 for three reasons. First, it is a lysine that was adenylylated in T4 RNA ligase (Thøgersen et al., 1985). Second, the AMP phosphoramide bond is known to be sensitive to hydroxylamine but resistant to snake venom phosphodiesterase (Shabarova, 1970; Rothberg et al., 1978). AMP is released from tRNA ligase by hydroxylamine but cannot be released by snake venom phosphodiesterase (Greer et al., 1983a). Third, complete trypsin digestion of the adenylylated enzyme resulted in a large radioactive peptide whose size is estimated to be ~4000 daltons by SDS gel electrophoresis. A tryptic peptide of this size could only occur if lysine-114 is blocked in the adenylylated enzyme (data not shown).

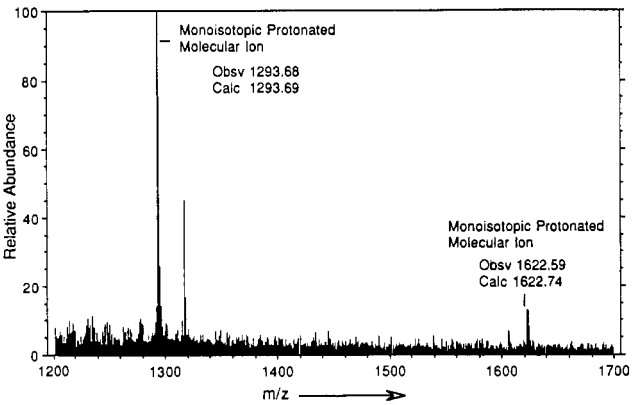

FIGURE 10: Mass spectrometry analysis of the adenylylated chymotryptic peptide. Adenylylated peptide from a digest of 1 mg of pure tRNA ligase, which was purified by a two-step procedure, was analyzed by positive-ion fast atom bombardment mass spectrometry as described under Materials and Methods.

DISCUSSION

It is clear from our results that segments of the 95-kDa tRNA ligase possess independent and nonoverlapping enzymatic activities. The 40-kDa N-terminal fragment can be adenylylated. The 25-kDa C-terminal fragment alone possesses phosphodiesterase activity. Although we do not have a fragment which is uniquely a polynucleotide kinase, the fact that the 65-kDa fragment has kinase activity while the 40-kDa N-terminal fragment does not suggests that this activity is located in the central domain. It is possible that the central domain interacts with the N-terminal domain to provide kinase activity. This brings us to an undoubtedly oversimplified view of tRNA ligase. In this view, the protein is organized into three domains with protease-sensitive sites between them. Several properties of tRNA ligase are not addressed by this model. It is known that while tRNA ligase can act to cyclize

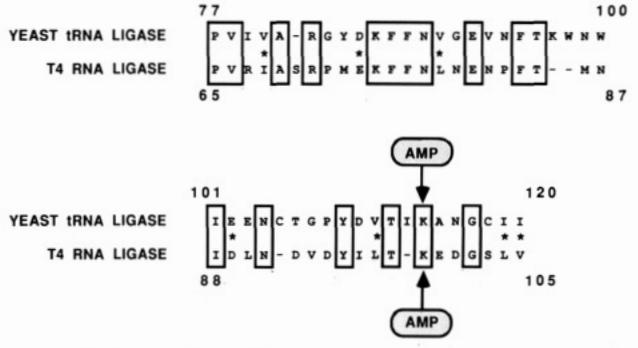

FIGURE 11: Comparison of the sequences of yeast tRNA ligase and T4 RNA ligase in the vicinity of the active site.

poly(A) it much prefers tRNA substrates. tRNA ligase can also interact with tRNA precursors to form a stable complex (C. Greer, personal communication). We have previously shown that the enzyme can be specifically cross-linked to bases within the intron (Tanner et al., 1988). Therefore, tRNA ligase must specifically recognize and interact with tRNA. These studies have not addressed where that interaction site might be. It could be solely a part of the ligase domain, or alternatively, it could be formed by the interaction of two or three domains. We are now preparing constructs which will allow us to express different portions of the ligase gene both in yeast and in *E. coli*. These should allow a much clearer and more comprehensive study of the organization of the enzyme.

