Nucleic Acids Research, 1993, Vol. 21, No. 1 51–57

Product release is a rate-limiting step during cleavage by the catalytic RNA subunit of *Escherichia coli* RNase P

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Received October 16, 1992; Revised and Accepted December 3, 1992

ABSTRACT

The kinetic constants for cleavage of the tRNA^{Tyr}Su3 precursor by the M1 RNA of *E.coli* RNase P were determined in the absence and presence of the C5 protein under single and multiple (steady state) turnover conditions. The rate constant of cleavage in the reaction catalyzed by M1 RNA alone was 5 times higher in single turnover than in multiple turnovers, suggesting that a rate-limiting step is product release. Cleavage by M1 RNA alone and by the holoenzyme under identical buffer conditions demonstrated that C5 facilitated product release. Addition of different product-like molecules under single turnover reaction conditions inhibited cleavage both in the absence and presence of C5. In the presence of C5, the K_I value for matured tRNA was approximately 20 times higher than in its absence, suggesting that C5 also reduces the interaction between the 5′-matured tRNA and the enzyme. In a growing cell the number of tRNA molecules is approximately 1000 times higher than the number of RNase P molecules. A 100-fold excess of matured tRNA over enzyme clearly inhibited cleavage *in vitro*. We discuss the possibility that RNase P is involved in the regulation of tRNA expression under certain growth conditions.

INTRODUCTION

RNase P is the enzyme responsible for 5′-maturation of tRNAs. The enzyme consists of an RNA subunit and a protein subunit, in *Escherichia coli* M1 RNA and C5 respectively. The RNA is the catalytic subunit and can under certain *in vitro* conditions cleave tRNA precursors correctly in the absence of the protein. However, C5 is absolutely required for activity *in vivo* (1, and references therein). Previous data have shown that RNase P cleavage is product inhibited (2–4). Furthermore, Reich *et al.* (4) proposed that a role of the C5 protein is to facilitate release of the product after cleavage. If this is true then the rate constant of cleavage (k_{cat}) by M1 RNA as measured under multiple turnover conditions should be lower that the rate constant of cleavage of the phosphodiester bond (k_{+2}) obtained with M1 RNA in excess over substrate *i.e.* single turnover [see scheme in the results section]. To test this hypothesis and to obtain further information about RNase P and its subunits we compared the rate constants of cleavage of the tRNA^{Tyr}Su3 precursor by M1 RNA alone and by the holoenzyme under identical buffer conditions as well as under optimal buffer conditions for the respective forms of the enzyme. The rate constants were determined under steady state (multiple turnover) and pre-steady state (single turnover) conditions.

Our results showed that in the absence of C5, k_{cat}, as measured in steady state, was about 5 times lower than k_{+2}, irrespective of the buffersystem used. By contrast, in the presence of C5, these rate constants were similar. We also showed that K_i for product-like molecules estimated under single turnover conditions, and K_m for cleavage of the tRNA^{Tyr}Su3 precursor were approximately the same in the absence of C5, whereas in its presence, K_i was 20 times higher than K_m. These results will be discussed with respect to the function of RNase P and its subunits.

MATERIALS AND METHODS

Preparation of substrate and enzyme

The construction of the gene encoding the precursor to tRNA^{Tyr}Su3 behind the phage T7 promoter has previously been described (5, 6). This gene construct carries a *Fok*I site downstream of the tRNA gene so that after cleavage with *Fok*I and transcription with DNA-dependent T7 RNA polymerase (7), the product indicated in Figure 1 was generated. The construction of the M1 RNA gene behind the T7 promoter and the purification of the C5 protein has been described elsewhere (5).

Reaction conditions

Buffers. The following reaction buffers were used to monitor M1 RNA and holoenzyme cleavage activities. Buffer IA (8) (our standard assay buffer in the absence C5); 50 mM Tris–HCl (pH 7.5), 100 mM NH_4Cl, 5% (w/v) polyethylene glycol 6000 and 100 mM $MgCl_2$. Buffer IB; as Buffer IA but 20 mM $MgCl_2$. Buffer II (4); 50 mM Tris-HCl (pH 8.0), 800 mM NH_4Cl, 0.05% Nonidet P-40 and 100 mM $MgCl_2$. Buffer III (3) (our standard assay buffer for the holoenzyme); 50 mM Tris–HCl (pH 7.5), 100 mM NH_4Cl and 10 mM $MgCl_2$.

Steady state conditions. The kinetic determinations were done according to Kirsebom and Altman (9) and Kirsebom and Svärd (6).

