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Studies on Thermal Inactivation of Transfer Ribonucleic Acid Nucleotidyltransferase from *Escherichia coli**

Jon P. Miller† and Georg R. Philipps‡

ABSTRACT: Thermal inactivation of apparently homogeneous tRNA nucleotidyltransferase and its protection by various substrates was investigated. All substrates protected the enzyme against thermal inactivation; complete protection was achieved with CTP and tRNA, while ATP protected the enzyme only 75%. The protection constants for all substrates were in the same range as their corresponding K_m values. The protection constants were the same for deacylated and aminoacylated tRNA which suggests that binding of aminoacylated tRNA to the enzyme was as efficient as binding of deacylated tRNA. No protection was observed with tRNA digested with ribonuclease T_1 under conditions which should give half-molecules, however, the fragments protected the enzyme after reannealing. When tRNA was digested with venom phosphodiesterase, the value of the protection constant increased with increasing numbers of nucleotides removed. A sharp increase was observed after the removal of, in the average, nine nucleotides per tRNA molecule. rRNA, 5S RNA, or synthetic polynucleotides did not protect the enzyme against thermal inactivation.

Transfer ribonucleic acid nucleotidyltransferase from *Escherichia coli* (EC 2.7.7.25) has been purified to apparent homogeneity (Miller and Phillips, 1971a). A single enzyme catalyzes the incorporation of one AMP and two CMP residues into tRNA previously digested with venom phosphodiesterase. The substrate for AMP incorporation is tRNA-X-C-C[1] while CMP is incorporated into both, tRNA-X-C and tRNA-X. The enzyme rapidly loses its activity when exposed for a short time to higher temperatures. Similar observations had been made previously with partially purified tRNA nucleotidyltransferase preparations from *E. coli* (Furth *et al.*, 1961) rabbit muscle (Starr and Goldthwait, 1963) and rat liver (Daniel and Littauer, 1963; Herbert and Canellakis, 1963). Thermal inactivation has also been reported for aminoacyl-tRNA synthetases. It has been shown that these enzymes may be stabilized by their substrates (Makman and Cantoni, 1966; Yaniv and Gros, 1969; Chlumecka *et al.*, 1970).

Since tRNA nucleotidyltransferase could be stabilized when stored in the presence of tRNA (Miller and Philipps, 1970), the protection of tRNA nucleotidyltransferase against thermal inactivation by various substrates was investigated in greater detail. Here we report that nucleoside triphosphates and tRNA in the deacylated as well as aminoacylated state protect the enzyme against heat inactivation.

Materials and Methods

Highly purified tRNA nucleotidyltransferase was prepared from *E. coli* strain B as described in greater detail elsewhere (Miller and Philipps, 1971a). Purification of unfractionated tRNA had been reported (Philipps, 1970). Purified $tRNA^{Val}$ (lot 1A), 89% pure, was obtained from A. D. Kelmers, Oak Ridge National Laboratory, through the National Institutes of Health. [^{14}C]Val-$tRNA^{Val}$ was prepared as described (Hirst-Bruns and Philipps, 1970). rRNA was prepared from *E. coli* ribosomes previously purified on Sephadex G-100 (Philipps, 1970). The RNA was extracted using the phenol method and rRNA was precipitated with 1.5 M NaCl. It was further purified by chromatography on Sephadex G-100 in 0.01 M Tris-HCl (pH 7.2)–0.1 M NaCl. Purification and characterization of 5S RNA will be described elsewhere.

Digestion of tRNA by Venom Phosphodiesterase. To obtain tRNA from which one, two, or three nucleotides had been removed the procedure described by Miller *et al.* (1970) was used. To prepare tRNA from which more than three nucleotides had been removed from the 3′-OH terminus, the amount of venom phosphodiesterase (Worthington Biochem. Corp.) was increased. A typical incubation mixture contained in 0.5 ml: 20 μmoles of glycine–NaOH (pH 8.7), 5 μmoles of magnesium acetate, 147 nmoles of tRNA, and 0.25 mg of venom phosphodiesterase (147 units/mg). Incubation was at 37° for 4, 6, 9, or 12 hr. Under these conditions, an average of 7, 9, 11, or 13 moles of total nucleotides was removed per mole of tRNA. The method used to determine these amounts by chromatography on Dowex 1-X8 has been described previously (Miller *et al.*, 1970).

Thermal Inactivation and Protection Assay. To study thermal inactivation of tRNA nucleotidyltransferase, a two-stage assay was used. The preincubation mixture contained in 50 μl: 0.01 μg of tRNA nucleotidyltransferase, 2.5 μmoles of glycine–NaOH (pH 9.2), 0.5 μmole of magnesium acetate, 0.5 μmole of glutathione, and varying concentrations of the par-

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[1] Abbreviations used are: tRNA, intact, deacylated tRNA; tRNA-X-C-C, tRNA-X-C, and tRNA-X, unfractionated tRNA from which one, two, or three nucleotides had been removed.