We are reasonably certain that there is a single adenylylation site in tRNA ligase at lysine-114. It is of interest to compare the sequence surrounding that site with the corresponding sequence of T4 RNA ligase. Overall, there is no convincing homology between the yeast tRNA ligase sequence and either T4 RNA ligase or T4 polynucleotide kinase. However, with knowledge of the location of the two adenylylation sites, an alignment between the N-terminal region of the two proteins can be discerned. This is shown in Figure 11. In the near vicinity of both sites, there are conserved Tyr, Thr, and Gly residues and upstream at position 86 a conserved Lys, Phe, Phe, Asn sequence. The possible functional significance of these sequences can be addressed by site-directed mutagenesis. This has been done for T4 RNA ligase (Heaphy et al., 1987), and we have begun by constructing mutations which alter Lys-114 in yeast tRNA ligase. These are presently being analyzed.

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REFERENCES

Aebersold, R. H., Teplow, D. B., Hood, L. E., & Kent, S. B. H. (1986) *J. Biol. Chem. 261*, 4229–4238.

DePauw, E., Pelzer, G., Dang, D. V., & Marien, J. (1984) *Biochem. Biophys. Res. Commun. 123*, 27–32.

Greer, C. L., Peebles, C. L., Gegenheimer, P., & Abelson, J. (1983a) *Cell 32*, 537–546.

Greer, C. L., Javor, B., & Abelson, J. (1983b) *Cell 33*, 899–906.

Heaphy, S., Singh, M., & Gait, M. J. (1987) *Biochemistry 26*, 1688–1696.

Konarska, M., Filipowicz, W., Domdey, H., & Gross, H. J. (1981) *Nature 293*, 112–116.

March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.

McCraith, S. M., & Phizicky, P. (1990) *Mol. Cell. Biol. 10*, 1049–1055.

Ogden, R. C., Lee, M.-C., & Knap, G. (1984) *Nucleic Acids Res. 12*, 9367–9382.

Peebles, C. L., Ogden, R. C., Knapp, G., & Abelson, J. (1979) *Cell 18*, 27–35.

Peebles, C. L., Gegenheimer, P., & Abelson, J. (1983) *Cell 32*, 525–536.

Phizicky, E. M., Schwartz, R. C., & Abelson, J. (1986) *J. Biol. Chem. 261*, 2978–2986.

Pick, L., & Hurwitz, J. (1986) *J. Biol. Chem. 261*, 6684–6693.

Reyes, V. M., & Abelson, J. (1987) *Anal. Biochem. 166*, 90–106.

Rothberg, P. G., Harris, T. J., Nomoto, A., & Wimmer, E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4868–4872.

Schwartz, R. C., Green, C. L., Gegenheimer, P., & Abelson, J. (1983) *J. Biol. Chem. 258*, 8374–8383.

Shabarova, Z. (1970) *Prog. Nucleic Acid Res. Mol. Biol. 10*, 145–182.

Shaltiel, S. (1974) *Methods Enzymol. 34*, 126–140.

Snyder, L. (1983) in *Bacteriophage T4* (Mathews, C. K., Kutter, E. M., Mosig, G., & Berget, P. B., Eds.) pp 351–355, American Society for Microbiology, Washington, D.C.

Tanner, N. K., Hanna, M. M., & Abelson, J. (1988) *Biochemistry 27*, 8852–8861.

Thøgersen, H. C., Morris, H. R., Rand, K. N., & Gait, M. J. (1985) *Eur. J. Biochem. 147*, 325–329.

Uhlenbeck, O. C., & Gumport, R. I. (1982) *Enzymes (3rd Ed.) 15*, 31–58.

Westaway, S. K., Phizicky, E. M., & Abelson, J. (1988) *J. Biol. Chem. 263*, 3171–3176.

Witten, J. L., Schaffer, M. H., O'Shea, M., Cook, J. C., Hemling, M. E., & Rinehart, K. L. (1984) *Biochem. Biophys. Res. Commun. 124*, 350–358.

Xu, Q., Phizicky, E. M., Greer, C. L., & Abelson, J. (1990) *Methods Enzymol.* (in press).