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Pre-steady state conditions. Determinations of the kinetic constants for the M1 RNA reactions were performed at 37°C or at the indicated temperatures with the final concentrations of reactants as follows: precursor tRNA 0.0025 pmol/μl, M1 RNA varied between 0.05 and 0.3 pmol/μl in Buffer IA and II and between 0.3 and 1.1 pmol/μl in Buffer IB. Determinations of the kinetic constants for the holoenzyme were performed at 37°C or at the indicated temperatures in Buffer III or IB with the final concentrations of reactants as follows: precursor tRNA 0.0025 pmol/μl, M1 RNA 0.005−0.035 pmol/μl and C5 at saturating amounts (10). The enzyme was preincubated at 37°C for 6 minutes prior to addition of substrate which had been preincubated at the same temperature for 2 minutes or prior to transfer to a different assay temperature followed by 6 minutes temperature equilibration before addition of substrate preincubated at that temperature for 2 minutes. Starting from the time of mixing enzyme and substrate, samples were withdrawn with 5 to 10 seconds intervals depending on buffersystem. The numbers given are based on three to four independent experiments within 20 to 30% deviation from the average. Conditions were adjusted so that the measurements were carried out in the linear range of the curve of kinetics of the cleavage reaction for the different concentrations of M1 RNA added. Quantitations were done on a PhosphorImager, Molecular Dynamics 400S (Company).

Inhibition experiments

The following RNA molecules (Fig. 1) were used to inhibit cleavage of pSu3; $tRNA^{Tyr}$ (purchased from Boehringer Mannheim), $tRNA^{Phe}$ (kindly provided by Dr N.Bilgin), pSu3-I: a $tRNA^{Tyr}$Su3 transcript starting at position +1, an *in vitro* transcript of the 5'-flanking sequence of pSu3, CopA: the antisense RNA from plasmid R1 (11), p4.5S RNA (12), Su3-CL: 5'-matured $tRNA^{Tyr}$Su3 prepared *in vitro* in the following way; the $tRNA^{Tyr}$Su3 precursor was cleaved by RNase P, the cleavage products were separated on an 8% polyacrylamide sequencing gel, the 5'-matured tRNA was visualized by uv-shadowing, excised and eluted from the gel and precipitated with ethanol. These inhibitors were added to the reaction together with the substrate at the concentrations indicated in Figure 5. The experiments were performed at 37°C under optimal buffer conditions for cleavage with M1 RNA alone or for the reconstituted holoenzyme, respectively.

RESULTS

Comparison of the kinetic constants under steady state and pre-steady state conditions

It has been proposed that a role of the protein subunit of RNase P, C5, is to facilitate product-release (4). This would imply that in the absence of C5, k_{+2}, the rate constant of cleavage of the phosphodiester bond, is higher than the rate constant k_{cat} as determined under steady state reaction conditions. To test this, we determined and compared the kinetic constants for cleavage by M1 RNA alone under steady state and pre-steady state reaction conditions. The rate constants are given by the following simplified scheme:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} EP \underset{k_{-3}}{\overset{k_{+3}}{\rightleftarrows}} E + P$$

where under steady state conditions $1/k_{cat} = 1/k_{+2} + 1/k_{+3}$ assuming that $k_{-2} >> k_{+2}$. The tRNA precursor we used as substrate was the well-characterized $tRNA^{Tyr}$Su3 precursor [pSu3 (Fig. 1)]. The experiments were performed in Buffer IA and Buffer II, where the latter has a higher ionic strength (Materials and Methods). Throughout this study we will refer to k_{+2} as the rate constant obtained under pre-steady state conditions and k_{cat} as the rate constant obtained under steady state conditions. Furthermore, $K_{m(pss)}$ is the concentration of M1 RNA at which half maximal rate is obtained in pre-steady state whereas K_m is the concentration of tRNA precursor at which half the maximal rate is obtained in steady state. The results are shown in Table 1.

The kinetic constants for cleavage of pSu3 by M1 RNA under steady state conditions have previously been determined in Buffer IA (6, 9, 13). The results reported here are in agreement with these (within experimental errors). Furthermore, k_{+2} and $K_{m(pss)}$ were significantly higher (>2-fold) than k_{cat} and K_m in both buffer systems. These results show that cleavage of pSu3 is inhibited by the cleavage product and that events occurring after cleavage of the phosphodiester bond limit the maximal steady state reaction rate; k_{cat}. If k_{+2} were the rate-limiting step in the M1 RNA cycle, the pre-steady state and steady state measurements would give the same rate: $k_{+2} = k_{cat}$. We also note that $k_{+2}/K_{m(pss)}$ and k_{cat}/K_m were approximately the same as expected, since they both measure the effective association rate of substrate to M1 RNA.

