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Phosphorylation of tRNA by T_4 polynucleotide kinase

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ABSTRACT

The phosphorylation of various intact tRNA species by T_4 polynucleotide kinase has been studied. The apparent Michaelis constant was on the average found to be 100 times lower than for some single-stranded DNAs previously studied. (J.R. Lillehaug and K. Kleppe, (1975) Biochemistry, 14, 1221). Conditions which result in complete phosphorylation of different tRNA species have also been established. Studies on equilibrium constants and the reversibility of the reaction revealed that the phosphorylation reaction is not a true equilibrium reaction under the conditions used in this work.

INTRODUCTION

The mechanism of action of T_4 polynucleotide kinase using various DNAs as substrate has been extensively studied in this laboratory[1-3]. Incomplete phosphorylation of double-stranded DNAs was found when the 5'-hydroxyl end group was situated in nicks on the DNA or when the DNA contained protruding 3'-hydroxyl end groups, using the same phosphorylation condition that allows complete phosphorylation of single-stranded DNAs[3]. In many ways the 5'-hydroxyl end group structure of various tRNAs resemble that of some of the DNAs previously studied. It was therefore of considerable interest to also study phosphorylation of intact tRNA species.

T_4 polynucleotide kinase has recently been used to label tRNA and the enzyme has become an important tool in the sequence analysis of tRNA on a microscale[4-6]. It is unclear whether complete phosphorylation was obtained in these studies. If conditions could be established that allows complete phosphorylation of all types of tRNA species the enzyme could prove to be a powerful tool in the study of modification of tRNA species

particularly those that are difficult to label in vivo. For example in the search for alterations of specific tRNAs from transformed cells such a method would seem very useful. The analysis of intact tRNAs by chromatography as well as electrophoresis could then be performed on a microscale.

In the present work we have examined the kinetic parameters for the phosphorylation of several tRNA species and also conditions that allow complete phosphorylation of tRNA.

MATERIALS AND METHODS

Enzymes. T_4 polynucleotide kinase was isolated according to Panet et al.[7] and the specific activity was as reported. Possible contamination with RNase was investigated by incubating the enzyme for various lengths of time with T_7 mRNA and also with mouse plasma cytoma cell mRNA. The RNA was then precipitated with trichloroacetic acid. In the case of the mouse plasma cytoma cell mRNA binding studies of this RNA to oligo-(dT)-cellulose was also carried out. No RNAase activity could be detected by these experiments. To further exclude any RNase contamination the phosphorylated products were degraded to mononucleotides by 0.3 M KOH hydrolysis and the products were separated by chromatography. Radioactivity was seen only in the mononucleotide expected from the sequence.

Bacterial alkaline phosphatase was obtained from Worthington Biochemical Corporation. Glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerat kinase were obtained from Boehringer Mannheim.

Nucleic acids and ATP. [^{32}P]-γ-ATP was prepared by a method slightly modified from that originally published by Glynn and Chappell[8]. The specific activity was approximately 100 Ci/mmole. Transfer ribonucleic acid N-formyl methionine specific ($tRNA_f^{Met}$), $tRNA^{Phe}$ and unfractionated tRNA, all from E.coli MRE 600, were products of Boehringer Mannheim. Based on amino acid incorporation of these tRNAs the purity was estimated to be at least 90 %. Unfractionated tRNA from mouse liver was a gift from Dr. B.H. Berg, Bergen; the amino acid accepting ability of this tRNA was as described[9]. The tRNAs were dephosphorylated using bacterial alkaline phosphatase as previously described[1]. The

RNA was then precipitated with ethanol at -20°C. The precipitate was collected by centrifugation, dissolved in 0.5 ml of 0.05 M triethylammonium bicarbonate pH 7.5 and further purified by gel filtration on a column of Sephadex G-50 equilibrated with the same buffer. After this treatment no phosphatase activity could be detected in the tRNA samples. The concentration of tRNA was estimated spectrophotometrically using an extinction coefficient of 2 nmole 5'-hydroxyl ends per 1 absorbance unit measured at 260 nm, dissolved in 1 ml and using 1 cm lightpath[10].

