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Characterization In Vitro of the Defect in a Temperature-Sensitive Mutant of the Protein Subunit of RNase P from *Escherichia coli*

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We have studied the assembly of *Escherichia coli* RNase P from its catalytic RNA subunit (M1 RNA) and its protein subunit (C5 protein). A mutant form of the protein subunit, $C5^{A49}$, has been purified to apparent homogeneity from a strain of *E. coli* carrying a thermosensitive mutation in the *rnpA* gene. The heat inactivation kinetics of both wild-type and mutant holoenzymes are similar, an indication of equivalent thermal stability. However, when the catalytic efficiencies of the holoenzymes were compared, we found that the holoenzyme containing the mutant protein had a lower efficiency of cleavage than the wild-type holoenzyme at 33, 37, and 44°C. We then explored the interaction of M1 RNA and C5 protein during the assembly of the holoenzyme. The yield of active holoenzyme obtained by reconstitution with wild-type M1 RNA and $C5^{A49}$ protein in vitro can be considerably enhanced by the addition of excess M1 RNA, just as it can be in vivo. We concluded that the Arg-46→His-46 mutation in the $C5^{A49}$ protein affects the ability of the protein to participate with M1 RNA in the normal assembly process of RNase P.

RNase P is an endoribonuclease which cleaves the 5′ ends of precursor tRNA molecules to create their mature 5′ ends. In *Escherichia coli*, it consists of a protein component, C5 (molecular weight, 13,800), and an RNA component, M1 (377 nucleotides in length) (1, 1b). The catalytic activity of the enzyme resides in the RNA (5). M1 RNA alone can carry out the cleavage reaction in vitro, in buffers containing >20 mM Mg^{2+}. In vivo and in buffers containing 10 mM Mg^{2+}, both M1 RNA and C5 protein are required for the RNase P reaction to proceed (15, 17). C5 protein increases the k_{cat} of the reaction catalyzed by M1 RNA 10- to 20-fold (1, 1b, 13) and also plays a role in the recognition of specific tRNA precursor substrates (9).

The *rnpA49* mutation in the *rnpA* gene, the gene coding for C5 protein in *E. coli* (17), results in an arginine-to-histidine alteration at position 46 in the C5 protein (9). Cells containing this mutation do not grow above 42°C on LB plates and show an accumulation of precursor tRNAs when shifted from a permissive to a nonpermissive temperature (18). Even under permissive growth conditions, cells bearing the *rnpA49* mutation show a modest accumulation of certain precursor tRNAs (18) and a reduced (though not zero) production of some mature tRNAs (8, 14, 18). Interestingly, cells bearing the *rnpA49* mutation will grow above 42°C when they contain a plasmid carrying the *rnpB* gene, which codes for M1 RNA (8, 11, 14). Presumably, complementation of the temperature-sensitive phenotype is related to the production of excess M1 RNA in the cells containing the plasmid (8, 14). It has been suggested that an increase in the efficiency of assembly of the holoenzyme itself is directly responsible for the complementation (11). To explore the validity of this explanation of the complementation phenomenon, we have studied aspects of the assembly in vitro of RNase P from its separated RNA and protein subunits.

Although the *rnpA49* mutation was first reported in 1973 (17), the RNase P holoenzyme containing C5 protein with the *rnpA49* mutation has been studied only in vitro with crude cell extracts or partially purified protein preparations (10, 18), both of which yield very little enzymatic activity. Additionally, while the enzymatic activity in crude extracts of *rnpA49* was temperature sensitive, no such behavior was observed with RNase P reconstituted with partially purified $C5^{A49}$ protein and M1 RNA (10). There is one report of temperature-dependent inactivation of RNase P partially purified from *rnpA49* (6), but no direct comparison of similar inactivation studies of wild-type RNase P was provided.

We have now cloned the *rnpA49* gene in a manner which allows the production of large quantities of $C5^{A49}$ protein. We have purified this protein to apparent homogeneity and have studied the activities in vitro of the RNase P holoenzyme formed from this protein and of M1 RNA made by transcription in vitro. We show that compared with the wild-type holoenzyme, RNase P made with $C5^{A49}$ protein has a much lower efficiency of cleavage at high temperatures. The ability of excess M1 RNA to complement the temperature sensitivity phenotype of *rnpA49* in vivo can be mimicked, in part, in vitro in reconstitution experiments. The latter experiments indicate that the assembly of RNase P holoenzyme from M1 RNA and $C5^{A49}$ protein is also defective in comparison with the assembly of the wild-type holoenzyme.