The influence of Mg^{++}-concentration on the kinetic constants in pre-steady state and steady state

The presence of Mg^{++} is important for the cleavage reaction (3). To further evaluate the importance of Mg^{++} in the cleavage of pSu3 by M1 RNA alone we determined the rate constants at different concentrations of Mg^{++} under steady and pre-steady state conditions. The experiments were performed in Buffer IA at varying Mg^{++}-concentrations as indicated in Figure 2.

The results showed that both K_m and $K_{m(pss)}$ increased when the concentration of Mg^{++} was reduced (Fig. 2). This suggests that the enzyme-substrate interaction is impaired at low Mg^{++}. In contrast, a decrease in the Mg^{++}-concentration resulted in an increase in k_{cat} whereas k_{+2} was essentially unchanged (Fig. 2). We also noted that $k_{+2}/K_{m(pss)}$ in pre-steady state increased with increasing Mg^{++} and reached a plateau at approximately 100 mM Mg^{++} (not shown), which is the concentration in our standard assay. The effect of Mg^{++} in pre-steady state using Buffer II was essentially the same as observed using Buffer IA, *i.e.* a reduction in the Mg^{++}-concentration resulted in an increase in $K_{m(pss)}$ whereas k_{+2} remained

Table 1. Cleavage of pSu3 by M1 RNA under steady state and pre-steady state reaction conditions in buffer IA or II at 37°C, as described in Materials and Methods

Assay conditions	k_{cat} # (k_{+2})	K_m # /10^{-8} ($K_{m(pss)}$)	$k_{cat}/K_m \times 10^7$ ($k_{+2}/K_{m(pss)}$)
steady state, buffer IA	0.6	1.1	6.0
pre-steady state, buffer IA	2.6	5.6	4.7
steady state, buffer II	3.0	8.0	3.8
pre-steady state, buffer II	8.4	17.1	4.8

k_{cat} and k_{+2} (min^{-1}), K_m and $K_{m(pss)}$ (M).

unchanged (data not shown). In conclusion, at low Mg^{++} k_{cat} approaches k_{+2}.

Comparison of the kinetic constants in the absence or presence of the C5 protein

To understand the role of the C5 protein in the reaction catalyzed by RNase P, we compared the kinetic constants for cleavage of pSu3 by M1 RNA and by the reconstituted holoenzyme under pre-steady state and steady state conditions. M1 RNA and the holoenzyme have different buffer requirements for optimal cleavage activity (Buffer IA for M1 RNA and Buffer III for the holoenzyme, see Materials and Methods). Therefore we decided to compare cleavage in the absence and presence of C5 under identical buffer conditions (Buffer IB). In this buffer, both forms of the enzyme can function, albeit with reduced efficiencies. The results are shown in Table 2.

Addition of C5 (Buffer IB) resulted in a significant increase in k_{cat} whereas K_m was only slightly changed (Table 2). A similar result was obtained when cleavage was performed under optimal buffer conditions (data not shown) in agreement with