Assay system. The assay system for T_4 polynucleotide kinase was as previously described[1]. The standard assay contained: 60 mM Tris/HCl pH 8.0, 9 mM $MgCl_2$, 15 mM β-mercaptoethanol. The concentrations of [γ-^{32}P]-ATP and tRNA are given in the legends. The temperature was 37°C.

Determination of equilibrium constants. These were determined essentially as previously described[3].

RESULTS

Kinetic parameters for different tRNAs. Numerous initial rate studies of phosphorylation of different tRNAs were performed and from these the apparent Michaelis constants were determined as shown in Table I. Also given here for comparison are the kinetic constants for calf thymus DNA. Compared with the latter DNA and also with the values previously determined for other DNA substrates, the apparent Michaelis constants obtained are strikingly low. Thus the constant for mouse liver was only 1/55 of that for calf thymus DNA and 1/175 of the apparent Michaelis constant for 3'-dTp[1]. The apparent maximal velocities deteremined were also lower than those reported for single-stranded DNAs and 3'-mononucleotides. The Michaelis constant for ATP using 3.2 μM *E.coli* tRNA as a substrate was found to be 37 μM, which is close to the true Michaelis constant for ATP (40 μM), 3'-dTp being the fixed substrate[1].

Completion of phosphorylation. As in the case of the phosphorylation of DNA and 3'-mononucleotides we also observed that the concentration of ATP and enzyme had a profound influence on the final plateau values of phosphorylation of tRNA.

Table I

Apparent Michaelis Constants and V_{max} Values for some tRNA*.

Substrate	Apparent Michaelis Constants (μM)	Apparent V_{max}, relative**
Total mouse liver tRNA	0.06	1.0
Total E.coli tRNA	1.35	1.5
E.coli $tRNA_f^{Met}$	0.12	2.9
E.coli $tRNA^{Phe}$	0.39	4.8
Calf thymus DNA	7.6	43.0***

* The concentration of ATP was 66 μM. Other conditions were as described in Materials and Methods.

** The apparent V_{max} for mouse liver tRNA was taken as unity.

*** Taken from J.R. Lillehaug and K. Kleppe (1975) Biochemistry, 14, 1221.

Figure 1 shows the time course of phosphorylation of $tRNA_f^{Met}$ and $tRNA^{Phe}$. The initial ATP concentration was in this case very low, 12.5 μM, which is approximately the same concentration used in many labelling experiments of tRNA and tRNA fragments. With this ATP concentration a plateau value was established after approximately 40 minutes. Addition of more ATP to a final concentration of 45 μM resulted in an immediate increase in the rate of phosphorylation and after approximately 80 minutes 50 % completion had been reached, and the reaction had not yet levelled off. Further experiments with effect of different ATP concentrations showed that to achieve more than 90 % phosphorylation with a tRNA concentration of 3.0 μM (5'-hydroxyl group ends) an ATP concentration of at least 66 μM was required.

The effect of enzyme concentration was also investigated and it was observed that in order to obtain complete phosphorylation, within a reaction period of approximately 1 hour and with a high ATP concentration, an initial enzyme concentration of at least 5 unit/ml should be used. An example employing such conditions is shown in Figure 2, the phosphorylation of $tRNA_f^{Met}$. Complete phosphorylation was here reached after approximately 50 minutes. Addition of more enzyme after 60 minutes did not