Preparation of $C5^{A49}$ protein. DNA coding for the *rnpA49* gene was initially isolated from genomic DNA of *E. coli* A49 and cloned into m13mp18 as previously described (9). The bacteriophage DNA was cut with *Eco*RI and *Pst*I, and the fragment which contains the *rnpA49* mutation and a corresponding downstream open reading frame was isolated and inserted in place of the wild-type fragment in pARE-7, a plasmid that contains the wild-type C5 gene cloned behind a T7 promoter (20). Potential problems arising from overproduction of a contaminating protein from the downstream open reading frame were avoided by replacing a *Hin*dIII fragment in the newly constructed plasmid with a similar fragment which lacks the downstream open reading frame from pARE-7 (pARE-7 was originally constructed in a manner that removed the downstream open reading frame).

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The new plasmid, pMBA49, contains the *rnpA49* gene cloned behind a T7 promoter and the strong ribosome-binding site of gene 10 of phage T7. The identity of this clone and the presence of the G-to-A transition characteristic of the *rnpA49* mutation were confirmed by dideoxy-DNA sequence analysis (16).

Cells containing the *rnpA49* mutation in their chromosomes and the gene for T7 RNA polymerase under control of the *lacUV5* promoter [BL21(DE3)A49 (20)] were transformed with pMBA49. On induction with isopropyl-β-D-thiogalactopyranoside, large amounts of $C5^{A49}$ protein accumulated in these cells (Fig. 1, lane 1). This strain ordinarily dies at 44°C on LB plates. However, in the presence of pMBA49, these cells survived at 44°C. Even in the absence of any inducer, the $C5^{A49}$ protein is produced to a small extent from the plasmid because of a constitutive level of mRNA transcribed by T7 RNA polymerase (20). Thus, it appears that not only the presence of excess M1 RNA but also the presence of excess $C5^{A49}$ protein allows the growth of an *E. coli* strain bearing the *rnpA49* mutation at high temperatures.

To obtain $C5^{A49}$ protein, BL21(DE3)A49 cells containing pMBA49 were grown overnight at 33°C and then diluted and grown at 37°C as previously described in the procedure for purification of wild-type C5 protein (20). The production of $C5^{A49}$ protein was induced with isopropyl-β-D-thiogalactopyranoside (20), and $C5^{A49}$ protein was then extracted and purified as follows. The crude cell extract was centrifuged at 30,000 × *g* (20), and the majority of the $C5^{A49}$ protein was found in the pellet (P30) rather than in the supernatant (S30) (Fig. 1). This is unlike the wild-type C5 protein, which is always found in the S30 and must be recovered subsequently from the P100 by washing with a solution with a high salt concentration (20).

The amount of $C5^{A49}$ protein found in the P30 relative to the amount of the S30 varies according to the temperature at which the cells are grown prior to and during the induction of $C5^{A49}$ protein production. When the cells are grown at 30 or 33°C after dilution from the overnight culture, more $C5^{A49}$ protein is found in the S30 than when the cells are grown at 37°C. (A similar temperature-dependent fractionation of phage T4 DNA polymerase during purification has also been reported [12].) Further purification of the $C5^{A49}$ protein was achieved by using the P30 rather than the S30. (Attempts to purify the $C5^{A49}$ protein from the S30 were unsuccessful, because the $C5^{A49}$ protein in this fraction stuck to the ribosomes and could not be recovered without disruption of the ribosomes and release of the ribosomal proteins.)