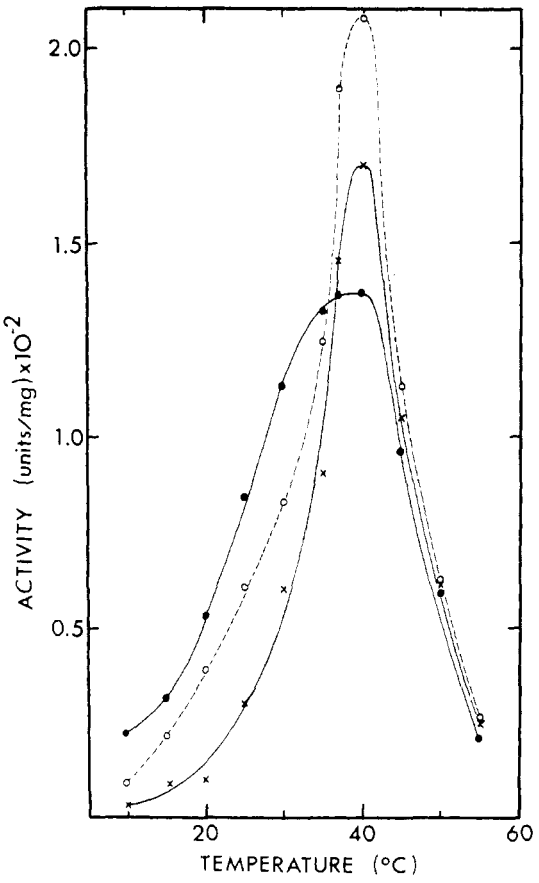

FIGURE 1: Effect of temperature on the activity of tRNA nucleotidyltransferase. The assays were performed as described in Methods except that the temperature was varied and 0.02 μg of enzyme was used. CMP incorporation into tRNA-X-C, O----O; CMP incorporation into tRNA-X, X—X; and AMP incorporation into tRNA-X-C-C, ●—●.

ticular substance being tested for its ability to protect the enzyme against inactivation. Incubation was at 40° for 10, 20, and 30 min after which the tubes were placed on ice. A nonpreincubated control was kept on ice for the same time. For the second stage, buffer and substrates were added to the preincubation mixture to obtain the standard reaction mixture which contained in 100 μl: 5.0 μmoles of glycine–NaOH (pH 9.2), 1.0 μmole of magnesium acetate, 1.0 μmole of glutathione, 20 μg of bovine serum albumin, and 1.6 nmoles of venom phosphodiesterase-treated tRNA. When CMP incorporation was determined, 20 nmoles (33 nCi) of [5-^{3}H]CTP and either tRNA-X-C or tRNA-X were added, for AMP incorporation 20 nmoles (25 nCi) of [8-^{3}H]ATP and tRNA-X-C-C were used. The standard reaction mixture was also used to determine the activity of nonpreincubated enzyme. To obtain proper control values, protector substances in the appropriate concentrations were added to the incubation mixtures. The mixtures were then incubated at 37° and aliquots of 20 μl each were removed at 10, 20, and 30 min. They were precipitated with trichloroacetic acid and the precipitates were collected on glass fiber filters. The radioactivity was determined as described by Miller *et al.* (1970). The initial rates for nucleotide incorporation were determined from these data.

Results

Rate of Nucleotide Incorporation into tRNA at Different Temperatures. The rate of incorporation of AMP into tRNA-X-C-C and of CMP into tRNA-X-C or tRNA-X catalyzed by tRNA nucleotidyltransferase was investigated at various temperatures. The results are shown in Figure 1. The amount of product formation increased linearly for at least 40 min, and the rate was determined from the data obtained after 20-min incubation. It can be seen that incorporation of AMP or CMP into the three tRNA substrates showed essentially the same temperature dependence under the conditions used. The results suggested that the activity for nucleotide incorporation into tRNA-X-C-C, tRNA-X-C, and tRNA-X was rapidly lost at temperatures above 40°.

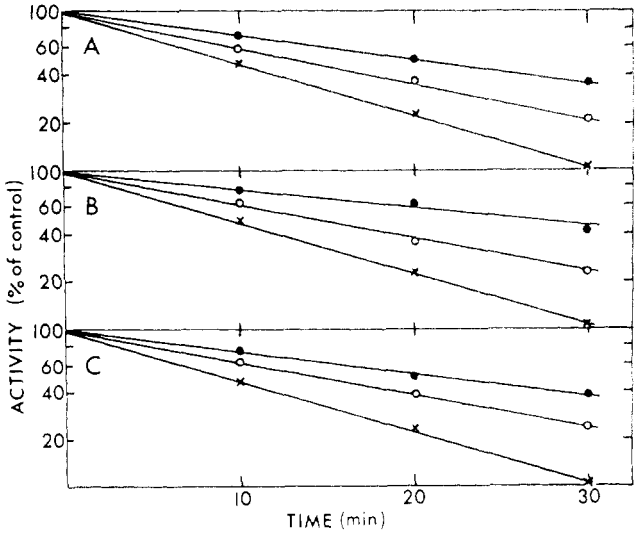

FIGURE 2: Spontaneous inactivation of tRNA nucleotidyltransferase at various temperatures. The assays were performed as described in Methods. In A, AMP incorporation into tRNA-X-C-C; in B, CMP incorporation into tRNA-X-C; and in C, CMP incorporation into tRNA-X. The temperatures used were 40°, ●—●; 42°, O—O; and 45°, X—X.

