Frenkel, K., Goldstein, M. S., & Teebor, G. W. (1981b) *Biochemistry 20*, 7566-7571.
Frenkel, K., Goldstein, M. S., & Teebor, G. W. (1982) *Proceedings of the 73rd Annual Meeting of AACR*, St. Louis, MO, Vol. 23, p 67 (Abstract 261), Cancer Research, Philadelphia, PA.
Frenkel, K., Chrzan, K., Troll, W., Teebor, G. W., & Steinberg, J. J. (1986) *Cancer Res. 46*, 5533-5540.
Hariharan, P. V., & Cerrutti, P. A. (1977) *Biochemistry 16*, 2791-2795.
Higgins, S., Frenkel, K., Cummings, A., & Teebor, G. W. (1986) *Proceedings of the 77th Annual Meeting of AACR*, Los Angeles, CA, Vol. 27, p 104 (Abstract 409), Cancer Research, Philadelphia, PA.
Hollstein, M. C., Brooks, P., Linn, S., & Ames, B. N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4003-4007.
Ide, H., Kow, Y. W., & Wallace, S. (1985) *Nucleic Acids Res. 13*, 8035-8052.
Iida, S., & Hayatsu, H. (1970) *Biochim. Biophys. Acta 213*, 1-13.
Iida, S., & Hayatsu, H. (1971) *Biochim. Biophys. Acta 240*, 370-375.
Latarjet, R., Ekert, B., Apelgot, S., & Rebeyrotte, N. (1961) *J. Chim. Phys. 58*, 1046-1057.
Mattern, M. R., Hariharan, P. V., Dunlap, B. E., & Cerutti, P. A. (1973) *Nature (London), New Biol. 245*, 230-232.
Murahashi, S., Yuki, H., Kosai, K., & Doura, F. (1966) *Bull. Chem. Soc. Jpn. 39*, 1559-1562.
Rouet, P., & Essigman, J. M. (1985) *Cancer Res. 45*, 6113-6118.
Scholes, G. (1976) in *Photochemistry and Photobiology of Nucleic Acids* (Wang, S. Y., Ed.) Vol. I, pp 521-577, Academic, New York.
Teebor, G. W., Frenkel, K., & Goldstein, M. S. (1982a) *Prog. Mutat. Res. 4*, 301-311.
Teebor, G. W., Frenkel, K., & Goldstein, M. S. (1982b) *Adv. Enzyme Regul. 20*, 39-54.
Teebor, G., Cummings, A., Frenkel, K., Shaw, A., Voiturez, L., & Cadet, J. (1987) *Free Radical Res. Commun. 2*, 303-309.
Teoule, R., Bonicel, A., Bert, C., Cadet, J., & Polverelli, M. (1974) *Radiat. Res. 57*, 46-58.
Teoule, R., Bert, C., & Bonicel, A. (1977) *Radiat. Res. 72*, 190-200.

Effect of Single Amino Acid Changes in the Region of the Adenylylation Site of T4 RNA Ligase

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ABSTRACT: Preparation and analysis of a series of mutants of bacteriophage T4 RNA ligase that carry single amino acid changes at or near the site of covalent reaction with ATP (adenylylation) are described. The mutant proteins were constructed by site-directed mutagenesis of the gene for T4 RNA ligase (g63) cloned in M13 vectors, transfer of the mutant genes into a λpL-containing expression plasmid, and subsequent expression in *Escherichia coli*. The results give further evidence that Lys-99 is the adenylylation site and that the residue is also important to step 3 in the RNA ligase mechanism (ligation between acceptor and adenylylated donor). Mutations at Glu-100 or Asp-101 have no effect on adenylylation, but Asp-101 is shown to be crucial to both step 2 (transfer of adenylyl to donor) and step 3.

Bacteriophage T4 RNA ligase (EC 6.5.1.3) is a useful enzyme that catalyzes a variety of inter- and intramolecular nucleic acid joining reactions (Uhlenbeck & Gumport, 1982). Of all currently known ligases, it is the only one that catalyzes the formation of a 3′-5′-phosphodiester bond between one nucleic acid strand containing a 5′-terminal phosphate and another containing a 3′-terminal hydroxyl group without needing a template. The enzyme has therefore been of particular value in the synthesis of defined-sequence oligoribonucleotides and in the 3′ labeling of RNA using nucleoside 3′,5′-bisphosphates (Uhlenbeck & Gumport, 1982).

The enzyme mechanism for joining reactions catalyzed by RNA ligase involves three reversible steps. First, the enzyme reacts with ATP to form a covalently adenylylated enzyme intermediate (E-pA) and pyrophosphate. In the second step, the adenylyl moiety is transferred to the 5′-terminal phosphate of a donor molecule [pN(pN)$_n$] to form an adenylylated donor. Finally, a 3′-5′-phosphodiester bond is formed by reaction of the 3′-hydroxyl group of an acceptor molecule [N(pN)$_m$pN] with the adenylylated donor releasing AMP.

$$E + ATP \rightleftarrows E\text{-}pA + PP_i \quad (1)$$

$$E\text{-}pA + pN(pN)_n \rightleftarrows [A\text{-}5'pp5'N(pN)_n]\cdot E \quad (2)$$

$$N(pN)_mpN + [A\text{-}5'pp5'N(pN)_n]\cdot E \rightleftarrows N(pN)_mpNpN(pN)_n + AMP + E \quad (3)$$

Adenylylated enzyme intermediates appear to be important to the mechanism of many ligase enzymes. A covalent phosphoramidate bond between the adenylyl moiety and enzyme has been shown to be formed in the case of T4 DNA ligase (Gumport & Lehman, 1971), T4 RNA ligase (Juodka et al., 1980; Juodka & Markuckas, 1985), and wheat germ RNA ligase (Pick et al., 1986). Similar adenylylated intermediates have been postulated for a yeast tRNA ligase (Phizicky et al., 1986) and an RNA ligase (Perkins et al., 1985) and a cyclase (Filipowicz et al., 1985) from HeLa cells. In the cases of T4 DNA ligase and T4 RNA ligase, a lysine residue has been implicated as the site of adenylylation. By

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fast atom bombardment (FAB)[1] mass spectrometric analysis of chymotryptic fragments of adenylylated enzyme, the lysine residues have been identified as Lys-99 for RNA ligase (Thøgersen et al., 1985) and Lys-221 for DNA ligase (H. C. Thøgersen, personal communication). Although considerable homology is apparent between the protein sequences around the adenylylation site of T4 DNA ligase and sequences in yeast DNA ligase and T7 DNA ligase (Barker et al., 1985), homology between T4 RNA ligase and T4 DNA ligase is extremely weak.

Inspection of the reaction mechanism of T4 RNA ligase shows that whereas step 2 (adenylylation of donor) requires adenylylated enzyme, step 3 (ligation) requires free enzyme. Therefore, in usage of the enzyme in joining reactions, it is necessary to carefully adjust the ATP concentration for optimal results. This is particularly important in the case of poor acceptors, where excess ATP is detrimental, since adenylylated donor can dissociate from the enzyme before ligation to acceptor occurs. If the free enzyme becomes adenylylated before the donor can rebind, the system can become "over-adenylylated", and ligation yields are reduced (Beckett & Uhlenbeck, 1984). The alternative strategy of reducing the ATP concentration has the effect of causing a substantial reduction in the rate of reaction.

