EVIDENCE FOR METALLOENZYME CHARACTER OF tRNA NUCLEOTIDYL TRANSFERASE

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SUMMARY

Previous studies (1-5) have shown that several nucleotidyl transferases are metalloenzymes and in a few cases (1-3) the metal has been identified as zinc. In all instances these enzymes are specifically inhibited by incubation with the chelating agent 1,10-phenanthroline but are not affected by the structurally similar 1,7-phenanthroline which does not chelate metals. We report here that tRNA nucleotidyl transferase from E. coli is inhibited by 1,10-phenanthroline and that only the initial rate of incorporation of AMP is affected; CMP incorporation is not specifically inhibited by this chelator. This finding is in conflict with a previous study (5) in which it was claimed that tRNA nucleotidyl transferase from Rous sarcoma virus and from yeast was unaffected by 1,10-phenanthroline and suggests that the E. coli tRNA nucleotidyl transferase is a metalloenzyme.

Several nucleotidyl transferases appear to be metalloenzymes and, in a few instances, the metal has been identified as zinc. Scrutton et al (1) reported that the DNA-dependent RNA polymerase from E. coli contains two atoms of bound zinc per molecule of enzyme while Slater et al (2) showed that the DNA polymerases from E. coli and sea urchin contain two and four molecules respectively of zinc per molecule of enzyme. More recently, Auld et al (3) have demonstrated the existence of approximately two moles of zinc per mole of the RNA-dependent DNA polymerase from avian myeloblastosis virus. In addition, the following enzymes are specifically inhibited by 1,10-phenanthroline: the terminal deoxynucleotidyl transferase from calf thymus gland (4), DNA dependent RNA polymerase from rat liver and sea urchin (5), and the RNA-dependent DNA polymerase from Rous sarcoma virus (5). On the basis of kinetic data, Chang and Bollum (4) postulated that the metal ion in terminal deoxynucleotidyl transferase is required for the binding of the deoxynucleotide substrate. The similarities in the reaction catalyzed between these various nucleotidyl transferases and the tRNA adenyl(cytidyl)trans-

ferase (E.C. 2.7 . 7.25) led us to analyze the latter for the presence of a heretofore undetected metal ion.

Materials and Methods

The tRNA adenyl(cytidyl)transferase was purified from E. coli B by a method which is to be published (10). The final specific activity of the purified enzyme used in these studies was 485 nanomoles AMP incorporated/min./mg of protein under optimum conditions at 37°C. Miller and Philipps (8) have previously reported a specific activity of 480 nanomoles AMP incorporated/min/mg of protein for a nearly homogeneous preparation, while Carre et al (9) claim a specific activity of 1,000 nanomoles AMP incorporated/min/mg of protein for their highly purified preparations. The standard assay contained in a final volume of 0.1 ml: 5.0 μmoles of Tris-HCl, pH 9.0, 1.0 μmole of $MgCl_2$, 1.0 μmole of β-mercaptoethanol, 2.0 nanomoles of yeast tRNA, 40.0 nanomoles (0.25 μCi) of 2,8-(^{3}H) ATP or 5-(^{3}H)CTP and 0.2 μg of protein. The reaction was stopped by the addition of 5.0 ml of cold 10% trichloroacetic acid. The precipitate was collected on glass fiber filters and washed with 5% trichloroacetic acid and 95% ethanol. The filters were dried and counted in a toluene based scintillator. All assays were done in triplicate and only the average values are reported. In all cases the average deviation of these multiple assays was less than 10%. The 1,7-phenanthroline was from the G. Frederick Smith Company and the 1,10-phenanthroline was from the Aldrich Chemical Company. The 2,8-(^{3}H) ATP and 5-(^{3}H) CTP were obtained from New England Nuclear while the yeast tRNA was from Schwarz-Mann.

Results and Discussion

Initial studies demonstrated that preincubation of the enzyme with 0.1 mM 1,10-phenanthroline in the presence of 10 mM Tris-HCl, pH 6.8, 10 mM MgCl, 2 mM β-mercaptoethanol and 10% glycerol led to a loss of nearly 40% of the enzyme activity as measured by AMP incorporation. However, when 1,10-phenanthroline was added directly to the standard assay system no significant inhibition was observed. In view of this discrepancy each of the components of the assay system

was examined to determine its effect on the extent of inhibition by 1,10-phenanthroline.