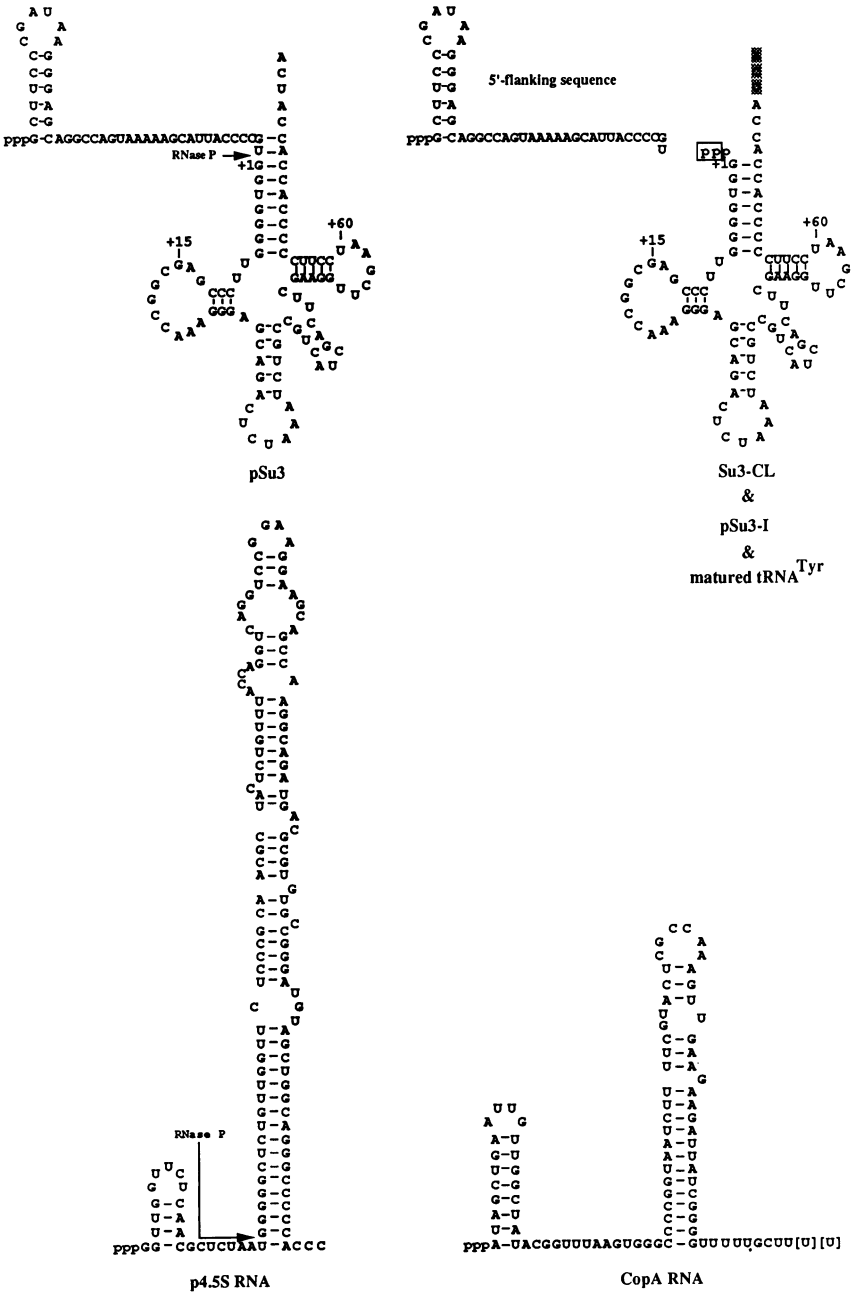

Figure 1. Predicted secondary structures of tRNATyrSu3 (pSu3), Su3-I, Su3-CL and CopA. The numbering of the precursor was according to Sprinzl *et al.*, (19). The boxed phosphates are not present after cleavage by M1 RNA or by the holoenzyme. Shaded nucleotides at the 3'-end of the tRNATyrSu3 precursor are not present in the matured tRNATyrSu3. The sites of cleavage are indicated by arrows.

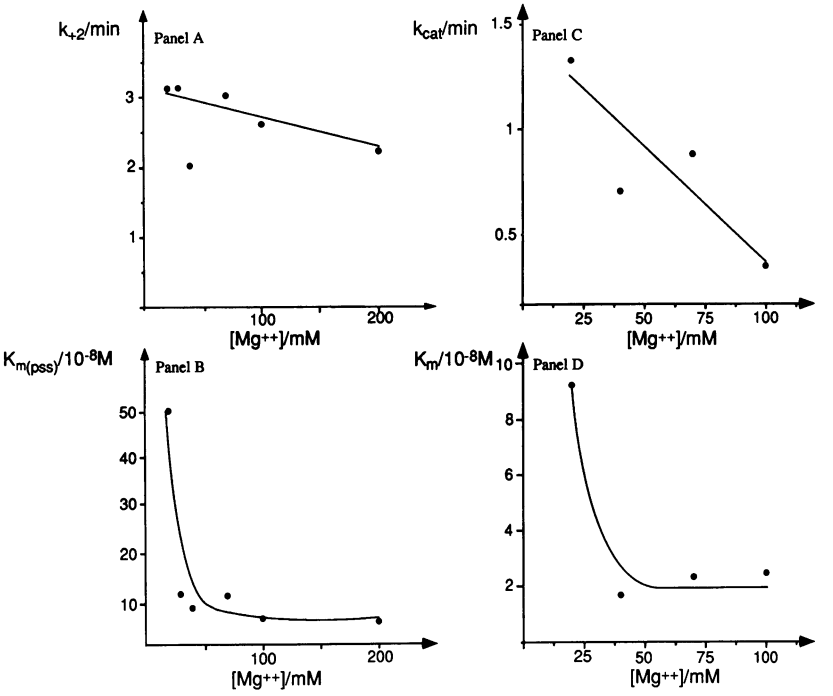

Figure 2. The kinetic constants for M1 RNA cleavage of pSu3 determined in steady state and pre-steady state as a function of Mg^{++}-concentration in buffer IA. The experiments were perfomed as outlined in Materials and Methods at the different Mg^{++}-concentrations *indicated.* **Panels A and B**: cleavage performed under pre-steady state conditions. **Panels C and D**: cleavage performed under steady state conditions.