Spontaneous Inactivation of tRNA Nucleotidyltransferase. The spontaneous inactivation of tRNA nucleotidyltransferase in the absence of substrate was measured between 40 and 45°. The activity remaining at each time was determined for each of the three nucleotide incorporating activities of the enzyme. As shown in Figure 2, the thermal inactivation of tRNA nucleotidyltransferase appeared to be a first-order process. Burton (1951) has defined the first-order rate constant of inactivation: $k = 1/t \ln (x_0/x')$, where x' is the enzymatic activity remaining after incubation at a fixed temperature for t min, and x_0 is the activity obtained with a control kept at 0°. It can be seen that the first-order rate constant of inactivation for each temperature was the same for each of the three nucleotide incorporating activities. The half-life of the enzymatic activity in 50 mM glycine–NaOH (pH 9.2), 10 mM magnesium acetate, and 10 mM glutathione was approximately 23 min at 40° and about 9 min at 45°. The magnitude of the rate constant was linearly related to the inactivation temperature as described by the Arrhenius equation (Figure 3). The slopes of the lines on the Arrhenius plot representing the three nucleotide-incorporating activities were approximately equal. These results show that each of the three activities have the same temperature dependence of their first-order rate constants.

Protection of the Enzyme against Thermal Inactivation. The effect of different substrates for tRNA nucleotidyltransferase on the thermal inactivation was then investigated. In the presence of ATP and CTP, thermal inactivation of tRNA nucleotidyltransferase at 40° still followed first-order kinetics,

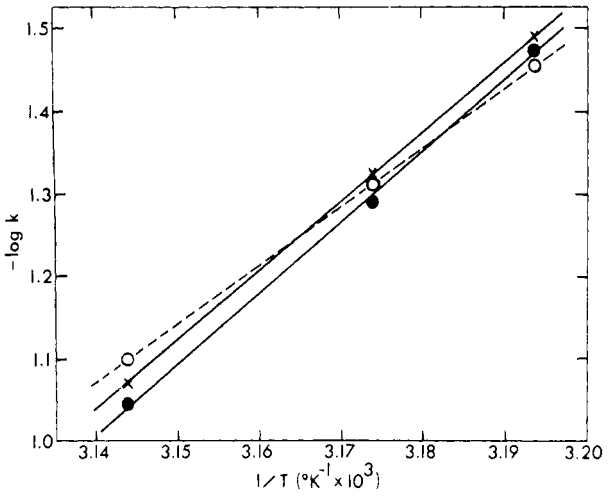

FIGURE 3: Arrhenius plots of nucleoside triphosphate incorporation into tRNA. AMP incorporation into tRNA-X-C-C, ●—●; CMP incorporation into tRNA-X-C, O----O; CMP incorporation into tRNA-X, ×—×.

however, the value of the rate constant of inactivation was reduced (Figure 4). This reduction was approximately proportional to the concentration of added substrates.

The effect of the three tRNA substrates on the thermal inactivation is shown in Figure 5A–C. Figure 5D depicts the effect of the product of the enzymatic reaction. It also shows that not only tRNA-X-C-C-A but also aminoacylated tRNA stabilized the enzyme. When tRNA is digested with venom phosphodiesterase beyond the third nucleotide from the 3′-OH terminus, the resulting tRNA cannot longer incorporate any nucleotides (Miller *et al.*, 1970). Protection by tRNA from which in the average nine nucleotides had been removed could be demonstrated, however, 3- to 5-fold higher concentrations were needed as compared to those needed for protection by tRNA substrates (Figure 5E). As shown in Figure 5F, protection of the enzyme was quite specific for tRNA. When rRNA or 5S RNA were present during the preincubation, no stabilization was observed even at much higher concentrations[2] than those used for complete protection by tRNA.

Determination of Protection Constants for ATP, CTP, and tRNA. Burton (1951) defined the protection constant π, as the concentration of protector substance at which $k = (1/2)(k_0 + k_\infty)$, where k_0 is the first-order rate constant of inactivation in the absence of protector and k_∞ is the minimum first-order rate constant of inactivation obtained by increasing the concentration of protector. In order to determine π for the various substrates, inactivation of tRNA nucleotidyltransferase was examined at 40° at varying concentrations of each of its substrates. The first-order rate constant of inactivation was then plotted as a function of the concentration of protector. The plot for the protection of the enzyme by CTP is shown in Figure 6. In A, the residual activity was determined in the presence of tRNA-X-C, while in B, tRNA-X was present. The rate of CMP incorporation into the two tRNA substrates at 37° at varying concentrations of CTP is shown in the same figure. CTP saturated the enzyme over a two order of magnitude change in CTP concentration. The course of protection of the enzyme, *i.e.*, the decrease in the value of k

[2] For the estimation of the RNA concentration: 22.0 A units was assumed to be equivalent to 1 mg of RNA in 0.01 M NaCl.