We recently reported the cloning of gene 63 of bacteriophage T4 into M13 vectors. The gene codes for a single polypeptide of 374 amino acids containing both RNA ligase and tail fiber attachment activities (Rand & Gait, 1984). The gene was engineered into an expression plasmid containing the *tac* promoter for high-level expression in *Escherichia coli*. With this strain (E/KR54), milligram quantities of RNA ligase could be prepared, and from FAB mass spectrometric analysis of chymotryptic fragments of adenylylated enzyme, Lys-99 was identified as the likely adenylylation site (Thøgersen et al., 1985).

To gain further insight into the adenylylation reaction, we now describe the construction and analysis of a number of mutants of RNA ligase that have single amino acid replacements at or near the presumed site of adenylylation. The mutant enzymes were prepared by site-directed mutagenesis of the gene for RNA ligase and expression in *E. coli*, new techniques that are proving useful for structure–function analysis of proteins (Fersht et al., 1984). One aim of this work was to see whether a mutant RNA ligase could be obtained which was blocked in its ability to be adenylylated, yet could still carry out step 3 (ligation). Such a mutant enzyme might be useful as an additive to enhance the rate of an overall ligation reaction catalyzed by wild-type enzyme, since the mutant would not be converted to the adenylylated form and would be available for step 3.

Materials and Methods

Restriction endonucleases were purchased from New England Biolabs; DNA polymerase I (Klenow subfragment) was from Boehringer. T4 DNA ligase was a gift from K. Nagai or purchased from New England Biolabs. The in vitro prokaryotic DNA-directed translation kit was purchased from Amersham International and used following the manufacturer's recommendations with L-[^{35}S]methionine as the radioactive tracer.

T4 RNA Ligase Expression Vector (pMG518) and E. coli Strains E/MG518 and E/MG526. A 1.15 kb *Mnl*I fragment from pUC9HS containing the T4 RNA ligase gene (g63) (Rand & Gait, 1984) was ligated into the *Hin*cII site of M13mp7 RF DNA and used to transfect *E. coli* strain TG1 (Carter et al., 1985). RF DNA from a recombinant phage isolate (M/KR95) was digested with *Bam*HI, and a 1.15 kb fragment containing g63 was purified by agarose gel electrophoresis. The fragment was ligated to the λpL-containing vector, pLmp10 (Nagai & Thøgersen, 1984), which had been digested with *Bam*HI and phosphatase treated, and used to transform *E. coli* strain QY13 (Nagai et al., 1985) containing a λ-lysogen with a temperature-sensitive repressor (cI857). An isolate was obtained (E/MG518) containing a plasmid in which the orientation of the insert is correct for expression of g63 from the λpL promoter. Plasmid from this strain (pMG518) was used also to transform another *E. coli* strain, MZ-1 (Nagai & Thøgersen, 1984), containing a λ-lysogen with a temperature-sensitive repressor to give *E. coli* strain E/MG526. The strains are best grown at 30 °C in rich media containing 100 μg/mL ampicillin. For production of RNA ligase, cells are grown to late log phase, brought rapidly to 42 °C for 10 min, and incubated at 37 °C for a further 50 min. Harvesting and isolation of RNA ligase are carried out by (a) cell lysis, (b) streptomycin sulfate precipitation of DNA, (c) ammonium sulfate precipitation, (d) DEAE-Sephacel chromatography, and (e) chromatography on Blue Dextran–agarose as previously described for strain E/KR54 (Thøgersen et al., 1985; Rand et al., 1985). With E/MG518, ca. 10–15 mg of >95% RNA ligase can be obtained per liter of induced cell suspension. E/MG526 has a somewhat longer lifetime when stored either on agar plates or in 50% glycerol suspension but produces slightly lower quantities of RNA ligase (note the viability of host strains QY13 and MZ-1 appears to be more rapidly reduced upon storage than many common *E. coli* strains).

Site-Directed Mutagenesis of g63. The 1.15 kb *Bam*HI fragment from M/KR95 containing g63 was ligated to M13K19 RF DNA (Carter et al., 1985) which had been cut with *Bam*HI, phosphatase treated, and used to transfect *E. coli* TG1. A phage isolate was obtained (M/MS152) that contained g63 orientated correctly as if for expression from the *lac* promoter. However, in order to maintain g63 in this construction stably, phage were stored and passaged in *E. coli* in the presence of 2 mg/mL Antiinducer, *o*-nitrophenyl β-D-fucopyranoside (ONPF), to reduce the possibility of induction of the *lac* promoter by media components and subsequent selection of deletion mutants (Thøgersen et al., 1985). Mutagenesis was carried out by using a two-primer approach on single-stranded M/MS152 DNA and *Eco*K/*Eco*B selection as previously described (Carter et al., 1985). The selection primer was SEL 2 (Carter et al., 1985). The mutagenesis primers were as listed below. Transfection of the primer-extended M/MS152 DNA was into repair-deficient *E. coli* HB2154 *mut*L using a lawn of repair + HB2151. The antiinducer ONPF was also used during plating. A total of 40–50 plaques were grown as colonies of infected bacteria and transferred to nitrocellulose, and the DNA was probed with mutagenesis oligonucleotide as previously described (Carter et al., 1985). The proportion of strongly hybridizing colonies varied from 5% to 40%. Mutant phage were plaque purified, and the complete DNA sequence of g63 and the polylinker

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; FAB, fast atom bombardment; kb, kilobase(s); ONPF, *o*-nitrophenyl β-D-fucopyranoside; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; PEG, poly(ethylene glycol); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; TFA, tail fiber attachment; RLi, RNA ligase.

region was sequenced by the chain termination procedure (Bankier & Barrell, 1983) using the six primers listed below.

Oligonucleotides. Oligonucleotide synthesis was carried out either by the manual phosphotriester method as previously described (Sproat & Gait, 1984) or on an Applied Biosystems 380B DNA synthesizer using the phosphoramidate procedure. Gene 63 sequencing primers were P1:M13 universal sequencing primer (Duckworth et al., 1981), P2:d(CAGAGCTCGGTCTAAGTAC), 907–926, P3:d(CACAGCAACATAGCCTTCG), 694–712, P4:d(GCTAATTCTTTAAGTCT), 449–465, P5:d(AAAACTTTTCCATAGGA), 229–245, and P6:d(CTTACGCTGCGAATCCTT), 53–70. The numbers refer to the nucleotide sequence numbers given for the g63-containing DNA fragment previously published (Rand & Gait, 1984).

Mutagenesis primers were as follows (asterisks denote and follow the mutagenic base): d(CCGTCTTCA*TTTGTTAG) for Lys-99-Asn, d(CGTCTTCTC*TTGTTAGAAT) for Lys-99-Arg, d(ACCCGTCTTG*TTTTGTT) for Glu-100-Gln, d(GACCCGTCTG*T*TTTTGTT) for Glu-100-Thr, d(AGACCCA*TT*TTCTTTTG) for Asp-101-Asn, d(AGACCCGC*T*TTCTTTTG) for Asp-101-Ser, and d(AGACCCT*TCTTCTTTTG) for Asp-101-Glu. Synthetic oligonucleotides were also prepared for use in construction of an *Xba*I–*Hin*dIII polylinker that includes sites for *Pst*I, *Eco*RV, and *Kpn*I: (top strand) d(CTAGACTGCAGATATCGGTACCA); (bottom strand) d(AGCTTGGTACCGATATCTGCAGT).

Expression of Mutant RNA Ligases. (A) Vector pMG524. pLmp10 (Nagai & Thøgersen, 1984) DNA was digested with *Xba*I and *Hin*dIII and electrophoresed on a 0.8% agarose gel. Material in the major band was eluted by the "glass beads/NaI" method (Vogelstein & Gillespie, 1979) as modified by D. Botstein (personal communication). This was ligated to an *Xba*I–*Hin*dIII polylinker, formed by annealing of top and bottom strands described above, and used to transform *E. coli* strain QY13. An isolate was obtained with a plasmid containing the desired insert (pMG524).