$C5^{A49}$ protein was recovered from the P30 by gentle suspension in buffer containing 50 mM Tris hydrochloride (pH 7.5), 10 mM magnesium acetate, 60 mM NH_4Cl, and 4 M urea, and then the suspension was gently agitated at 4°C for 45 min. The resulting solution was then centrifuged for 30 min at 30,000 × *g*. The supernatant recovered after centrifugation, the P30 wash, contained at least half of the total $C5^{A49}$ protein produced in the cells. The P30 wash was then dialyzed at 4°C against a buffer (1× PA) containing 100 mM NH_4Cl, 10 mM MgCl, and 50 mM Tris hydrochloride (pH 7.5). During dialysis, the $C5^{A49}$ protein precipitated. (Wild-type C5 protein is prepared by dialysis of a P100 wash against the buffer used to precipitate $C5^{A49}$ [20].) The precipitated $C5^{A49}$ protein was then suspended in buffer containing 50 mM sodium acetate (pH 7.2), 10 mM $MgCl_2$, and 7 M urea and was further purified by CM Sephadex C50 chromatography as previously described for the purification of the wild-type C5 protein (20). Both wild-type and mutant C5 proteins were stored in the same buffer containing 0.3 M NaCl, the approximate salt concentration at which the proteins were eluted from the CM Sephadex column. Analysis of the purified protein by sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis revealed the presence of only a single band, with a molecular size of 13,800 daltons (Fig. 1, lane 7). The initial fractionation properties of $C5^{A49}$ protein and the need for the presence of 4 M urea to free it from the P30 indicate that the protein is not in its native conformation in the absence of an association with M1 RNA and that, as a consequence of polymerization in its nonnative state, it becomes part of an insoluble aggregate.

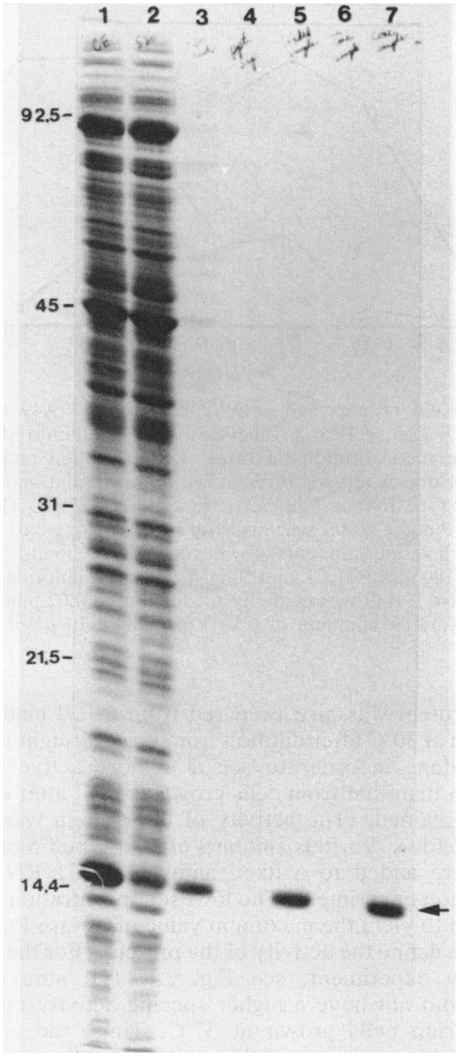

FIG. 1. Purification of $C5^{A49}$ protein from *E. coli rnpA49*. $C5^{A49}$ protein was purified as described in the text. Portions of the different fractions were analyzed on a 12.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and 4 M urea, and the gel was stained with Coomassie blue. Lane 1, Crude cell extract; lane 2, S30; lane 3, 4 M urea wash from P30; lane 4, dialysate of P30 wash after removal of precipitated protein; lane 5, precipitate from P30 wash; lane 6, peak fraction from chromatography on CM Sephadex before concentration; lane 7, peak fraction from chromatography on CM Sephadex after concentration. Numbers on the left indicate the positions of molecular weight (in thousands) standards. The arrow indicates the position of the $C5^{A49}$ protein.