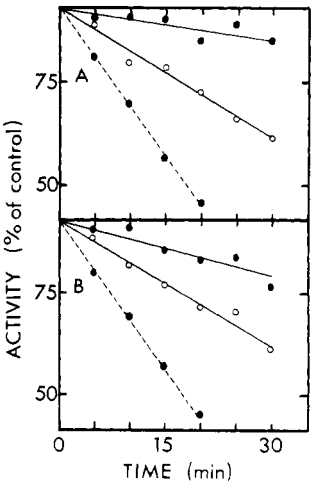

FIGURE 4: Effect of ATP and CTP on the thermal inactivation of tRNA nucleotidyltransferase at 40°. The assays were performed as described in Methods. In each case the dashed line (●----●) represents the spontaneous inactivation in the absence of protectors. In A, protection by ATP; 0.13 mM, O—O; and 0.40 mM, ●—●. The residual activity was measured by AMP incorporation into tRNA-X-C-C. In B, protection by CTP; 9.0 μM, O—O; and 13 μM, ●—●. The residual activity was measured by CMP incorporation into tRNA-X-C.

with increasing concentrations of protector, also occurred over a two order of magnitude change in CTP concentration. The protection constants for CTP evaluated from these plots were for $\pi_{CTP}^{tRNA\text{-}X\text{-}C} = 6.0$ μM and for $\pi_{CTP}^{tRNA\text{-}X} = 14$ μM, where $\pi_{CTP}^{tRNA\text{-}X\text{-}C}$ referred to the protection con-

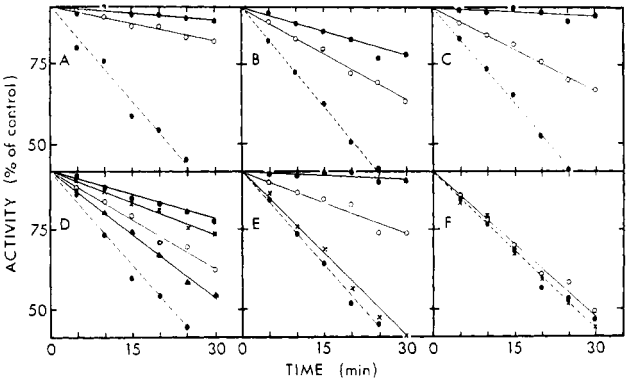

FIGURE 5: Effect of various RNAs on the thermal inactivation of tRNA nucleotidyltransferase at 40°. Assays were performed as described in Methods. In each case the dashed line (●----●) represents the spontaneous inactivation in the absence of protectors. A, protection by tRNA-X; 0.30 μM, O—O; and 0.60 μM, ●—●. The residual activity was measured by CMP incorporation into tRNA-X. B, protection by tRNA-X-C; 0.30 μM, O—O; and 0.40 μM, ●—●. The residual activity was measured by CMP incorporation into tRNA-X-C. C, protection by tRNA-X-C-C; 0.40 μM, O—O; and 0.95 μM, ●—●. The residual activity was measured by AMP incorporation into tRNA-X-C-C; D, protection by tRNA-X-C-C-A; 1.7 μM, O—O; 2.7 μM, ●—●; and protection by Val-tRNA^{Val}; 1.8, ▲—▲; and 3.3 μM, ×—×. In both cases the residual activity was measured by CMP incorporation into tRNA-X-C. E, protection by tRNA digested with venom phosphodiesterase to remove a total of 9 moles of nucleotide per mole of tRNA; 0.5 μM, X—X; 1.5 μM, O—O; and 3.0 μM, ●—●. The digestion of this tRNA is described in Methods. F, effect of 10 μM rRNA, O—O; and 10 μM 5S RNA, ×—×; on the thermal inactivation of the enzyme. The residual activity in both cases was measured by CMP incorporation into tRNA-X-C.

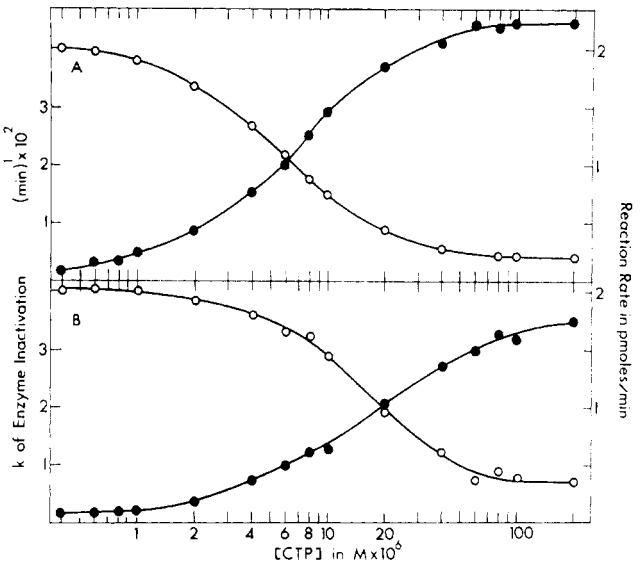

FIGURE 6: Protection of tRNA nucleotidyltransferase by CTP. The assays were performed as described in Methods. In A, residual activity determined in the presence of tRNA-X-C. In B, residual activity determined in the presence of tRNA-X. Open circles (○—○) represent the values of k at 40° and closed circles (●—●) represent the reaction rate at 37° with the nonpreincubated enzyme.