(B) g63 Mutant Double-Stranded DNA. Single-stranded M13 DNA containing the desired g63 mutation was prepared as for dideoxy sequencing (Bankier & Barrell, 1983) except that just prior to the phenol extraction step small RNA primers were eliminated by digestion of the resuspended PEG pellet with 0.5 mg/mL RNase at 37 °C for 30 min. From 5 × 1.5 mL aliquots of M13-infected cell supernatant, a total of ca. 15 μg of single-stranded DNA was obtained. For second-strand synthesis, single-stranded DNA (5 μg) was mixed with M13 sequencing primer P1 (20 pmol) in 20 μL of TM buffer [10 mM Tris·HCl (pH 7.4) and 10 mM $MgCl_2$] and annealed by cooling from 80 to 20 °C over 1 h. To this was added 5 mM dNTPs (2 μL), 0.1 M DTT (2 μL), 10 × TM buffer (2 μL), water (12 μL), and finally DNA polymerase I (Klenow) (2 μL, 10 enzyme units). After incubation at 20 °C for 20 min followed by 70 °C for 5 min, 60 μL of TE buffer (10 mM Tris·HCl, pH 7.4, and 0.1 mM EDTA) was added and the DNA precipitated by addition of 13% PEG and 1.6 M NaCl (100 μL). The PEG pellet was dissolved in TM buffer and digested sequentially with *Kpn*I (40 enzyme units) for 1 h, 37 °C, and *Xba*I (40 enzyme units) for 1 h, 37 °C, after adjustment of the salt to 50 mM Tris·HCl, pH 7.4, and 100 mM NaCl. The material was electrophoresed on a 0.8% agarose gel, and the 1.15 kb band, corresponding to the mutant g63, eluted.

(C) Cloning and Expression of g63 Mutants in pMG524. pMG524 DNA was digested exhaustively with *Kpn*I and *Xba*I (note that it is essential that *E. coli* strain QY13 be used for the preparation of pMG524 DNA since in strain MZ-1 the *Xba*I site appears to be methylated and is not digested by *Xba*I) and electrophoresed on a 0.8% agarose gel. The material in the major band was eluted and ligated to mutant g63 double-stranded DNA and used to transform *E. coli* MZ-1 (note that MZ-1 gives a higher transformation frequency than QY13). Recombinants containing mutant g63 were selected by restriction analysis of plasmid DNA. Expression of g63 mutants in *E. coli* was carried out as for wild-type enzyme (see above).

Purification of Mutant RNA Ligases. Three types of sample preparation were used: (a) Adenylylation assays (step 1) were initially carried out on crude cell extracts after sonication in 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, 0.5 mM PMSF, 1 mM benzamidine, and 0.07% β-mercaptoethanol. Further adenylylation assays were carried out on RLi (Arg-99) and RLi (Asn-99) after purification by method c. (b) Overall assays (pCp addition to tRNA; see below) were carried out on rapid protein "minipreps" as follows. The cells from a 10-mL scale growth were sonicated 3 times in 3 × 300 μL of 50 mM Tris·HCl (pH 7.4), 1 mM EDTA, 0.5 mM PMSF, 1 mM benzamidine, and 0.07% β-mercaptoethanol. After centrifugation, the combined supernatants (ca. 1 mL) were taken, DNA was removed by streptomycin sulfate precipitation, and the protein was precipitated at 50% ammonium sulfate saturation as previously described (Thøgersen et al., 1985; Rand et al., 1985) except that microfuge tubes were used for precipitation and centrifugation. The ammonium sulfate pellet was dissolved in 0.5 mL of buffer A (20 mM Hepes/NaOH, pH 7.5, 1 mM DTT, and 0.1 mM EDTA), applied to a column of DEAE-Sephacel (3 × 1 cm), and eluted with buffer A at ca. 0.4 mL min^{-1}. When the A_{280} of the eluate returned to the base line, the column was eluted with buffer A + 400 mM NaCl. Material in the eluting peak was collected (ca. 1 mg) and was sufficiently pure for the overall assay described below. (c) Step 2 and step 3 assays were carried out on >95% pure material as judged by SDS–polyacrylamide gel electrophoresis. Purifications were carried out on cells obtained from a 2-L culture and followed the procedures of ammonium sulfate precipitation, DEAE-Sephacel chromatography, and Blue Dextran–agarose chromatography as previously described (Thøgersen et al., 1985; Rand et al., 1985). The purifications were monitored by SDS–polyacrylamide gel electrophoresis.

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out on 11% minigels (10 × 10 × 0.05 cm) using a 4% stacking gel (Laemmli, 1970). Polyacrylamide gel electrophoresis was carried out on 10% or 12.5% native gels (20 × 15 × 0.15 cm) buffered with 0.375 M Tris·HCl and 0.125 mM EDTA (pH 8.8) using 25 mM Tris–glycine (pH 8.3) in the reservoirs (Blackshear, 1984). Urea gradient gel electrophoresis, with the urea gradient perpendicular to the direction of electrophoresis, was carried out by the method of Creighton (1979) except that gels were prepared with a gradient of acrylamide of 10–8% in the reverse direction to the urea gradient (0–6 M) and 2 mM mercaptoacetic acid was used in the reservoir buffer to prevent oligomerization of denatured proteins. Samples were loaded in the presence of 8 M urea, but no difference was observed for wild-type RNA ligase if the urea was omitted.

Determination of Protein Concentration. Total cellular protein was estimated by using the Bio-Rad dye reagent using BSA as standard. RNA ligase and mutants were estimated spectroscopically by using a value of 1.3 A_{280}/mg of protein

(Uhlenbeck & Gumport, 1982). RNA ligase concentrations determined by the Bio-Rad reagent gave values ca. 1.8-fold higher than those determined spectroscopically.

Polyclonal Antiserum to RNA Ligase and Western Blotting. A mixture of 1.5 mL of Freunds complete adjuvant and 1.5 mL of RNA ligase (0.5 mg/mL) was injected in multiple subcutaneous sites into each of two New Zealand white rabbits. On day 14, a booster injection of 2 mL of incomplete adjuvant and 2 mL of RNA ligase (0.5 mg/mL) was given to each rabbit. On day 24, the rabbits were bled, and after clotting for 7 h at 37 °C and centrifugation, the serum from each was collected and stored at −20 °C in aliquots. The sera were tested for antibodies to RNA ligase in an assay using ^{125}I-labeled protein A, the best giving maximal binding at 1/64 dilution and still better than half-maximal at 1/2000. Western blot transfers of protein from SDS–polyacrylamide gels to nitrocellulose were carried out as previously described (Towbin et al., 1979) except that the gels were smaller (10 × 10 × 0.05 cm). The filters were washed for 8–15 h with (a) 1/64 dilution of anti-RNA ligase serum in 0.25% BSA, 0.15 M NaCl, 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, and 0.1% sodium azide and (b) ^{125}I-labeled protein A (0.08 μCi mL^{-1}) in the same buffer as well as with intermediate washings. After being dried, the filters were autoradiographed for ca. 3 h at −70 °C without preflash.