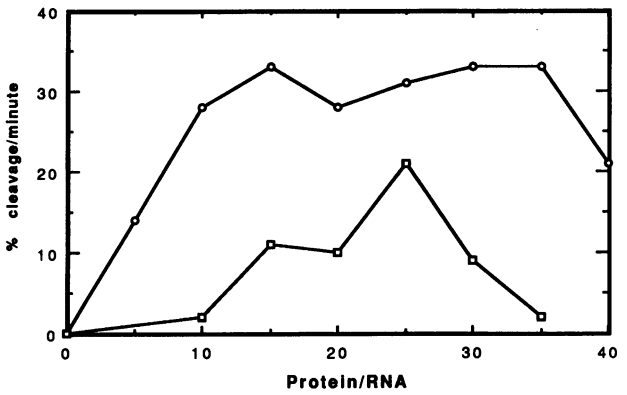

FIG. 2. Yield of cleavage activity of RNase P-A49 (□) and wild-type (○) RNase P as a function of the protein-to-M1 RNA ratios in the reconstitution mixtures. Reconstitution and assays were carried out at temperatures at which maximum activity occurred (wild-type RNase P, 44°C; RNase P-A49, 37°C). The wild-type RNase P holoenzyme was made by reconstituting 0.01 pmol of M1 RNA with various amounts of wild-type C5 protein in 10 μl of 10 mM $MgCl_2$, 100 mM NH_4Cl, and 50 mM Tris hydrochloride (1× PA buffer). RNase P-A49 was made by reconstituting 0.02 pmol of M1 RNA with various amounts of $C5^{A49}$ protein in 10 μl of 1× PA buffer.

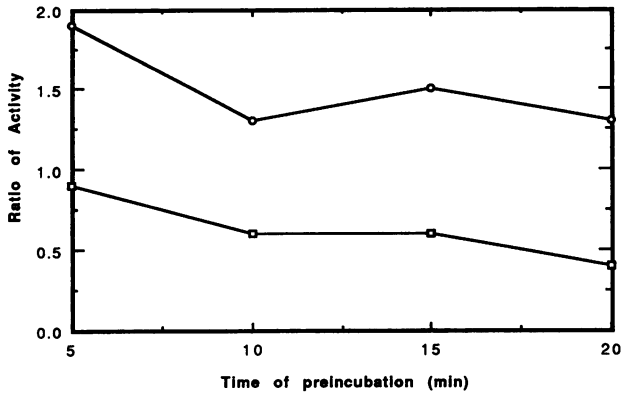

FIG. 3. Inactivation of RNase P by preincubation at different temperatures. M1 RNA and C5 or $C5^{A49}$ protein were mixed together in 1× PA buffer (see text) and incubated for various periods at either 44 or 37°C. Substrate (pTyr) was added, and the activities of these preincubated enzymes were then assayed by measuring the cleavage of pTyr under standard conditions (2). The ratio of the cleavage activity at 44°C to that at 37°C of RNase P-C5 (○) and RNase P-A49 (□) after preincubation at these temperatures for various periods is shown. All assays were carried out in the linear range of kinetics.

$C5^{A49}$ protein was also prepared from a P30 made from cells grown at 30°C after dilution from the overnight culture. This was done in order to see if a more active protein preparation than that from cells grown at 37°C after dilution could be obtained. (The activity of the protein was determined as follows. Various amounts of wild-type C5 or $C5^{A49}$ protein were added to a fixed amount of M1 RNA in a reconstitution experiment. The lowest concentration of protein needed to yield the maximum value of RNase P activity was used to define the activity of the protein. [For the results of such an experiment, see Fig. 2.]) The pure protein recovered did not have a higher specific activity than that prepared from cells grown at 37°C. Thus, the cleavage activity resulting from use of the purified $C5^{A49}$ protein does not appear to be altered by growth of the cells at 37°C. Since more $C5^{A49}$ protein can be recovered from the P30 when cells are grown at 37°C, this was chosen as the standard growth temperature.

Properties of RNase P-A49 at various temperatures. The RNase P holoenzyme was made by reconstitution in vitro, as previously described (20). M1 RNA produced in vitro by transcription with T7 polymerase (20) was mixed with either purified wild-type C5 protein (gift from A. Vioque) or purified $C5^{A49}$ protein to produce the holoenzymes RNase P-C5 and RNase P-A49, respectively. To ensure a high yield of activity of the reconstituted holoenzymes, a molar excess of protein to M1 RNA of 20:1 or 25:1 was used (Fig. 2). We note that both the wild-type and mutant proteins were exposed to buffers containing 7 M urea during their preparation. Furthermore, all calculations of the concentration of the reconstituted holoenzyme prepared in the presence of excess protein were made on the assumption that all the M1 RNA is in the holoenzyme complex under the conditions of reconstitution we used (20- to 25-fold excess of protein [Fig. 2]).