As can be seen in table 1, nearly 90% of the AMP incorporating activity is lost in a five minute incubation at 37°C with 0.25 mM 1,10-phenanthroline. This inhibition is independent of whether the incubation is done at pH 6.8 or pH 9.0 in the presence or absence of ATP. If the incubation is done on ice, or at 37°C in the presence of yeast tRNA, then 30% of the activity remains after five minutes. However, if β-mercaptoethanol is included, even in the absence of tRNA, then over 70% of the original activity remains. A similar phenomenon has been reported by Valenzuela et al (5) for RNA polymerase II from rat liver. They found that inclusion of 14.3 mM β-mercaptoethanol decreased the inhibition observed with 1,10-phenanthroline by 49%.

TABLE I. EFFECT OF ASSAY COMPONENTS ON INHIBITION OF tRNA-NUCLEOTIDYL TRANSFERASE BY 1,10 PHENANTHROLINE

CONDITIONS	% INHIBITION
pH 9.0	92
pH 9.0, 0°	69
pH 6.8	87
+ 0.4 mM ATP	91
+ 0.5 mg/ml tRNA	70
+ 10 mM ESH	30

Table 1: All incubation mixtures contained 50 mM Tris-HCl, 10 mM $MgCl_2$, and 0.25 mM 1,10-phenanthroline. Other additions are as indicated above. After incubation for 5 min., and at 37°, pH 9.0 unless otherwise indicated, samples were withdrawn and assayed in the standard system minus β-mercaptoethanol (ESH). Control samples without 1,10-phenanthroline were run for each set of conditions and the % inhibition was determined by comparison to these controls.

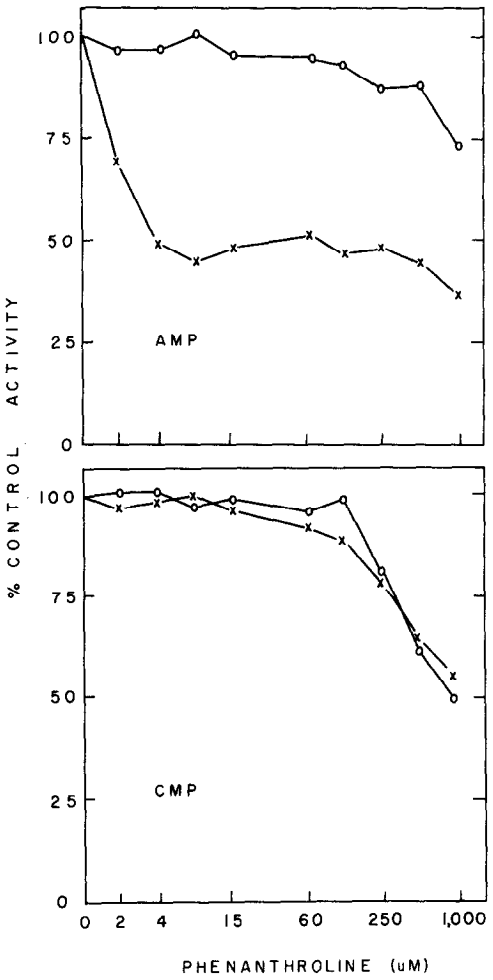

Fig. 1 Inhibition by phenanthroline. Samples were assayed either for AMP incorporation (top graph) or for CMP incorporation (lower graph) using the standard assay without β-mercaptoethanol. In addition, all assays contained the indicated concentrations of phenanthroline and 2% methanol. 0-0 1,7-phenanthroline; X-X 1-10-phenanthroline.

The effect was further studied by investigating the concentration dependence of inhibition by 1,10-phenanthroline and by assaying for CMP as well as AMP incorporation. As shown in figure 1, maximal inhibition of AMP incorporation is reached at about 4-8 μM 1,10-phenanthroline which is similar to the value of 10 μM reported for maximal inhibition of Rous sarcoma virus reverse transcriptase (5). Under the conditions cited in figure 1, 50% of the AMP incorporating activity is lost; however, more extensive inhibition is observed by preincubation