Overall Assay of RNA Ligase Activity. Reactions were carried out in microtiter wells in 20-μL volumes containing 1.66 pmol of [^{32}P]pCp [300 μCi pmol^{-1} prepared by T4 polynucleotide kinase catalyzed reaction of cytidine 3′-monophosphate (P-L Pharmacia) with [γ-^{32}P]ATP (Amersham, 3000 Ci mmol^{-1})], 0.2 mg mL^{-1} yeast tRNA, 5 μM rATP, 50 mM Hepes/NaOH (pH 8.0), 20 mM $MgCl_2$, 10 μg mL^{-1} BSA, 3.3 mM DTT, 10% Me_2SO, and varying concentrations of protein (0.02–0.1 mg mL^{-1}; Bio-Rad) at 4 °C for 16 h. The reaction mixtures were spotted on Whatman GF/C filter paper disks. Filters were dried and washed (all filters simultaneously) 3 times in 10% trichloroacetic acid solution, dipped briefly in water, dried, and counted by liquid scintillation in 3 mL of Aquasol-2 (New England Nuclear).

Adenylylation Assays (Step 1). Adenylylation was carried out at 1 mg/mL protein concentration in 50 mM Tris·HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, and 1 mM rATP [in some cases [^{3}H]rATP, 2 Ci mmol^{-1}, was used] for 15 min at room temperature with or without 0.005 enzyme unit μL^{-1} inorganic pyrophosphatase (Sigma). For the pH profile of RNA ligase adenylylation, 50 mM Hepes/NaOH, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM rATP buffer was used and the pH increased by addition of NaOH. Samples were applied together with loading dye to an SDS–polyacrylamide gel (see above) which was either (a) stained with Coomassie blue or (b) transferred to nitrocellulose by Western blotting and probed with anti-RNA ligase serum. (c) In the case of [^{3}H]ATP reactions, after Coomassie staining and destaining, the gel was washed with Amplify (Amersham) for 30 min, dried between cellophane sheets, and fluorographed with preflashed film at −70 °C. Scanning of gels was carried out on a Camag electrophoresis scanner.

HPLC Assays for Steps 2 and 3. HPLC was carried out by using a Waters gradient system consisting of two model 510 pumps, a Model 680 gradient programmer, a Model 481 variable-wavelength UV detector, a Model 730 data module, and a Rheodyne 7125 injector. Columns were of Partisil 10-SAX (250 × 4.6 mm; Whatman), and chromatography was carried out at ambient temperature at a flow rate of 2 mL min^{-1}. Buffers were prepared from a stock solution of 1 M KH_2PO_4 adjusted to pH 5.0 with KOH to give (A) 1 mM and (B) 0.4 M. Gradients for step 2 assay were 2 min, 0% B; 4 min, 0–30% B; 20 min, 30–50% B. For step 3 assay, gradients were 4 min, 0% B; 12 min, 0–30% B; 9 min, 30–100% B.

Step 2 Assay: Adenylylation of Donor. Reactions were carried out in 0.1-mL volumes at 37 °C containing 50 mM Hepes/NaOH (pH 8.5), 20 mM $MgCl_2$, 5 mM DTT, 10 μg mL^{-1} BSA, 0.2 mM pCp, 0.5 mM rATP, and 60 μg mL^{-1} enzyme. Twenty-microliter aliquots were taken at appropriate time intervals, diluted with 20 μL of 0.1 M phosphate buffer (pH 4.5), and frozen in dry ice/2-propanol. Thawed samples were injected onto the HPLC column which was monitored at 260 nm, 0.2 full-scale deflection. Peak areas of pCp and AppCp were determined and corrected for the extinction coefficient and the ratio calculated to assess the percent completion.

Step 3 Assay: Ligation. Reactions were carried out in 0.1-mL volumes at 37 °C containing 50 mM Hepes/NaOH (pH 8.5), 20 mM $MgCl_2$, 5 mM DTT, 10 μg mL^{-1} BSA, 0.2 mM AAG, 0.4 mM AppGp, and 20 μg mL^{-1} enzyme. Twenty-microliter aliquots were treated as for the step 2 assay. Peak areas for ApApG and ApApGpGp were determined and corrected for the extinction coefficient and the ratio calculated to assess the percent completion.

Results

Some initial mutagenesis experiments have been previously carried out on g63 cloned in M13 vectors (Rand et al., 1985). Such vectors are useful in that they are suitable for several mutagenesis procedures (Smith, 1985) and also allow rapid DNA sequencing by the chain termination method (Bankier & Barrell, 1983). M13 DNA containing mutant g63 was used to infect *E. coli* cells and protein synthesized by induction of the *lac* promoter. In vivo complementation assays for RNA ligase (RLi) and tail fiber attachment (TFA) activities were developed for assaying M13-infected *E. coli* clones. In this way, it was shown by deletion analysis that a mutant that would give rise to a polypeptide lacking 74 amino acids at the C-terminus maintained its RLi activity but TFA activity was abolished. In another construct, a point mutation that would give rise to a protein with a single Lys to Asn change at position 99 was phenotypically RLi$^-$, TFA$^+$.

These assay procedures have disadvantages, however. Although the role of TFA activity in T4 infection is well established (Wood & Henninger, 1969), the physiological role of RNA ligase activity is not clearly understood. Most *E. coli* can be infected with RLi$^-$ T4, and the assay was therefore based on an unusual *E. coli*, CTr5X (Depew & Cozzarelli, 1974), which is restrictive to RLi$^+$ T4. The role of RNA ligase here is thought to be involved with the religation of a nuclease-cleaved anticodon in a host Lys tRNA (Kaufmann et al., 1986). The in vitro joining activities of RNA ligase are better characterized (Uhlenbeck & Gumport, 1982). We recently reported the cloning of gene 63 of bacteriophage T4 into M13 vectors. The gene codes for a single polypeptide of 374 amino acids containing both RNA ligase and tail fiber attachment activities (Rand & Gait, 1984). The gene was engineered into an expression plasmid containing the *tac* promoter for high-level expression in *E. coli*. By use of this strain (E/KR54), milligram quantities of RNA ligase could be prepared, and from FAB mass spectrometric analysis of chymotryptic fragments of adenylylated enzyme, Lys-99 was identified as the likely adenylylation site (Thøgersen et al., 1985). However, since these joining activities are highly inhibited by components in *E. coli* lysates (Rand & Gait, 1984), it was important that sufficient quantities of purified mutant

proteins be obtained. This would also enable assays to be carried out for the three individual reaction steps of the joining mechanism as well as for the overall reaction. Unfortunately, the level of RNA ligase obtainable from M13-infected *E. coli* is insufficient to ensure reliable identification, isolation, and in vitro assay of mutants. It was therefore necessary to transfer mutants obtained in M13 vectors into a high-level expression plasmid.

Experience with E/KR54 had shown that care was needed in storage and passage to avoid inadvertent induction of the *tac* promoter by media components and subsequent selection of deletion mutants (Thøgersen et al., 1985). This danger coupled with the cost of chemicals for induction and antiinduction made this system impractical for routine preparation of mutant RNA ligases. Plasmid vectors containing the λpL promoter have been used successfully for the production of proteins when used with *E. coli* hosts harboring a λ-lysogen with the CI857 temperature-sensitive repressor (Remaut et al., 1981). At 30 °C, the repressor is active, and transcription from pL is fully repressed. When cell growth is shifted to 42 °C, cloned genes are fully transcribed [e.g., see Nagai & Thøgersen (1984)]. Accordingly, we constructed a new vector, pMG518, which consists of g63 together with its ribosome binding site cloned in pLmp10 (Nagai & Thøgersen, 1984). *E. coli* strains QY13 and MZ-1 (which carry the defective λ-lysogen) containing plasmid pMG518 produce on induction 5–10% of the soluble protein as RNA ligase (a similar level to E/KR54) as judged by SDS–polyacrylamide gel electrophoresis (data not shown). These strains (E/MG518 and E/MG526) show no danger of loss of RNA ligase producing ability on storage (see note on viability under Materials and Methods).