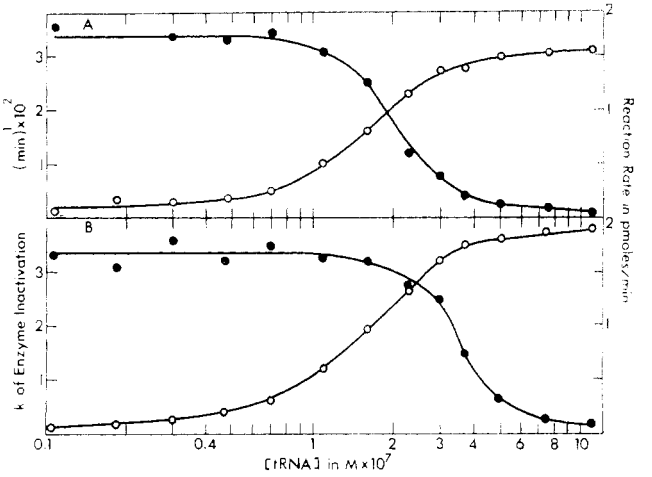

FIGURE 8: Protection of tRNA nucleotidyltransferase by tRNA-X and tRNA-X-C. The assays were performed as described in Methods. In A, protection of the enzyme by tRNA-X; in B, protection by tRNA-X-C. In both cases, the residual activity was determined in the presence of CTP. Closed circles (●—●) represent the values of k at 40° and open circles (○—○) represent the reaction rate at 37° with the nonpreincubated enzyme.

stant for CTP when the residual activity was determined in the presence of tRNA-X-C; while for $\pi_{CTP}^{tRNA\text{-}X}$ the residual activity was measured with tRNA-X. The K_m values for CTP as determined from Lineweaver–Burk plots were $K_{m,CTP}^{tRNA\text{-}X\text{-}C} = 7.0\ \mu M$; and $K_{m,CTP}^{tRNA\text{-}X} = 15\ \mu M$.

The effect of varying ATP concentrations on the thermal inactivation of the enzyme and on the rate of AMP incorporation into tRNA-X-C-C is shown in Figure 7. From this plot, the protection constant was calculated $\pi_{ATP} = 75\ \mu M$. The K_m for ATP, evaluated from the linear portion of the Lineweaver-Burk plot in Figure 7 was 95 μM. As can be seen, ATP did not completely protect the enzyme against heat inactivation when saturating concentrations were used. Seventy-five per cent protection was the most that could be achieved with ATP. In this respect, it was interesting to note that the lowest concentration of ATP which inhibited the enzyme, about 0.4 mM, was about the same concentration at which the k of inactivation ceased to decrease.

The protection of the enzyme by the three tRNA substrates was also investigated. Figure 8A depicts the course of protection and the kinetics of saturation of the enzyme with tRNA-X, Figure 8B shows the data with tRNA-X-C. Both tRNA substrates saturated the enzyme over a one order of magnitude change in tRNA concentration. The course of protection occurred over the same range of tRNA concentrations. The protection constants as evaluated from these plots were for $\pi_{tRNA\text{-}X\text{-}C} = 0.35\ \mu M$ and for $\pi_{tRNA\text{-}X} = 0.20\ \mu M$. The corresponding K_m values as determined from Lineweaver–

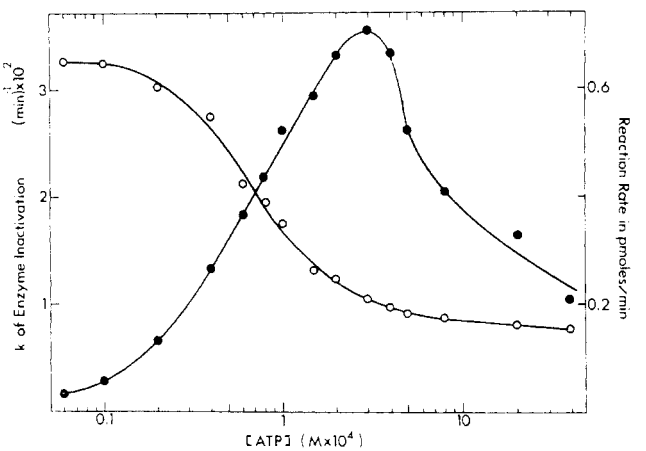

FIGURE 7: Protection of tRNA nucleotidyltransferase by ATP. The assays were performed as described in Methods. The residual activity was determined in the presence of tRNA-X-C-C. Open circles (○—○) represent the value of k at 40° and closed circles (●—●) represent the reaction rate at 37° with the nonpreincubated enzyme.

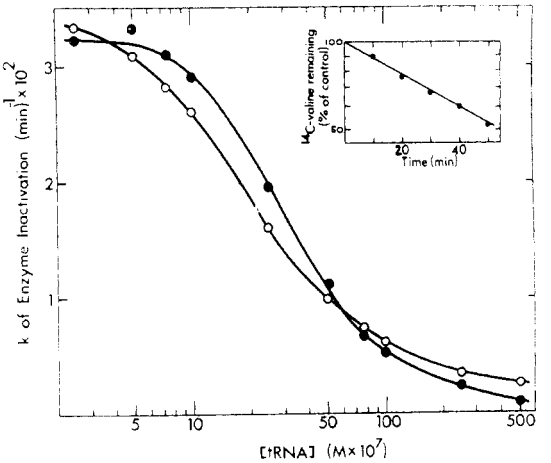

FIGURE 9: Protection of tRNA nucleotidyltransferase by unfractionated tRNA and Val-tRNA ^{Val}. The assays were performed as described in Methods. Protection by tRNA-X-C-C-A, ○—○; and by Val-tRNA^{Val}, ●—●. The residual activity was determined after addition of tRNA-X-C and CTP. The inset shows the course of deacylation of [^{14}C]Val-tRNA^{Val} under the conditions of the assay for preincubation.