of the enzyme with 1,10-phenanthroline as was shown in table 1. In order to achieve the same level of inhibition of the rate of CMP incorporation, the 1,10-phenanthroline concentration must be increased by at least two orders of magnitude To determine if the inhibition was due to the metal chelating ability of 1,10-phenanthroline a similar series of experiments was done using the non-chelating analogue 1,7-phenanthroline. As seen in figure 1, 1,7-phenanthroline exhibits significant inhibition of AMP incorporation only at concentrations in excess of 0.2 mM which is at least 20X the concentration of 1,10-phenanthroline required to obtain maximal inhibition. Hence the inhibition of AMP incorporation by 1,10-phenanthroline can best be explained on the basis of its metal chelating ability. In contrast, both 1,10 and 1,7-phenanthroline show the same extent of inhibition of CMP activity with varying phenanthroline concentration and this inhibition is probably due to a non-specific interaction of the aromatic phenanthroline ring system with the enzyme. A similar phenomenon has been reported by Anderson et al (11) for yeast alcohol dehydrogenase and by Scrutton et al (1) for the DNA-dependent RNA polymerase from E. coli.

It has long been known that tRNA nucleotidyl transferase requires 5-10 mM $MgCl_2$ *in vitro* for maximal activity (7). In addition, Miller and Philipps (8) reported that the enzyme from E. coli required Mg^{++} for its stability, since purified fractions that were dialyzed against Mg^{++}-free buffers lost all activity even though Mg^{++} was subsequently present in the assay mixture. Carre and Chapeville (9) have since shown that the tRNA - tRNA nucleotidyl transferase complex dissociates in the absence of Mg^{++} or on the addition of EDTA. However, the inhibition that we observe in the presence of 1,10-phenanthroline cannot be due to Mg^{++} chelation because of the low affinity of this chelator for Mg^{++}, the pK is 1.5 (12), and also because in all instances where we have exposed the enzyme to 1,10-phenanthroline, Mg^{++} has been present in excess. The maximal inhibition of AMP incorporation observed at 4-8 μM 1,10-phenanthroline is due to metal chelation since the non-chelating analogue 1,7-phenanthroline has no significant effect on the AMP incorporating activity unless it is present in at

least twenty times this concentration. The inhibition is not readily reversible because dilution or dialysis of 1,10-phenanthroline treated enzyme fails to give any significant increase in activity. It appears that the enzyme requires a transition metal ion, possibly zinc, for maximal rates of AMP incorporation. Recently Carre and Chapeville (6) have also reported that sulfhydryl blocking reagents inhibit AMP incorporation but are without effect on the incorporation of CMP into tRNA. The fact that only AMP incorporation is sensitive to both sulfhydryl reagents and 1,10-phenanthroline strongly supports the notion of separate binding sites on the enzyme for ATP and CTP. We are currently investigating the kinetics of 1,10-phenanthroline inhibition and are attempting to identify the particular transition metal involved and determine its function in tRNA nucleotidyl transferase.

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REFERENCES

1. Scrutton, M. C., Wu, C. W. and Goldthwait, D. A. (1971), Proc. Nat. Acad. Sci. USA 68, 2497.
2. Slater, J. P., Mildvan, A. S. and Loeb, L. A. (1971), Biochem. Biophys. Res. Comm. 44, 37.
3. Auld, D. S., Kawaguchi, H., Livingston, D. M. and Vallee, B. L. (1974), Biochem. Biophys. Res. Comm. 57, 967.
4. Chang, L. M. and Bollum, F. J. (1970), Proc. Nat. Acad. Sci. USA 65, 1041.
5. Valenzuela, P., Morris, R. W., Faras, A., Levinson, W. and Rutter, W. J. (1973), Biochem. Biophys. Res. Comm. 53, 1036.
6. Carre, D. S., and Chapeville, F. (1974), Biochim. Biophys. Acta 361, 176.
7. Daniel, V. and Littauer, V. Z. (1963), J. Biol. Chem. 238, 2102.
8. Miller, J. P. and Philipps, G. R. (1971), J. Biol. Chem. 246, 1274.
9. Carre, D. S., Litvac, S. and Chapeville, F. (1974), Biochim. Biophys. Acta 361, 185.
10. Williams, K. R. and Schofield, P., manuscript in preparation.
11. Anderson, B. M., Reynolds, M. L. and Anderson, C. D. (1966), Biochim. Biophys. Acta 113, 235.
12. Sone, K., Krumholz, P. and Stammreich, H. (1955), J. Amer. Chem. Soc. 77, 777.