Site-Directed Mutagenesis and Production of Mutant RNA Ligases. Carter et al. have recently described an improved procedure for oligonucleotide-mediated site-directed mutagenesis using M13 vectors (Carter et al., 1985). The method relies on the use of two mismatched primers: one to make the desired mutation within the gene of interest and a second selection primer to alter an *Eco*K site to an *Eco*B site within a linker region immediately adjacent to the gene. Simultaneously priming on plus-strand M13 template and extension using DNA polymerase I (Klenow) are followed by transfection into an *E. coli* K strain which is also repair deficient to reduce the chance of mismatch repair. This combined use of strand selection and repair-deficient *E. coli* gives substantially improved mutation frequencies.

Gene 63 was therefore cloned into the *Bam*HI site of M13 K19 (Carter et al., 1985) to give M/MS 152. M13 K19 is equipped with a cassette of restriction sites (*Hin*dIII, *Sph*I, *Pst*I, *Xho*I, *Eco*K, *Nru*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sst*I, and *Eco*RI). Thus, mutated g63 could be conveniently excised from M/MS 152 by using *Xba*I and *Kpn*I. Oligonucleotide-directed mutagenesis was carried out by using M/MS 152 and a series of seven oligonucleotides designed to produce mutation at positions 99, 100, and 101 of the RNA ligase protein sequence (Figure 1). M13 clones containing the desired mutation were selected first by hybridization with the mutagenic oligonucleotide under stringent conditions and subsequently by DNA sequencing of the entire coding and polylinker sequence. To aid in hybridization screening, in those cases where the mutagenic mismatch was a G-T pair, a second mutation was made in the primer by altering the third base position of a codon either immediately adjacent (two cases) or separated by one base (one case), so that no amino acid mutation resulted from such a change. Of 40–50 clones

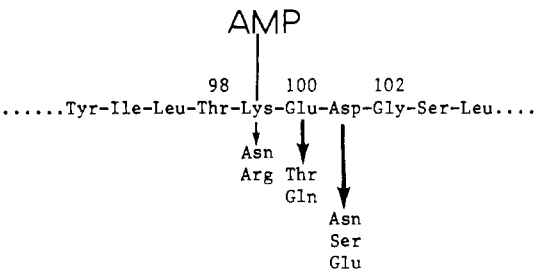

FIGURE 1: Single amino acid replacements at or near the adenylylation site of T4 RNA ligase.

screened for each mutagenesis, the proportion of strongly hybridizing clones varied from 5% to 41%. In most cases, clones selected for further analysis showed only the desired DNA sequence alteration. However, in about 25% of the clones selected, an unwanted deletion or deletion plus insertion was found at another site as well as the desired mutation. No undesired point mutations were found. The reason for this unusually high proportion of spurious deletions is not clear.

A double-stranded *Xba*I–*Kpn*I restriction fragment containing the mutant gene was obtained in each case by use of the universal M13 sequencing primer and DNA polymerase I (Klenow) to prepare a complementary strand from single-stranded M13/g63 mutant (Figure 2), digestion with *Xba*I and *Kpn*I, and purification of the fragment by agarose gel electrophoresis. The fragment was ligated to the expression vector pMG524 (prepared from pLmp10 by replacement of the *Xba*I–*Hin*dIII small fragment by a synthetic linker containing sites for *Xba*I, *Pst*I, *Eco*RV, *Kpn*I, and *Hin*dIII), which had been cut with *Xba*I and *Kpn*I. After transformation of *E. coli* MZ-1, recombinants were isolated capable of expressing the mutant g63 from the λpL promoter. Essentially similar techniques were used to isolate mutants as for wild-type RNA ligase (Thøgersen et al., 1985).

Characterization and Assay of RNA Ligase Mutants. The identity of RNA ligase mutants on SDS–polyacrylamide gels was established by use of "Western blotting" (Towbin et al., 1979) using polyclonal antisera raised against wild-type RNA ligase. This was particularly useful since the mobilities of some of these mutants were quite variable (Figure 3). Mutants made at other positions of RNA ligase (Singh et al., 1986) have shown the same mobility of the mutant protein as wild type (data not shown). Another anomaly was that in the case of RLi (Arg-99), two very closely running bands on SDS–PAGE were visible in the purified material instead of a single band obtained with the remaining mutants (Figure 4a). By scanning densitometry of the Coomassie blue stained gel, it was found that 15–20% was in the slower band and 80–85% in the faster band. Protein isolated from several independent clones gave essentially the same result. Moreover, plasmid DNA was used to retransform *E. coli* MZ-1, and the same result was again seen when protein was isolated from individual clones.

To assess which of the mutants would be important to assay in more detail, a rapid screening procedure for overall RNA ligase activity was developed based on the addition of [^{32}P]pCp to tRNA (Bruce & Uhlenbeck, 1978). For this assay, a partially purified preparation of the mutant protein was sufficient. This preparation could be obtained very rapidly from 10 mL of induced cell suspension, and the majority of inhibitors of the assay were removed by this procedure. It was found that RLi (Asn-99) and all three mutants at Asp-101 were completely inactive and that RLi (Glu-100) showed essentially wild-type activity but RLi (Arg-99) and RLi (Thr-100) had intermediate activity (Table I). This assay has proved useful

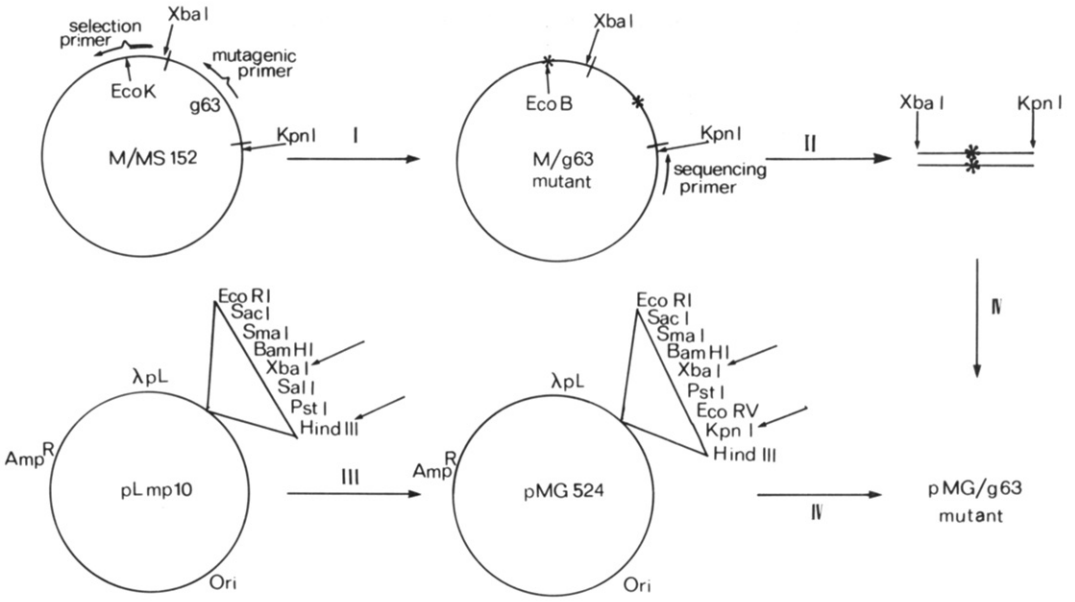

FIGURE 2: Construction of expression plasmids containing g63 mutants. (I) Two-primer mutagenesis on single-stranded M/MS152. (II) Second-strand synthesis and excision of g63 mutant. (III) Construction of expression vector pMG524 using *Xba*I–*Hin*dIII polylinker. (IV) Cloning of g63 mutant into pMG524 using *Xba*I–*Kpn*I sites.