The ability of the reconstituted holoenzymes to cleave the precursor to $tRNA^{Tyr}$ from *E. coli* (pTyr) made by transcription in vitro (M. Baer and C. Guerrier-Takada, unpublished data) was assayed as previously described (13) at various temperatures and after various times of preincubation of the reconstituted complexes prior to the addition of substrate. After preincubation of the reconstituted complexes at 33 or 37°C for 20 min, there were no changes in the activities of either mutant or wild-type enzymes. At 44°C both the *rnpA49* and the wild-type holoenzymes were inactivated to some extent by preincubation, but the extent of the inactivation of both enzymes after 20 min of preincubation was similar (Fig. 3). Thus, there is no difference in the heat inactivation kinetics of wild-type and mutant enzymes at 44°C. However, the efficiency of catalysis (k_{cat}/K_m ratio) of the mutant holoenzyme was lower at 44 than at 37°C, in contrast to that of the wild-type RNase P, which increased at 44 compared with 37°C (see below and Fig. 3). Therefore, the temperature optima of the reactions with each holoenzyme are different, and RNase P-A49 is more thermosensitive in this regard only.

To probe the heat inactivation properties of the $C5^{A49}$ protein per se, the protein was incubated for various periods at 44°C in the absence of M1 RNA and pTyr. It was then mixed with M1 RNA and pTyr, which had also been preincubated at 44°C. Assays for RNase P activity were then carried out in the standard fashion, and comparisons were made with wild-type C5 protein treated in the same way. As the time of preincubation of the protein was prolonged, both $C5^{A49}$ and the wild-type C5 protein gave less-active holoenzymes. However, the percent decrease in activity of RNase P-A49 under these conditions was the same as the percent decrease in activity of wild-type RNase P.

We first demonstrated in earlier studies of the cleavage reaction of M1 RNA alone that a lag period is seen before the rate of the reaction becomes linear (1a). If the reaction rates of the holoenzymes RNase P-C5 and RNase P-A49 are studied after preincubation times varying from 30 s to 5 min, a short lag period is also seen before the reaction rate becomes linear. At 44°C, the wild-type RNase P holoenzyme shows a lag time of 1.0 min, while RNase P-A49 shows a lag time of about 1.75 min (Fig. 4). The small but reproducible difference in the lag time between the wild-type and mutant

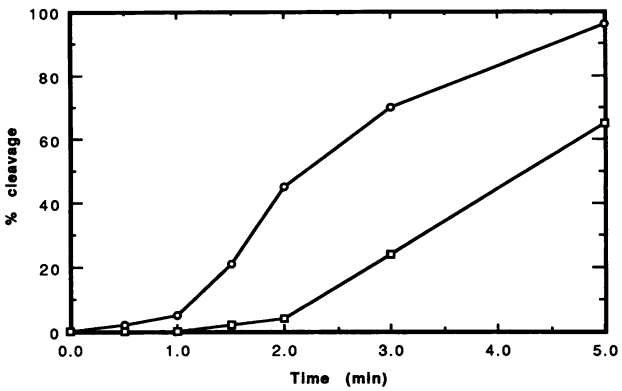

FIG. 4. Kinetics of RNase P activity at 44°C. The figure shows the cleavage of pTyr as a function of time of incubation at 44°C under standard conditions with wild-type RNase P holoenzyme (○), made by reconstituting 0.005 pmol of M1 RNA with 0.1 pmol of wild-type C5 protein (20), and RNase P-A49 (□), made by reconstituting 0.02 pmol of M1 RNA with 0.4 pmol of $C5^{A49}$ protein.

holoenzymes at 44°C could be related to an increased time for full assembly of the active A49 holoenzyme.

At 37 and 33°C, the lag times for both the wild-type and mutant holoenzymes are the same, 1.75 and 3.4 min, respectively. We note, however, that the lag period for the reaction with M1 RNA alone is longer (2.5 min at 43°C, 4.5 min at 37°C, and 50 min at 30°C [1a]) than that for the holoenzyme, suggesting that this phenomenon is due to a requirement for M1 RNA to assume a correct conformation prior to cleavage and that C5 protein, in addition to having other functions, enhances the rate of this process. Presumably, $C5^{A49}$ protein promotes the conformational change less rapidly than the wild-type C5 protein.