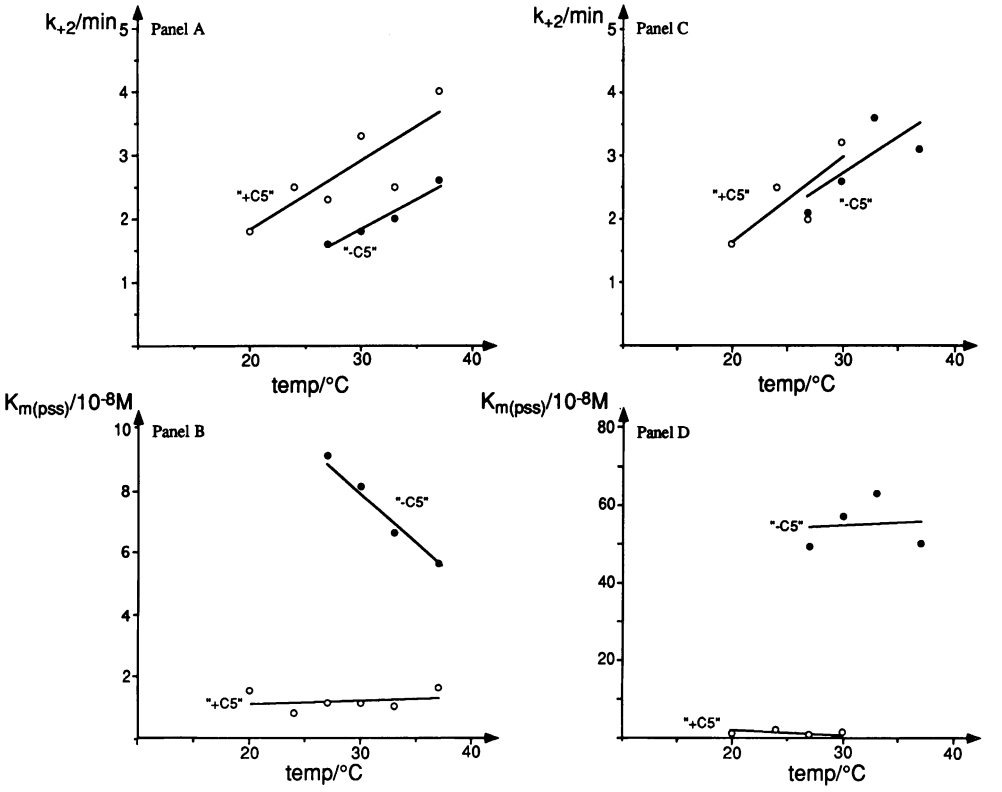

Figure 3. Comparison of the kinetic constants as a function of temperature for cleavage of pSu3 by M1 RNA and by the reconstituted holoenzyme under optimal or identical buffer conditions during pre-steady state as described in Materials and Methods. **Panels A and B**: cleavage performed under optimal buffer conditions, Buffer IA for M1 RNA alone and Buffer III for the holoenzyme. **Panels C and D**: cleavage performed under identical buffer conditions, Buffer IB.

previous reports (3, 6, 9, 13). In the pre-steady state reaction, a significant increase in $K_{m(pss)}$ was observed whereas k_{+2} remained essentially unchanged as a result of addition of C5. Taken together, these results suggest that the C5 protein plays a role after cleavage of the phosphodiester bond.

We also performed cleavage under pre-steady state conditions in the absence and presence of C5 under identical and optimal buffer conditions at different temperatures. The results are shown in Figure 3. A change in temperature did not affect the $K_{m(pss)}$ values for the holoenzyme, irrespective of the reaction buffer used (Buffer IB or Buffer III). In contrast, an increase in temperature under optimal buffer conditions (Buffer IA) resulted in a significant (>2-fold) decrease in $K_{m(pss)}$ for M1 RNA alone. No significant change in $K_{m(pss)}$ was observed under identical buffer conditions (Buffer IB). However, these values were considerably higher compared to the values determined under optimal buffer conditions. A rise in temperature lead to increased k+2 values both in the absence and presence of C5. We plotted $\ln k_{+2}$ versus 1/T (Arrhenius plot) to obtain activation energies (E_a) in the presence and absence of C5 (Fig. 4). The E_a values for the reactions catalyzed by M1 RNA and by the holoenzyme were approximately the same irrespective of the buffer systems used. The E_a value for the M1 RNA alone cleavage reaction as determined in this report is in keeping with Altman and Guerrier-Takada (14). We conclude that addition of C5 does not affect the activation energy for the cleavage reaction.