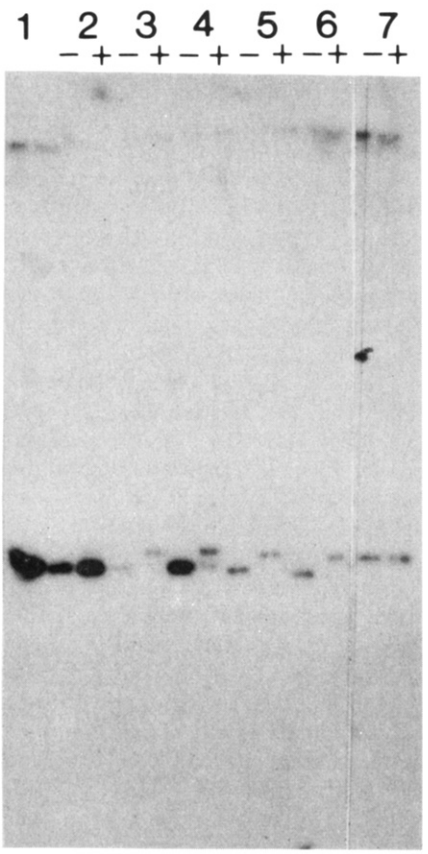

FIGURE 3: Autoradiograph of Western blot after SDS–PAGE of RNA ligase mutants in crude *E. coli* extracts with (+) and without (−) adenylylation. Note that the adenylylated form of RNA ligase migrates slower than free enzyme (Higgins et al., 1977). Lane 1, wild type; lane 2, RLi (Arg-99); lane 3, RLi (Asn-101); lane 4, RLi (Ser-101); lane 5, RLi (Gln-100); lane 6, RLi (Thr-100); and lane 7, RLi (Asn-99). The adenylylation reactions did not contain inorganic pyrophosphatase.

for a variety of mutations at other sites (Singh et al., 1986; unpublished results).

In contrast to the overall reaction, the adenylylation step of RNA ligase is not unduly affected by other components in crude *E. coli* extracts. The reaction of enzyme with ATP

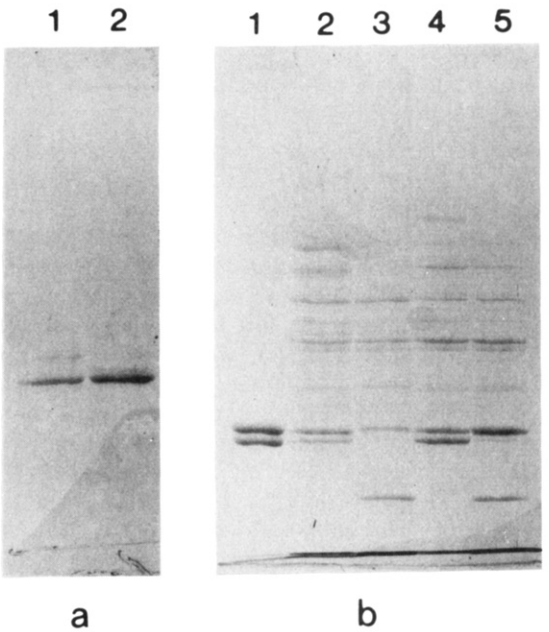

FIGURE 4: (Panel a) Coomassie blue stained SDS–PAGE of purified RLi (Arg-99). Lane 1, with adenylylation; lane 2, without adenylylation. (Panel b) Coomassie blue stained SDS–PAGE. Lane 1, control mixture of purified RNA ligase with and without adenylylation; lane 2, partially purified wild-type RNA ligase from E/MG518 showing both forms; lane 3, same after adenylylation; lane 4, partially purified RLi (Glu-101); lane 5, same after adenylylation. In lanes 3 and 5, the extra band near the bottom of the gel is inorganic pyrophosphatase.

Table I: Activity of RNA Ligase Mutants[a]

amino acid change	overall ligase activity	step 1	step 2	step 3
Lys-99 to Asn	−	−	−	−
Lys-99 to Arg	+	+	+	+
Glu-100 to Gln	++	++	ND	ND
Glu-100 to Thr	+	++	ND	ND
Asp-101 to Asn	−	++	−	−
Asp-101 to Ser	−	++	−	−
Asp-101 to Glu	−	++	−	−

[a] Abbreviations: (−) no activity; (+) intermediate activity; (++) similar activity to wild-type enzyme; ND, not determined.

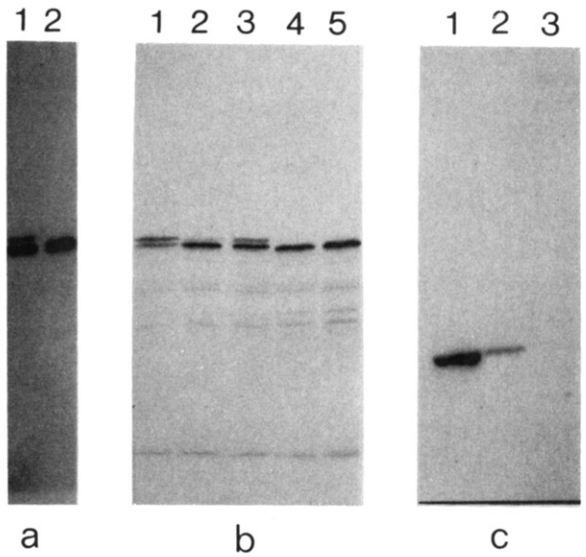

FIGURE 5: (Panel a) Autoradiograph of Western blot of RLi (Arg) in crude *E. coli* extract. Lane 1, with adenylylation in the presence of inorganic pyrophosphatase; lane 2, without adenylylation. (Panel b) Fluorograph of products from cell-free transcription–translation system. Lane 1, wild-type RNA ligase from E/MG518; lane 2, same but with sodium pyrophosphate; lane 3, same with adenylylation; lane 4, RLi (Arg-99) with adenylylation; lane 5, RLi (Arg-99) with sodium pyrophosphate. (Panel c) Fluorograph of SDS–PAGE of RNA ligase mutants after adenylylation with [^{3}H]rATP. Lane 1, wild-type RNA ligase; lane 2, RLi (Arg-99); lane 3, RLi (Asn-99).

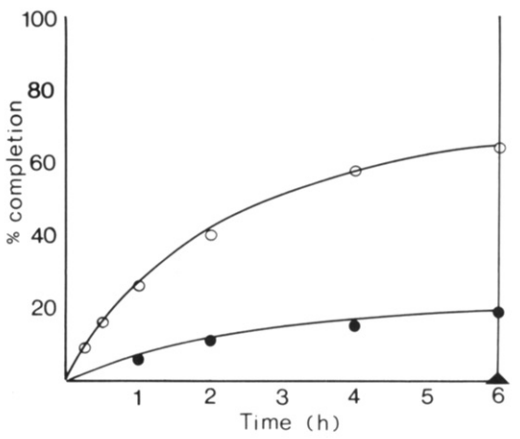

FIGURE 6: Step 2 assay: graph of percent completion against time; for conditions, see Materials and Methods. (○) Wild type; (●) RLi (Arg-99); (▲) RLi (Asn-99), RLi (Ser-101), RLi (Asn-101), and RLi (Glu-101).

requires Mg^{2+} and gives equimolar quantities of adenylylated enzyme and pyrophosphate. The apparent K_m for ATP has been estimated to be ca. 12 μM (Cranston et al., 1974). The reaction is completely reversible and reaches equilibrium rapidly (Uhlenbeck & Gumport, 1982). We have found that in the case of purified RNA ligase the equilibrium position is influenced significantly by pH. Under the conditions used (see Materials and Methods), maximum adenylylation is achieved above pH 8.7. Alternatively, adenylylation can be pushed to completion at lower pH by addition of inorganic pyrophosphatase (Cranston et al., 1974). The extent of adenylylation of RNA ligase and mutants can be easily assessed by SDS–PAGE since the adenylylated form of RNA ligase migrates substantially slower (Higgins et al., 1977). Figure 3 shows a Western blot of protein obtained from crude cell extracts of several RNA ligase mutants with and without adenylylation, and Figure 4b shows a Coomassie blue stained gel of the adenylylation of wild-type RNA ligase and a partially purified RLi (Glu-101) mutant. It can be seen that all mutants have essentially wild-type adenylylation except RLi (Asn-99) which is completely inactive and RLi (Arg-99) which shows only marginal activity.