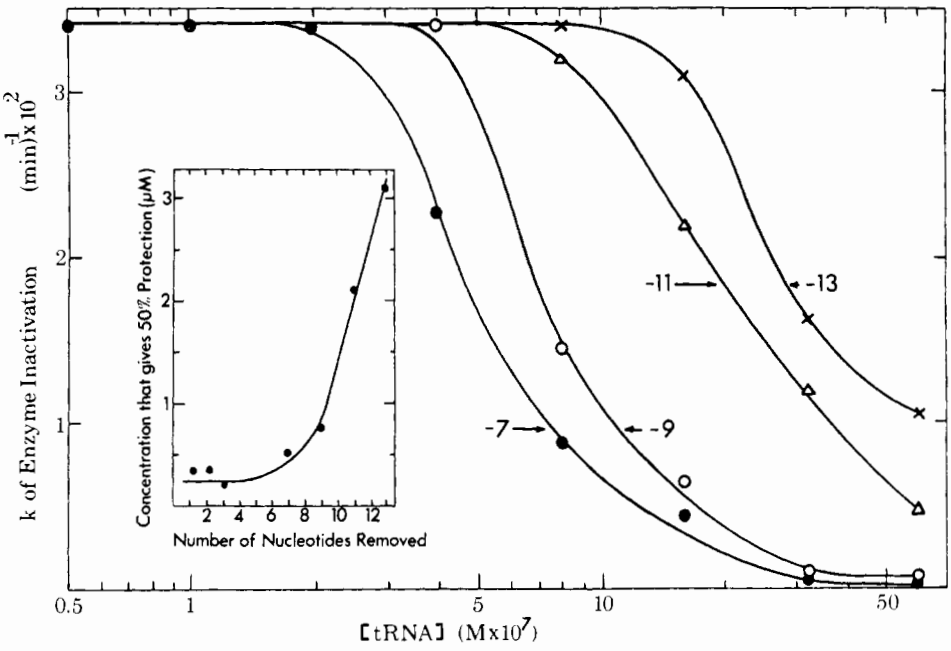

FIGURE 10: Protection of tRNA nucleotidyltransferase by tRNA digested beyond the third nucleotide from the 3′ terminus. The assays were performed as described in Methods. The average amount of nucleotides removed per mole of tRNA was as follows: 7 (●—●), 9 (○--○), 11 (△—△), and 13 (×—×) moles. Nucleotides were separated from tRNA on Sephadex, and the nucleotides quantitated as described in Methods. The value of π for each of the digested tRNAs was determined from the graph and then plotted as a function of the number of nucleotides removed (inset).

Burk plots were: $K_{m,tRNA\text{-}X\text{-}C}$ = 0.18 μM and $K_{m,tRNA\text{-}X}$ = 0.20 μM. Similar determinations were performed for tRNA-X-C-C as substrate. This tRNA substrate saturated the enzyme over a two order of magnitude change in tRNA concentration and the course of protection of the enzyme followed the same range of tRNA concentrations. The values for π and K_m determined from these data were $\pi_{tRNA\text{-}X\text{-}C\text{-}C}$ = 0.40 μM and $K_{m,tRNA\text{-}X\text{-}C\text{-}C}$ = 0.21 μM.

Protection by Deacylated and Aminoacylated tRNA. In order to see if tRNA nucleotidyltransferase can discriminate between deacylated tRNA and aminoacylated tRNA, the course of protection of the enzyme by these two tRNAs was determined (Figure 9). The inset in Figure 9 shows the time course of deacylation of [^{14}C]Val-tRNA^{Val} in the assay mixture at 40°. The half-life of the charged tRNA was 54 min under these conditions. The longest time used to measure protection was 30 min, at which time 65% of the tRNA^{Val} was still aminoacylated. After preincubation, the enzymatic activity was measured using the incorporation of CMP into tRNA-X-C. It can be seen that π was approximately the same for both tRNA substrates: $\pi_{tRNA\text{-}X\text{-}C\text{-}C\text{-}A}$ = 2.6 μM and $\pi_{Val\text{-}tRNA^{Val}}$ = 3.6 πM.

Protection by tRNA Digested Beyond the Terminal Trinucleotide. The effects of the removal of increasing numbers of nucleotides from the 3′-OH terminus of tRNA on its ability to protect the enzyme against thermal inactivation is shown in Figure 10. tRNA from which in the average 7, 9, 11, and 13 moles of nucleotides were removed per mole of tRNA were used. With increasing numbers of nucleotides removed, the value of the protection constant increased. In the inset in Figure 10, the protection constant is plotted against the average number of nucleotides removed per tRNA; also included are the values of π for tRNA from which one, two, or three nucleotides had been removed. The value of π remained essentially the same up to a point where 3 nucleotides were removed. It increased slightly with the removal of 7 or 9 nucleotides. However, when more than 9 nucleotides were removed, a rapid increase in the value of π was observed which remained linear up to a point where in the average 13 nucleotides had been removed.