Table 2. Comparison of the cleavage of pSu3 in steady state and pre-steady state by M1 RNA and by the reconstituted holoenzyme under identical buffer conditions (buffer IB) at 30°C.

Assay conditions	k_{cat} # (k_{+2})	K_m #/10^{-8} ($K_{m(pss)}$)	$k_{cat}/K_m \times 10^7$ ($k_{+2}/K_{m(pss)}$)
steady state, M1 RNA+C5	5.7	2.6	28.2
pre-steady, M1 RNA+C5	3.3	1.1	32.1
steady state, M1 RNA	0.4	4.3	1.0
pre-steady state, M1 RNA	2.6	57.1	0.5

k_{cat} and k_{+2} (min^{-1}), K_m and $K_{m(pss)}$ (M).

Inhibition by addition of product-like molecules

To further investigate the inhibition by the cleavage product, we decided to study the single turnover reaction catalyzed by M1 RNA and by the reconstituted holoenzyme in the presence of matured tRNA or different product-like molecules (see Materials and Methods and Fig. 1). The results are shown in Figure 5.

Our results showed that $tRNA^{Tyr}$, $tRNA^{Phe}$, pSu3-I and Su3-CL all inhibited cleavage of the $tRNA^{Tyr}$Su3 precursor, both in the absence and presence of C5. This finding is consistent with previous reports (2–4). To test if this inhibition is due to unspecific binding between two RNA molecules, we studied the reaction in the presence of the *in vitro* transcribed 5′-flanking sequence of pSu3 and an unrelated two stem-loop structured RNA of approximately the same size as a tRNA, CopA [Fig. 1 (11)]. Neither of these molecules inhibited cleavage of pSu3 by M1 RNA alone when added to the reaction together with the substrate under the conditions tested. In addition, another RNA, p4.5S RNA (which is a natural substrate for RNase P), did not inhibit cleavage of pSu3 in the absence of C5 (data not shown) when added to the reaction under conditions where cleavage of p4.5S RNA is negligible due to a high K_m value (12). These results suggest that the product-like molecules used in this study inhibit the cleavage reaction specifically.

From Figure 5 we also estimated K_i in the M1 RNA and holoenzyme catalyzed reactions for some of the product-like molecules. Determination of K_i under single turnover conditions is preferred to the standard method where K_i is obtained from steady state kinetic measurements since under single turnover conditions additional inhibition due to the accumulation of product is avoided (15). The K_i value in the M1 RNA catalyzed reaction was approximately 2×10^{-8} M independent of which of the product-like molecules we added to the reaction together with the substrate. This indicates that the product of the cleavage reaction interacts with M1 RNA as efficiently as the substrate, since K_i is approximately equal to K_m (Table 1). In the presence of the C5 protein the inhibition was clearly reduced and K_i for $tRNA^{Tyr}$ (or $tRNA^{Phe}$) was approximately 36×10^{-8} M. Since the corresponding K_m was approximately 2×10^{-8} M (6, 9, 13) we conclude that the product interacts with the holoenzyme about 20 times less efficiently than the substrate.

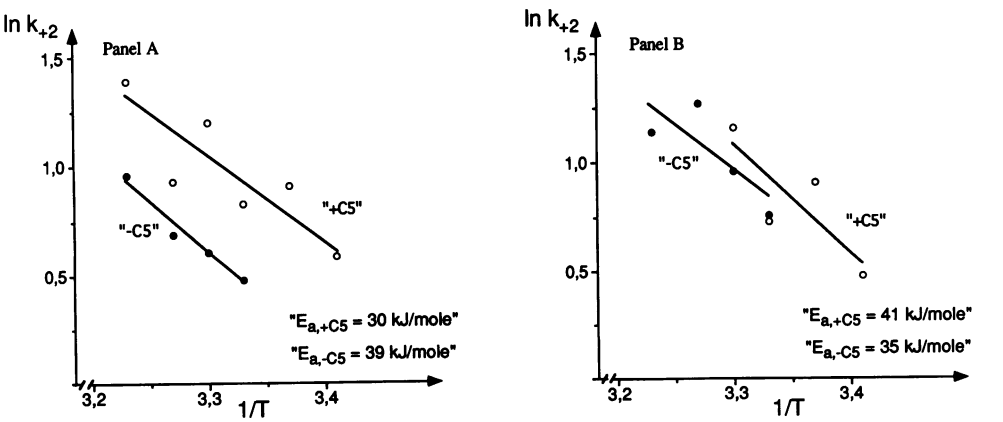

Figure 4. Arrhenius plots of the temperature dependence of k_{+2} for M1 RNA and the holoenzyme cleavage of pSu3 under identical buffer conditions in pre-steady state. (The k_{+2} values are given per minute and the temperatures are in Kelvin degrees). **Panel A**: k_{+2} as measured under optimal reaction conditions (Buffers IA and III). **Panel B**: k_{+2} as measured under identical reaction conditions (Buffer IB).