More careful study of RLi (Arg-99) by Western blotting (Figure 5a) shows that only the slower of the two closely running bands (the minor component) is altered in mobility on adenylylation. To rule out the possibility that RLi (Asn-99) and RLi (Arg-99) are adenylylated but not altered in mobility on the SDS-gel, adenylylation was carried out using [^{3}H]ATP. After electrophoresis, the gel was fluorographed (Figure 5c). Whereas wild-type adenylylated RNA ligase showed a single strong band, RLi (Arg-99) showed only a weak band, and there was no band in the case of RLi (Asn-99). The possibility that RLi (Asn-99) and the major (inactive) material in RLi (Arg-99) are denatured forms can be discounted since the mobility of both mutants was similar to wild-type enzyme when electrophoresed on native polyacrylamide gels (results not shown). Electrophoresis of the two mutants on gradient urea gels (Creighton, 1979) showed that they denature and renature rapidly and with similar characteristics to wild-type enzyme (results not shown), suggesting that the overall conformations of the mutants are not grossly distorted. Moreover, when plasmid DNA containing the gene for RLi (Arg-99) was used in a cell-free transcription–translation system and the protein obtained subjected to SDS–PAGE, fluorography showed only one band for RLi (Arg-99) corresponding to the major (inactive) band in the cellular system (Figure 5b). No alteration in mobility was seen under conditions of adenylylation.

Proteins with mutations at positions 99 and 101 were purified to >95% homogeneity for assay for steps 2 and 3 of the RNA ligase mechanism (Table I). The step 2 assay involved reaction of the good donor, pCp, with adenylylated enzyme to give AppCp. Free RNA ligase is inactive (McLaughlin et al., 1985). Thus, only those mutants which can be adenylylated normally would be likely to have activity in step 2. As expected, therefore, RLi (Asn-99) was inactive. Conditions for the assay were chosen to be similar to those used previously (McLaughlin et al., 1985) with high ATP concentration (to ensure rapid adenylylation) and pCp concentration close to the reported K_m. Figure 6 shows that for wild-type enzyme the reaction was 64% complete in 6 h whereas all mutants at position 101 had no detectable product formation in the same time period. RLi (Arg-99) showed 19% reaction in the same time period [kinetic parameters were not measured for RLi (Arg-99) since the mutant was not a single species].

For step 3 assays, the good acceptor ApApG (Romaniuk et al., 1982) and preadenylylated donor (A(5′)pp(5′)Gp (McLaughlin et al., 1985) were used as substrates. Measurement of the initial velocity of reaction proved difficult, since formation of product ApApGpGp as a function of time rapidly deviated from linearity, possibly due to inhibition by product or due to the presence of a large molar excess of adenylylated donor over enzyme which might result in a rapid decrease of the concentration of free enzyme by adenylylating it through reversal of the second step. Such deviation from linearity had not been reported for the analogous reaction of ApApA with A(5′)pp(5′)Gp (McLaughlin et al., 1985). Lineweaver–Burk plots based on our best measurement of the initial velocity gave an apparent K_m of 0.30 ± 0.15 mM for A(5′)pp(5′)Gp in the presence of 0.5 mM ApApG and a V_{max} of 16 000 ± 4000 nmol min^{-1} mg^{-1}. The apparent K_m for ApApG in the presence of 0.5 mM A(5′)pp(5′)Gp was 0.08 ± 0.04 mM, and the V_{max} was 5000 ± 2000 nmol min^{-1} mg^{-1}. Because of the high inaccuracies of these measurements, it seems unlikely that a meaningful comparison of kinetic pa-

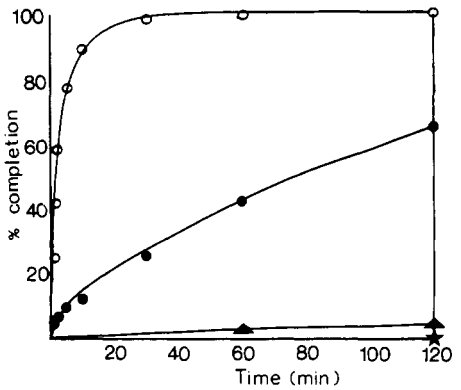

FIGURE 7: Step 3 assay: graph of percent completion against time; for conditions, see Materials and Methods. (○) Wild type; (●) RLi (Arg-99); (▲) RLi (Asn-99); (★) RLi (Ser-101), RLi (Asn-101), and RLi (Glu-101).

rameters is possible between wild-type and mutant enzymes. Accordingly, we report the results in graphical form (Figure 7). Under conditions where wild-type RNA ligase gave 99% completion for this reaction in 30 min, all three mutations at position 101 showed less than 2% reaction in 4 h. RLi (Asn) showed ca. 4% and RLi (Arg-99) 67% reaction in 4 h.

Discussion

The lack of activity of RLi (Asn-99) in the adenylylation reaction is consistent with Lys-99 being the site of adenylylation of RNA ligase as predicted by previous FAB mass spectrometric analysis (Thøgersen et al., 1985). The adenylylation reaction is not affected by mutations at positions 100 and 101 or by mutations at a number of other sites (Singh et al., 1986; unpublished results). In addition, RLi (Asn-99) has only slight activity in step 3 (ligation), suggesting that Lys-99 also plays an important role in this step. It was hoped that measurement of the activities of RLi (Arg-99) would shed light on the question of whether the catalytic role of Lys-99 in step 3 was due to its basic character. However, the results are complicated by the finding of two species that migrate slightly differently on SDS–PAGE. We have taken pains to establish that this is not the result of contamination of the *E. coli* clone with a second plasmid containing wild-type gene nor is the inactive material a denatured form of the protein. The most likely interpretation is that the slower (minor) band is the correct RLi (Arg-99) mutant, fully active in all three steps, and the faster (major) band results from a posttranslational modification to the mutant resulting in a totally inactive protein. The minor band material clearly can be adenylylated, and the partial activity of the mixture in steps 2 and 3 is consistent with the minor band material being the only active part.

Moreover, in in vitro cell-free protein synthesis using a cleared *E. coli* lysate, only the major (inactive) band is seen. This would also fit the intepretation, since complete modification would be expected in the in vitro system where the amount of mutant RLi made is low. In *E. coli* using a high-copy plasmid, the high level of protein synthesis of the mutant may swamp the modification machinery.

We have tried several techniques in an attempt to preparatively separate the two species so that their activities can be measured independently, but so far, we have not been successful. However, if the interpretation is correct, based on the activity of the minor band material, Arg can replace Lys as a site for adenylylation, despite the increase in the pK_a of Arg over Lys. Moreover, the basicity of Lys at position 99 is clearly also important for activity in step 3. Thus, it seems unlikely that any mutant could be found at position 99 which is inactive in adenylylation but fully active in step 3.