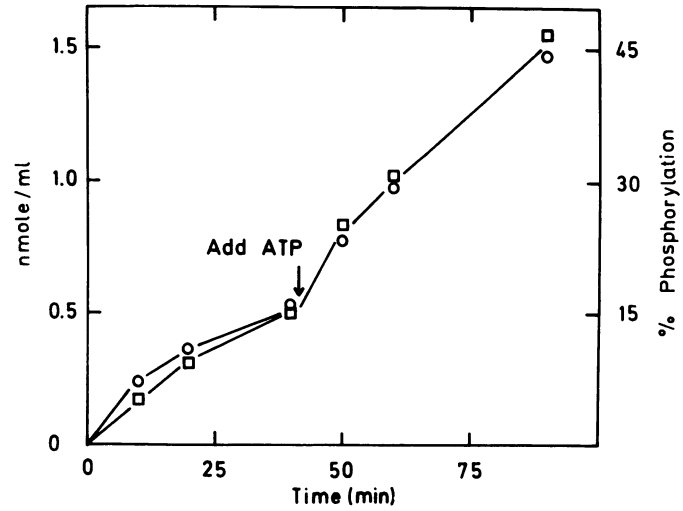

Figure 1. Phosphorylation of $tRNA_f^{Met}$ and $tRNA^{Phe}$. The assay conditions were as described in Methods. The initial ATP concentration was 12.5 μM and after 42 minutes the ATP concentration was increased to 45 μM. The tRNA concentration was 3.1 μM 5'-hydroxyl ends for both tRNAs used, and the enzyme concentration 0.7 units/ml.

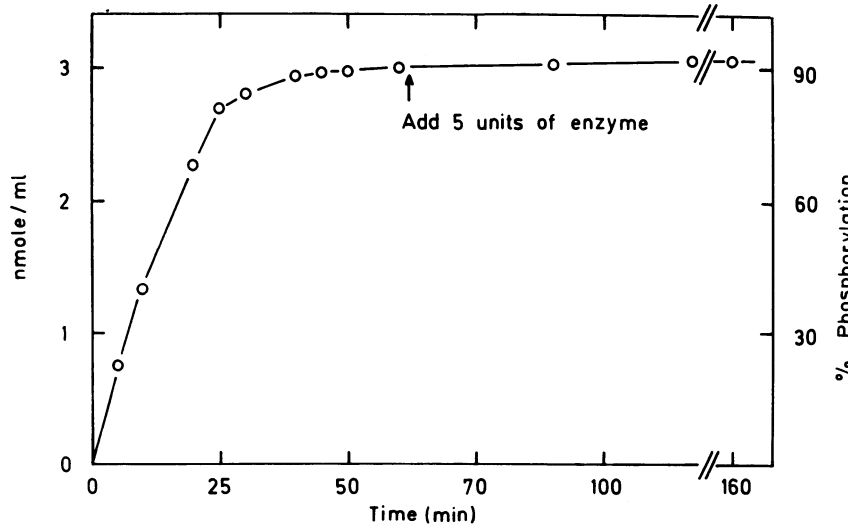

Figure 2. Complete phosphorylation of $tRNA_f^{Met}$. Assay conditions were as described in Methods. The ATP and tRNA concentrations were 66 and 3.1 μM respectively, and the initial enzyme concentration used was 5.5 units/ml. Total assay volume was 640 μl. When more enzyme was added, β-mercaptoethanol equivalent to 10 mM was also added. Aliquots of 50 μl were removed at the times indicated.

increase the yield. Similar results were also obtained with $tRNA^{Phe}$ and total mouse liver tRNA.

Equilibrium constant. The phosphorylation of both DNA and RNA is a reversible reaction. In the case of tRNA the phosphorylation reaction can be written:

$$HO\text{-}tRNA + ATP \rightleftharpoons P\text{-}tRNA + ADP$$

Chaconas et al.[11] have used the exchange nature of this reaction to label phosphorylated tRNA. In the case of single-stranded DNAs, 3'-mononucleotides and double-stranded DNAs containing protruding 5'-hydroxyl groups ends a true thermodynamic equilibrium is established[3]. This is not so with double-stranded DNAs containing protruding 3'-hydroxyl group ends. In the latter case it appears that enzyme-DNA complexes are formed that inhibit further reaction[3]. With regard to tRNA the forward equilibrium constant,