Kinetic parameters of the RNase P reaction. Although the wild-type and mutant proteins and holoenzymes did not differ in their rates of inactivation after incubation at 44°C for increasing periods, there was a difference in the temperature dependence of the activities of the two holoenzymes when they were assayed after a fixed period of incubation at 44°C compared with 37 or 33°C. The wild-type enzyme was always more active at 44°C than at 37 or 33°C, but the mutant enzyme was always less active at 44°C than at 37 or 33°C (Table 1). An examination of the kinetic parameters of the two reconstituted holoenzymes reveals that at all temperatures, the apparent K_m values for the mutant and wild-type holoenzymes are similar, while the apparent k_{cat} of the mutant holoenzyme is always lower than the k_{cat} of the wild type. (Because the holoenzymes are reconstituted in the presence of a large excess of protein, we assume that the concentration of holoenzyme is determined by the concentration of M1 RNA. This must be a maximum estimate of concentration, and therefore, the k_{cat} values we give are minimum estimates.) The k_{cat}/K_m ratio of the wild-type holoenzyme is greater at 44°C than at 37 or 33°C, while the k_{cat}/K_m ratio of the mutant holoenzyme, which is comparatively low even at 37°C, decreases further as the temperature is raised. The catalytic efficiency of the holoenzyme made with $C5^{A49}$ protein has a temperature dependence different from that of the wild-type holoenzyme. The mutant enzyme is less active than the wild-type enzyme at high temperatures. (In these experiments, the holoenzymes were preincubated for 10 min at the given temperatures before substrate, preheated at 37°C, was added.)

TABLE 1. Kinetic parameters of the RNase P reaction

Protein component of enzyme[a]	Kinetic parameter at[b]:								
	33°C			37°C			44°C		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
C5	1.4	9.7	6.9	0.9	10.0	11	2.0	32.7	16.4
$C5^{A49}$	0.7	3.2	4.5	1.4	6.9	4.9	2.9	8.4	2.9

[a] Holoenzyme was made with M1 RNA and the protein listed.
[b] Values are reported as follows: K_m, 10^{-8} M; k_{cat}, moles of product per minute per mole of enzyme (these are minimum estimates; assays were carried out as described previously [13]); k_{cat}/k_m, 10^{-8} M.

From our experiments we concluded that the $C5^{A49}$ protein, alone or in combination with M1 RNA to form a holoenzyme, does not have heat inactivation kinetics different from that of the wild type. Nonetheless, the final yield of active holoenzyme from the reconstitution process (see below) and its intrinsic k_{cat}/K_m ratio is lower with RNase P-A49 than with wild-type RNase P.

Assembly of RNase P. The reasons for the inviability of the cells bearing the *rnpA49* gene at restrictive temperatures are a decreased k_{cat}/K_m ratio of RNase P-A49 and possibly a low yield of the assembly of the holoenzyme. We infer the latter, because in the presence of excess M1 RNA or excess $C5^{A49}$, protein cells bearing the *rnpA49* gene will grow at the formerly nonpermissive temperature, thus indicating that an excess of one or the other component may push the equilibrium of the assembly reaction toward formation of more holoenzyme.

We have studied the effects of the addition of excess M1 RNA in vitro on the yield of RNase P activity from reconstitution reactions with $C5^{A49}$ or wild-type C5 protein. After initially determining the amount of protein that was limiting the rate of the reconstitution reaction with a fixed amount of M1 RNA (Fig. 2), we added an excess of M1 RNA to the reconstitution mixtures. We found that even a fivefold increase in the amount of M1 RNA in the reaction, in the presence of an apparently limiting amount of $C5^{A49}$ protein, gave a marked increased in cleavage activity at 44°C (Fig. 5). Similar effects were seen at 37 and 33°C. When a similarly limiting amount of wild-type C5 protein was incubated in the presence and absence of excess M1 RNA, no increase in cleavage activity was seen (Fig. 5). The addition of an excess of an RNA unrelated to M1 RNA or a mutant form of M1 RNA with little or no activity in vitro resulted in no stimulation of cleavage activity. Rather, a decrease in cleavage activity was seen.