Protection by tRNA Fragments. Preliminary results were also obtained with fragments of unfractionated tRNA which were prepared according to the procedure of Hashimoto *et al.* (1969). As shown by these authors, digestion of tRNA^{Tyr} under these conditions gave tRNA half-molecules. After digestion, the RNA was chromatographed on Sephadex G-100 in the presence of urea. The elution profile showed a major peak which was eluted after tRNA and represented tRNA fragments a sizable number of which were believed to be half-molecules as suggested from their migration in polyacrylamide gels. The ability of these fragments to protect tRNA nucleotidyltransferase against thermal inactivation was investigated under two sets of conditions; the RNA pooled from fractions containing fragments was heated to 90° for 3 min and then cooled either rapidly or very slowly. The results showed that when the tRNA fragments were quickly cooled they did not protect the enzyme. On the other hand, when the fragments were heated to 90° and cooled slowly to allow some of the corresponding halves to reanneal, some protection of the enzyme was observed.

Effect of RNA Other Than tRNA. As had been shown in Figure 5F neither rRNA nor 5S RNA protected tRNA nucleotidyltransferase against thermal inactivation at a concentration of about 10 μM. We also investigated oligonucleotides derived from tRNA after digestion with ribonuclease. For this purpose, 12 mg of rRNA was digested in 0.02 M Tris-HCl (pH 7.0) for 10 min at 4° with 0.1 μg of ribonuclease. The RNA was extracted using the phenol method and oligonucleotides of various sizes were separated on Sephadex G-100 in 4 M urea. No protection of tRNA nucleotidyltransferase against thermal in-

activation was observed at concentrations 10- to 20-fold higher than those of tRNA which gave complete protection. The following synthetic polynucleotides were also tested: poly(U), poly(A), poly(GU), poly(AG), poly(AU), and poly(CU). None gave any protection of tRNA nucleotidyltransferase against thermal inactivation at concentrations of about 0.1-mg/100 μl assay. Also, none of these different RNAs inhibited the enzyme reaction using tRNA-X-C and CTP as substrates.

Discussion

tRNA nucleotidyltransferase from *E. coli* is susceptible to thermal inactivation but may be stabilized by the addition of substrates. The first-order rate constant of inactivation was the same whether AMP incorporation into tRNA-X-C-C or CMP incorporation into tRNA-X-C or tRNA-X were determined. These results lent further support to the observation that one enzyme is responsible for the addition of all three terminal nucleotides to tRNA. As we will show in greater detail elsewhere (Miller and Philipps, 1971a), all three activities copurified and were found in one protein with a molecular weight of about 50,000.

With the exception of ATP, all substrates at saturating concentrations completely protected the enzyme against thermal inactivation at 40°. ATP was also the only substrate which exhibited substrate inhibition at concentrations of 0.4 mM and higher. At about the same concentration of ATP, protection against thermal inactivation ceased to increase. This suggests that whatever process is causing inhibition of the enzymatic activity at concentrations of ATP above 0.4 mM may be the same which is preventing further protection of the enzyme by ATP. For each of the substrates, the protection constants were approximately equal to their K_m values. This indicates that all substrates are protecting the enzyme by interaction at the active site. The protection constant of the product of the enzymatic reaction, *i.e.*, tRNA-X-C-C-A, was in the same range as its K_i value (Miller and Philipps, 1971b). Since the product inhibits the enzymatic reaction with various tRNA substrates competitively, protection by intact tRNA appears to involve the site on the enzyme for tRNA binding.

As will be shown in greater detail elsewhere (Miller and Philipps, 1971b), there is only one binding site for all three tRNA substrates on tRNA nucleotidyltransferase. The data presented here on the sequential removal of nucleotides from the 3′-OH terminus of tRNA suggest that the amino acid acceptor stem of tRNA is involved in this binding. It is generally believed that venom phosphodiesterase attacks tRNA at random (Preiss *et al.*, 1959; Nihei and Cantoni, 1963). If this is so, we may assume that under the conditions used in the experiment of Figure 10, all tRNA chains should have lost some of the 3′-OH nucleotides, the degree of digestion probably varied somewhat around the mean value plotted in Figure 10 (Holley *et al.*, 1964). Since, as shown here, protection of tRNA nucleotidyltransferase from thermal inactivation is accomplished by binding of the substrates to the active site of the enzyme it may be concluded that binding of tRNA to the enzyme ceases when in the average more than nine nucleotides had been removed. The amino acid acceptor stem of all tRNA molecules contains 11 nucleotides, 7 of which are hydrogen bonded or buried in the hydrogen-bonded part (see Philipps, 1969). Thus we may conclude that binding of the enzyme ceases only when the whole amino acid acceptor stem is removed. The nucleotide sequence in this part of the tRNA molecule varies considerably between different species of tRNA. However, the K_m value for unfractionated tRNA is essentially the same as the K_m values for five purified tRNA species so far tested (Miller and Philipps, 1971b). Binding of tRNA to tRNA nucleotidyltransferase therefore cannot be due to a particular nucleotide sequence but the enzyme must recognize the secondary or tertiary structure of tRNA. Since rRNA, 5S RNA, or synthetic polynucleotides were not able to stabilize the enzyme, it may be concluded that the primary and/or secondary structure of the amino acid acceptor stem alone is not sufficient to secure binding of tRNA to the enzyme but that some other parts of the tRNA molecule must also be involved in the binding reaction. However, as shown in the experiments of Figure 10, the size of the stem region is critical. Our data further suggest that the particular conformation of that part of the tRNA molecule which is required for the binding to tRNA nucleotidyltransferase remains unchanged after the attachment of an amino acid. This follows from the observation that the values for π were the same whether tRNA was in the deacylated or aminoacylated state, but they were larger by one order of magnitude than for tRNA from which one to three nucleotides had been removed. Of the various models proposed for the conformation of tRNA (see Levitt, 1969), the model of Cramer *et al.* (1968) appears to satisfy best the requirements for binding of tRNA nucleotidyltransferase, since it proposes that the amino acid acceptor stem is folded onto the dihydro-U loop and hydrogen bonds are formed between the two cytidyl residues of the stem and the two guanosyl residues of the dihydro-U loop. Since the protection constant for intact tRNA was larger by one order of magnitude than for tRNA from which a limited number of nucleotides had been removed, we may assume that the binding site on tRNA for tRNA nucleotidyltransferase becomes more accessible upon the removal of a few nucleotides from the 3′ terminus. Thus we may speculate that a conformational change, possibly a loosening of the tertiary structure of tRNA occurs upon the removal of nucleotides from the 3′ end.