The nature of the posttranslational modification of RLi (Arg-99) is unknown. The slightly faster mobility on SDS–PAGE does not necessarily infer a shorter polypeptide. Truncation at the C-terminus has no effect on RNA ligase activity (Rand et al., 1985), and we have also shown that alteration of the amino acid sequence in the adenylylation region can lead to a significant change in mobility. Characterization of this modification will require detailed analysis beyond the scope of the present study.

Mutation of Glu-100 to Gln has clearly little or no effect on adenylylation or the overall activity. RLi (Thr-100) is reduced in its overall activity but not in adenylylation. This may be due to the increased bulk of Thr interfering with step 2 or step 3. In general, however, Glu-100 does not appear to be playing an important role.

Mutations at Asp-101 have a dramatic effect on both step 2 and step 3, whereas adenylylation is unaffected. This loss of activity is not just an effect of removal of the charge [e.g., RLi (Asn-101)] since the RLi (Glu-101) is also inactive. The precise stereochemical positioning of the carboxylic moiety is therefore clearly critical. The fact that both step 2 and step 3 are affected would indicate that Asp-101 interacts in some important way with the donor molecule rather than the acceptor or the adenylyl moiety.

We have found the site-directed mutagenesis procedures, as well as the introduction of more random alterations at specific sites by means of "misincorporation mutagenesis" (Singh et al., 1986), to be generally useful in delineating amino acids important to the RNA ligase activity. For example, we have found that alterations at His-140, Arg-141, Arg-143, and Asp-144 do not affect RNA ligase activity (unpublished results). In another study, we have altered Cys residues in RNA ligase. It is known that absence of DTT or addition of cysteine-blocking agents causes serious reduction in RNA ligase activity (Snopek et al., 1977). Three out of the five cysteines in RNA ligase are within the C-terminal 74 amino acids and are therefore unlikely to be the sensitive sites. Of the remaining two sites, a Cys-53 to Ser change gave rise to a fully active RNA ligase whereas Cys-12 to Ser gave a highly unstable protein that could not be seen by SDS–PAGE of induced cell supernatants but was visible in the cell-free translation system and is unable to be adenylylated (unpublished results). It seems likely, therefore, that Cys-12 is the sensitive site.

In summary, the introduction of site-specific changes in the amino acid sequence of RNA ligase has helped to confirm the role of Lys-99 in adenylylation and to show that both positions 99 and 101 are important to other steps in the RNA ligase joining mechanism. However, more precise assessment of their roles in RNA ligase catalysis must await the availability of high-resolution structural data.

Added in Proof

In view of the report (Yang & Frey, 1979) that in the galactose-1-phosphate uridyltransferase reaction pathway a uridylyl group is covalently bonded to N-3 of a histidyl residue, we recently prepared RLi (His-99). The mutant RNA ligase was inactive both in adenylylation (step 1) and in ligation (step 3), and this gives further evidence of the importance of Lys-99 in both these steps.

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Registry No. RNA ligase, 37353-39-2; ATP, 56-65-5; Lys, 56-87-1; Asp, 56-84-8; Cys, 52-90-4.

REFERENCES

Bankier, A. T., & Barrell, B. G. (1983) *Tech. Life Sci: Biochem. B5*, 1–34.

Barker, D. G., White, J. H. M., & Johnston, L. H. (1985) *Nucleic Acids Res. 13*, 8323–8337.

Beckett, D., & Uhlenbeck, O. C. (1984) in *Oligonucleotide Synthesis: A Practical Approach* (Gait, M. J., Ed.) pp 185–197, IRL Press, Oxford, U.K.

Blackshear, P. J. (1984) *Methods Enzymol. 104*, 237–255.

Bruce, A. G., & Uhlenbeck, O. C. (1978) *Nucleic Acids Res. 5*, 3665–3667.

Carter, P., Bedouelle, H., & Winter, G. P. (1985) *Nucleic Acids Res. 13*, 4431–4443.

Cranston, J. W., Silber, R., Malathi, V. G., & Hurwitz, J. (1974) *J. Biol. Chem. 249*, 7447–7456.

Creighton, T. E. (1979) *J. Mol. Biol. 129*, 235–264.

Depew, R. E., & Cozzarelli, N. R. (1974) *J. Virol. 13*, 888–897.

Duckworth, M. L., Gait, M. J., Goelet, P., Hong, G. F., Singh, M., & Titmas, R. C. (1981) *Nucleic Acids Res. 9*, 1691–1706.

Fersht, A. R., Shi, J.-P., Wilkinson, A. J., Blow, D. M., Carter, P., Waye, M. M. Y., & Winter, G. P. (1984) *Angew. Chem. 23*, 467–473.

Filipowicz, W., Strugala, K., Konarska, M., & Shatkin, A. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1316–1320.

Gumport, R. I., & Lehman, I. R. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2559–2563.

Higgins, N. P., Geballe, A. P., Snopek, T. J., Sugino, A., & Cozzarelli, N. R. (1977) *Nucleic Acids Res. 4*, 3175–3186.

Juodka, B. A., & Markuckas, A. Yu. (1985) *Bioorg. Khim. 11*, 821–825.

Juodka, B. A., Markuckas, A. Yu., Snechkute, M. A., Zilinskiene, V. J., & Drigin, Yu. F. (1980) *Bioorg. Khim. 6*, 1733–1734.

Kaufmann, G., David, M., Borasio, G. D., Teichmann, A., Paz, A., Amitsur, M., Green, R., & Snyder, L. (1986) *J. Mol. Biol. 188*, 15–22.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

McLaughlin, L. W., Piel, N., & Graeser, E. (1985) *Biochemistry 24*, 267–272.

Messing, J., Crea, R., & Seeburg, P. H. (1981) *Nucleic Acids Res. 9*, 309–321.

Nagai, K., & Thøgersen, H. C. (1984) *Nature (London) 309*, 810–812.

Nagai, K., Perutz, M. F., & Poyart, C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7252–7255.

Perkins, K. K., Furneaux, H., & Hurwitz, J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 684–688.

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

Pick, L., Furneaux, H., & Hurwitz, J. (1986) *J. Biol. Chem. 261*, 6694–6704.

Rand, K. N., & Gait, M. J. (1984) *EMBO J. 3*, 397–402.

Rand, K. N., Singh, M., Thøgersen, H. C., & Gait, M. J. (1985) *Proc. Int. Symp. Biomol. Struct. Interact., Suppl. J. Biosci. 8*, 89–106.

Remaut, E., Stanssens, P., & Fiers, W. (1981) *Gene 15*, 81–93.

Romaniuk, E., McLaughlin, L. W., Nielson, J., & Romaniuk, P. J. (1982) *Eur. J. Biochem. 125*, 639–643.

Singh, M., Heaphy, S., & Gait, M. J. (1986) *Protein Eng. 1*, 75–76.

Smith, M. (1985) *Annu. Rev. Genet. 19*, 423–462.

Snopek, T. J., Wood, W. B., Conley, M. P., Chen, P., & Cozzarelli, N. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3355–3359.

Sproat, B. S., & Gait, M. J. (1984) in *Oligonucleotide Synthesis: A Practical Approach* (Gait, M. J., Ed.) pp 83–115, IRL Press, Oxford, U.K.

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

Towbin, H., Staehlin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

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

Vogelstein, B., & Gillespie, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 615–619.

Wood, W. B., & Henninger, M. (1969) *J. Mol. Biol. 39*, 603–618.

Yang, S.-L., & Frey, P. A. (1979) *Biochemistry 18*, 2980–2984.