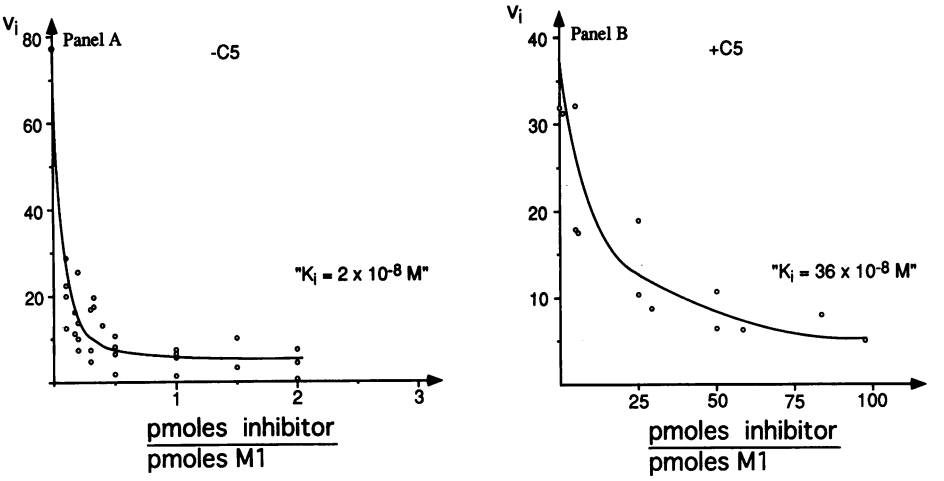

Figure 5. Panel A: Cleavage of pSu3 by M1 RNA during pre-steady state with increasing concentration of inhibitors ($tRNA^{Tyr}$, $tRNA^{Phe}$, Su3-I and Su3-CL). The experiments were performed in Buffer IA as outlined in Materials and Methods. **Panel B**: The cleavage of pSu3 by the reconstituted holoenzyme during pre-steady state with increasing concentration of matured $tRNA^{Tyr}$ and $tRNA^{Phe}$. The experiments were performed in Buffer III as outlined in Materials and Methods.

In conclusion, the combined data suggest that cleavage of the $tRNA^{Tyr}Su3$ precursor by RNase P is product inhibited and that one function of C5 is to reduce this inhibition. This will be further discussed below.

DISCUSSION

Product release is a rate-limiting step during cleavage of the $tRNA^{Tyr}Su3$ precursor by M1 RNA alone

It has been proposed that a role of the C5 protein of *E.coli* RNase P is to increase product release after cleavage (4). This predicts that the rate constant of cleavage for M1 RNA in the absence of C5 under multiple turnover conditions (k_{cat}), should be lower than the rate constant of cleavage of the phosphodiester bond as measured under single turnover conditions (k_{+2}). Here we studied cleavage of the $tRNA^{Tyr}Su3$ precursor under steady state (multiple turnover) and pre-steady state (single turnover) conditions. We showed that k_{+2}, the rate constant of cleavage of the phosphodisester bond, for M1 RNA alone was significantly higher than the rate constant from steady state measurements, k_{cat}. This shows that events occuring after cleavage of the phosphodiester bond are rate-limiting in the cleavage of the $tRNA^{Tyr}Su3$ precursor by M1 RNA in the absence of C5. In steady state, using the simplified scheme (see results section), $1/k_{cat} = 1/k_{+2} + 1/k_{+3}$ assuming that $k_{-2} << k_{+2}$. It is therefore likely that the rate-limiting step is product release *i.e.* k_{+3}. However, an effect on the overall reaction rate by k_{-3} can not be excluded under conditions where products accumulate (see below).

A decrease in the Mg^{++}-concentration resulted in an increase in k_{cat} whereas k_{+2} remained unchanged in the absence of C5. Thus, the rate of hydrolysis of the phosphodiester bond is independent of the concentration of Mg^{++} between 20 mM and 200 mM. In the absence of C5, M1 RNA interacts with the tRNA precursor and the 5'-matured tRNA with approximately the same affinity (16, this report). The K_m values increased with decreasing Mg^{++}-concentration both in steady state and in pre-steady state, indicating a less efficient interaction between enzyme and substrate at low Mg^{++}. Thus, our results suggest that the relative effect of product inhibition increases with the Mg^{++}-concentration. Consequently, k_{cat} increases with a reduction in the Mg^{++}-concentration as a result of less efficient product binding to M1 RNA at low Mg^{++} and hence k_{cat} approaches k_{+2}. This is consistent with the results discussed above as well as with the results of Smith *et al.* (16).