Conclusions. Our results show that *E. coli rnpA49* may die at the restrictive temperature through a combination of effects. The catalytic efficiency of the mutant holoenzyme has a lower temperature optimum than the wild type, and assembly of the mutant holoenzyme is apparently defective compared with that of the wild type. The end result is that the amount of active RNase P-A49 is insufficient to meet the required demands for mature tRNA production needed to sustain growth. Maturation of some specific tRNA precursors may fail to keep up with the needs of an increased metabolic rate at the high temperature. We note that the lower catalytic efficiency of the mutant holoenzyme at high temperatures and the apparent low yield of the reconstituted holoenzyme from its subunits may both be consequences of an abnormal interaction of the $C5^{A49}$ protein with M1 RNA. No distinctive heat inactivation kinetics were exhibited by the holoenzyme or by the A49 protein itself. An effect of a thermosensitive mutation in a subunit on the assembly of a

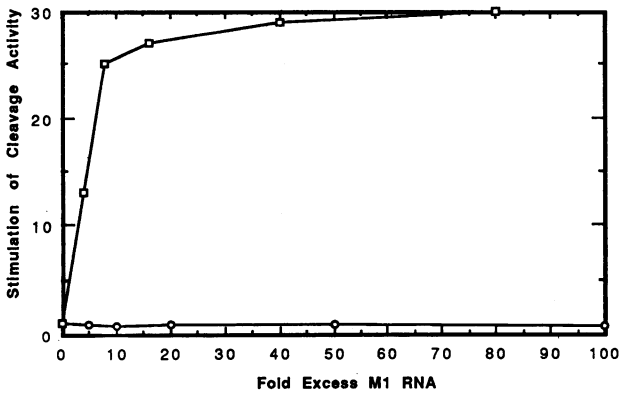

FIG. 5. Stimulation of cleavage activity by addition of increasing amounts of M1 RNA to a limiting amount of wild-type C5 (○) or $C5^{A49}$ (□) protein. Increasing amounts of M1 RNA were added to a standard reconstitution mixture containing 0.01 pmol of M1 RNA and 0.05 pmol of C5 or $C5^{A49}$ protein in 1× PA buffer on ice. The mixtures were transferred to 44°C, and after 10 min βTyr was added and samples were taken at specified intervals thereafter and analyzed for the presence of cleavage product. The ordinate represents the factor by which cleavage activity was stimulated by the addition of excess M1 RNA to the standard reconstitution mixture. The abscissa shows the fold excess of M1 RNA (compared with 0.01 pmol) added to the standard reconstitution mixture.

larger structure with a well-defined function in vivo was also seen by King and his collaborators in their studies of phage P22 tail assembly (3, 4, 19). In our studies, the defect can be overcome by driving the reaction with an excess of the wild-type M1 RNA subunit to improve the yield of holoenzyme in vitro or in vivo. We also cannot be certain, however unlikely it seems, that the enzyme made in the presence of excess M1 RNA is not in some way intrinsically more active than that made under normal conditions.

Although an increase in the amount of active holoenzyme during assembly may be part of the reason for the viability of cells bearing the *rnpA49* gene at 44°C when excess M1 RNA is present, it may not be the only effect of excess M1 RNA. Some increased production of protein may also occur through a regulatory interaction involving M1 RNA and the regulatory regions of the $C5^{A49}$ operon (7, 20). M1 RNA in which uracil 333 has been changed to cytosine 333 (2), for example, while able to form as active as holoenzyme in vitro with $C5^{A49}$ as wild-type M1 RNA is, does not complement the *rnpA49* mutation in vivo. This may be due to its inability to act like wild-type M1 RNA in vivo in its hypothetical regulatory capacity (2).

Finally, a lag period ascribed to the need for M1 RNA to undergo a conformational change to an active form is evident in the kinetics of the enzymatic reaction (Fig. 4). One explanation for the lag may be that during formation of the active holoenzyme complex, the RNA and protein subunits interact in a manner that facilitates the transition of each from an inactive to an active conformation. Our results indicate that $C5^{A49}$ protein is defective in carrying out this process, although further experiments are needed to elucidate the details of this interaction between a catalytic RNA and its protein cofactor.

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