An interesting observation is that the protection constants for tRNA were essentially the same whether three or seven nucleotides had been removed. It is known that tRNA from which four nucleotides had been removed cannot serve as substrate for the enzyme (Preiss *et al.*, 1961; Daniel and Littauer, 1965; Miller *et al.*, 1970). As we will show elsewhere (Miller and Philipps, 1971a), apparently homogeneous tRNA nucleotidyltransferase incorporates only two CMP and one AMP residues into tRNA-X while less purer enzyme preparations can make "mistakes," *e.g.*, they may incorporate UMP or two CMP residues into tRNA-X-C. The question then arises, how is the specificity of the incorporation accomplished which leads to the restoration of the -pC-C-A terminus? The two guanosyl residues in the dihydro-U loop are constant for all tRNA species (Philipps, 1969) which in the tRNA model of Cramer *et al.* (1968) are hydrogen bonded to the cytidyl residues of the -pC-C-A terminus. They are preceded in almost every tRNA by a dihydrouridyl residue which is substituted by a cytidyl residue only in $tRNA^{Ala}$ and $tRNA^{Val}$ from yeast and in $tRNA^{Tyr}$ and $tRNA^{Phe}$ from *E. coli;* in *E. coli* $tRNA^{fMet}$ an uridyl residue is found in this position (see Philipps, 1969). We may then speculate that the high degree of specificity of tRNA nucleotidyltransferase may be the result of the transcription of the internal G-G or D-G-G sequence of the dihydro-U loop. However, more data are needed to support this hypothesis, but it should be noted that nowhere else in the tRNA structure is found a constant (U/D)-G-G sequence.

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Extrinsic Cotton Effects in Complexes of Creatine Phosphokinase with Adenine Coenzymes*

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ABSTRACT: Rotatory dispersion and circular dichroic properties of creatine phosphokinase (CPK) and its complexes with adenine coenzymes were compared between 190 and 600 mμ. Binding of ADP, dADP, or ATP, either alone or in combination with bivalent metal ions and substrates, generates a large positive *extrinsic Cotton effect* at the absorption band of the bound chromophores, but does not alter the far-ultraviolet rotatory dispersion of CPK. The extrinsic Cotton effects contribute to the rotatory dispersion in the near-ultraviolet and visible region accounting for the increased dextrorotation of the enzyme–coenzyme–substrate complex reported previously (Samuels, A. J., Nihei, T., and Nada, L. (1961), *Proc. Nat. Acad. Sci. U.S. 47*, 1992). The amplitude of the Cotton effect increases in proportion to the extent of complex formed until 2 moles of coenzyme is bound per mole of CPK. The maximum total amplitude, $[M]_{278} - [M]_{243}$ of the CPK · ADP complex is 95,000° compared to the maximum span of only 5000° for the negative Cotton effect of the free coenzyme. Binding of Mg^{2+} and substrates to the complex does not affect the extrinsic Cotton effect, but substitution of Mg^{2+} by Mn^{2+} tends to reduce its amplitude slightly. The Cotton effect of the CPK · dADP complex is similar in amplitude to that induced by ADP, but, in contrast, that of the CPK · ATP complex is 30–40% smaller. The differences are considered to reflect different modes of attachment of the coenzymes to CPK. The association of these Cotton effects with a strong absorption band of the coenzyme and their accompaniment by bathochromic and hypochromic changes of this band are suggestive of their generation by a dipole coupling mechanism (Kuhn–Kirkwood). Theoretical considerations show that they could arise from intermolecular coupling of the adenine transition of the coenzyme and strongly allowed transitions in the protein. An interaction with farultraviolet transitions of a juxtaposed tryptophanyl residue is proposed to account for the extrinsic Cotton effects in this system.

Generation of rotatory power by asymmetric binding of chromophoric molecules to macromolecules is well recognized (Ulmer and Vallee, 1965). In proteins and polypeptides, such Cotton effects have been termed extrinsic Cotton effects to distinguish them from those of the peptide backbone and of amino acid side chains which are designated as intrinsic Cotton effects (Blout, 1964). In enzymes, such extrinsic Cotton effects can arise from noncovalent complexes with chromophoric coenzymes, prosthetic groups, metal ions,

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