We emphasize that the results reported here were obtained using the $tRNA^{Tyr}Su3$ precursor as substrate. A previous report showed that K_m and k_{cat} in cleavage of a precursor to ^{tRNAHis} and the tRNATyrSu3 precursor by M1 RNA alone differed significantly (6). Our preliminary data suggest that product release is not a rate-limiting step in cleavage of a $tRNA^{His}$ precursor in the absence of C5. Therefore, it appears that the rate-limiting step in cleavage by M1 RNA alone is dependent on the identity of the substrate.

Product inhibition and the role of the C5 protein

Bulk tRNA and other product-like molecules inhibit cleavage both in the absence and presence of C5 (2–4, this report). Cleavage in the presence of $tRNA^{Tyr}$ or $tRNA^{Phe}$ yielded an estimated K_i of 36×10^{-8} M in the presence of C5 compared to 2×10^{-8} M for cleavage by M1 RNA alone. In the absence of C5, K_i for different product-like inhibitors was approximately the same as K_m for cleavage of the $tRNA^{Tyr}Su3$ precursor. This indicates that M1 RNA interacts with the $tRNA^{Tyr}Su3$ precursor and the 5'-matured tRNA with approximately the same affinity. The change in K_i for different product-like inhibitors as a result of addition of the C5 protein suggests that under conditions where the product accumulates, one role of C5 is to reduce the interaction between the 5'-matured tRNA and the enzyme. This would mean a change in k_{-3} and/or k_{+3} in the simplified reaction scheme (see results section). In addition, the finding that the observed rate of cleavage at high concentration of matured tRNA approaches zero indicates that the tRNA precursor and the matured tRNA compete for the same site on the enzyme. A similar product inhibition pattern was observed for the L21-*Sca*I ribozyme derived from the *Tetrahymna* intervening RNA sequence (15).

From the discussion above it is clear that it is not possible to distinguish whether addition of matured tRNA to the cleavage reaction results in a change in k_{+3} and/or k_{-3}. Neither is it

possible to understand the role of the C5 protein if cleavage is performed under optimal buffer conditions for the respective form of the enzyme. Therefore, we studied cleavage of the $tRNA^{TyrSu3}$ precursor in the absence and presence of C5 under identical buffer conditions. Under these conditions we showed that addition of C5 to the cleavage reaction resulted in no difference between k_{cat} and k_{+2}. Thus, we conclude that one role of C5 is to facilitate product-release (increase in k_{+3}), as proposed by Reich *et al.* (4). Consequently, this could be one reason why the C5 protein is essential for cleavage activity *in vivo*.

We recently showed that addition of C5 during cleavage of a precursor to $tRNA^{His}$ resulted in a significant decrease in the K_m value (6). In contrast, only a slight decrease in the K_m value for the $tRNA^{TyrSu3}$ precursor was observed (6, 9, 13, this report). Here we showed that $K_{m(pss)}$ as determined under limiting substrate conditions decreased significantly under identical reaction conditions as a result of addition of C5. Therefore, the C5 protein appears to play a role in the enzyme-substrate interaction in a substrate identity dependent manner as well as under conditions where the substrate is limiting. It is conceivable that C5 stabilizes the folding of M1 RNA to ensure optimal enzyme-substrate interaction.

Implication of product inhibition *in vivo*

There are approximately 200 molecules of RNase P within an *E.coli* cell whereas the number of matured tRNA molecules is three orders of magnitude higher (5, 17). Addition of a 100-fold excess of matured tRNA over M1 RNA in the presence of C5 resulted in a strong inhibition of the cleavage reaction (Fig. 5, panel B). This implies that under growth conditions where there is an accumulation of free tRNA there would be an impairment in RNase P processing. Indeed, an accumulation of tRNA precursors was observed as a result of methionine starvation (18). Taken together, it appears that RNase P is involved in the regulation of tRNA expression at least under certain growth conditions.

ACKNOWLEDGEMENTS

We wish to thank Drs. S.Burnett and M.Ehrenberg for critical reading and suggested improvements of the manuscript. Miss U.Kagardt is acknowledged for her skillfull technical assistance. This work was supported by a research grant to L.A.K. from the Swedish Research Council.

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