$$K_{eq.forw.} = \frac{[P\text{-}tRNA][ADP]}{[HO\text{-}tRNA][ATP]}$$

and the reverse equilibrium constant

$$K_{eq.rev.} = \frac{[HO\text{-}tRNA][ATP]}{[P\text{-}tRNA][ADP]}$$

were determined for $tRNA^{Phe}$. The value obtained were $K_{eq.forw.} = 0.69$ and $K_{eq.rev.} = 6 \times 10^{-3}$. For a true thermodynamic equilibrium $1/K_{eq.forw.} = K_{eq.rev.}$. In the present case $1/K_{eq.forw.} = 1.45$ and thus the phosphorylation of tRNA by T_4 polynucleotide kinase appears not to be a true equilibrium reaction under the conditions used in this work.

DISCUSSION

The phosphorylation of various tRNAs differs in many ways from the results obtained with DNAs and 3'-mononucleotides previously studied[1]. The low apparent Michaelis constant obtained for tRNAs as compared with various DNAs suggests that the enzyme has a high affinity for tRNA molecules. The reason for this is not clear, but probably it is related to the unique 3-dimensional structure of the acceptor stem region.

The 3-dimensional structure recently elucidated for the yeast $tRNA^{Phe}$[2] shows the acceptor stem region to be a separate entity protruding almost at a right angle from the rest of the molecule. It is probable that other tRNA species possess similar 3-dimensional structures. The compact bulky nature of the tRNA molecules may also prove advantageous when binding to the enzyme as it could provide more groups for interactions with the enzyme. The difference in apparent Michaelis constant between $tRNA^{Phe}$ and $tRNA_f^{Met}$ may be due to the fact that $tRNA_f^{Met}$ has a nonhydrogen-bonded cytosine residue at the 5'-hydroxyl group end whereas $tRNA^{Phe}$ contains a hydrogen-bonded guanine residue in this position.

The phosphorylation of tRNA was found not to be a true thermodynamic equilibrium reaction and it is thus similar to that observed for double-stranded DNAs containing protruding 3'-hydroxyl group ends. The reason for this is probably that dead-end complexes are formed analogous to those previously suggested for certain DNAs[3]. From the apparent equilibrium constant determined for $tRNA^{Phe}$, 0,69, it be seen that in order to achieve 95 % phosphorylation at least 25 fold excess of ATP must be present in the reaction mixture. From the data given in this work it is probable that the apparent equilibrium constants for other tRNA species also are of the same order of magnitude. Thus in order to obtain complete phosphorylation of tRNA it is of prime importance to have a large excess of ATP over the concentration of free 5'-hydroxyl groups.

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REFERENCES

1. Lillehaug, J.R. and Kleppe, K. (1975) Biochemistry 14, 1221-1225.
2. Lillehaug, J.R. and Kleppe, K. (1975) Biochemistry 14, 1225-1229.
3. Lillehaug, J.R., Kleppe, R.K. and Kleppe, K. (1975) Biochemistry 15, 1858-1865.

4. Szekely, M. (1971) Procedures in Nucleic Acid Research Vol. 2, 780-795.
5. Simsek, M., Ziegelmeyer, J., Heckman, J. and RajBhandary, U.L. (1973) Proc. Nat. Acad. Sci. USA 70, 1041-1045.
6. Szeto, K.S. and Søll, D. (1974) Nucleic Acids Research 1, 1733-1738.
7. Panet, A., van de Sande, J.H., Lowen, P.C., Khorana, H.G., Raae, A.J., Lillehaug, J.R. and Kleppe, K. (1973) Biochemistry 12, 5045-5050.
8. Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147-149.
9. Berg, B.H. (1975) Biochem. Biophys. Acta 414, 93-98.
10. Stern, R. and Littauer, U.Z. (1971) Procedures in Nucleic Acid Research Vol. 2, 565-587.
11. Chaconas, G., van de Sande, J.H. and Church, R.B. (1975) Biochem. Biophys. Res. Comm. 66, 962-969.
12. Sussman, J.L. and Kim, S.H. (1976) Science 192, 853-858.