TERTIARY STRUCTURE INTERACTIONS OF 7-METHYLGUANOSINE IN YEAST $tRNA^{Phe}$ AS STUDIED BY BOROHYDRIDE REDUCTION

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1. Introduction

According to the recently published crystallographic analyses of yeast $tRNA^{Phe}$ the positively charged m^7G in the extra loop contributes to the stabilization of the tertiary structure by hydrogen bonding to the C13–G22 base pair of the dihydrouridine stem [1–4]. The possibility of an additional electrostatic interaction of m^7G with the closely neighbouring phosphate of A9 has been noted [4]. We have been interested in m^7G as a point of specific chemical modification and cleavage of $tRNA^{Phe}$ [5]. Previous [6,7] and new results of $NaBH_4$ reduction experiments prompted us to investigate the accessibility of m^7G in $tRNA^{Phe}$ under a number of conditions.

In the present study aniline catalyzed chain scission [5,8] was used as a sensitive method for the detection of m^7G modification by $NaBH_4$. The fragments Phe 1–45 and Phe 47–76 are formed in this reaction, which can also be used in a preparative scale. The $NaBH_4$ modification of m^7G does not affect the activity of $tRNA^{Phe}$ in a number of biochemical assay systems.

At low ionic strength m^7G in $tRNA^{Phe}$ is reduced rather slowly as compared to its reactivity in the fragment Phe 38–76. With increasing ionic strength, however, the rate of reduction of m^7G in $tRNA^{Phe}$ is greatly accelerated and approaches the rate observed in the case of Phe 38–76. This result is direct evidence for an electrostatic interaction of m^7G with another part at the $tRNA^{Phe}$ molecule which is present, however, only under certain conditions.

Abbreviations: m^7G, 7-methylguanosine; Phe 47–76, fragment of $tRNA^{Phe}$ with nucleotides 47 and 76 of $tRNA^{Phe}$ at the 5′- and 3′-terminus, respectively.

2. Materials and methods

$tRNA^{Phe}$ was isolated from brewer's yeast tRNA (Boehringer, Mannheim) by chromatography on BD-cellulose [9] and rechromatography on a column of DEAE-Sephadex A 25 using a linear gradient of 0.3 to 0.7 M sodium chloride in 0.05 M sodium acetate, pH 5.0, 0.01 M $MgCl_2$, 7 M urea. The acceptor activity was 1.5 nmol Phe/A_{260} unit. Phe 38–76 was prepared as in ref. 10. Sodium borohydride (Merck, Darmstadt) was 97% pure according to iodometric determination [11]. Aniline (Merck) was distilled from zinc powder under reduced nitrogen pressure before use. In the analytical experiments two procedures of $NaBH_4$ reduction were used: (A) $tRNA^{Phe}$ was preincubated for 15 min at 37°C in the buffer specified in the legend of fig.2. The cooled solution was added to a freshly prepared ice-cold solution of $NaBH_4$ in the same buffer to give final concentrations of 20–60 μM tRNA and 0.5 M $NaBH_4$. The reduction was performed at 0°C under subdued light. The reaction was terminated by pipetting aliquots of the reaction mixture into one tenth vol 6 N acetic acid and subsequent ethanol precipitation. (B) 30 μM solutions of $tRNA^{Phe}$ or Phe 38–76, which for analytical experiments had been exhaustively dialyzed [5], were incubated for one min at 80°C and 15 min at 37°C in the buffer given in the legend of fig.3. After cooling in ice the solutions were made 2.5 mM in

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$NaBH_4$ by the addition of 25 mM $NaBH_4$ in cold 0.01 N KOH. Unless otherwise stated the reduction was stopped after 30 min at 0°C by mixing with an equal volume of 0.5 M sodium acetate buffer, pH 4, and subsequent ethanol precipitation.

The aniline treatment was performed by incubating the reduced tRNA (approx. 20 A_{260} units/ml) in 0.3 M aniline·HCl, pH 4.5, for 3 h at 25°C. In analytical experiments the samples were then subjected to disc electrophoresis in 16% polyacrylamide gels [12]. The extent of m^7G conversion was calculated from the molar amount of Phe 47–76 in the densitograms (cf. fig.1). The amount of Phe 1–45 was not used in the calculation since this fragment was preferentially degraded during aniline treatment.

In preparative experiments 150 A_{260} units of $tRNA^{Phe}$ were reduced in a KCl containing buffer according to procedure B (no prior dialysis necessary). The products of the aniline treatment were precipitated with ethanol and chromatographed on a column of DEAE-Sephadex A 25 (cf. fig.5b in ref. 5). Pure fragment Phe 47–76 was isolated in 70% yield.

3. Results

3.1. *Chain scission in reduced $tRNA^{Phe}$ at the m^7G position*

When $tRNA^{Phe}$ is treated with $NaBH_4$ and subsequently with aniline at acidic pH, the tRNA is split into two fragments (fig.1). The electrophoretic mobility of the fragments suggests that they are formed by a chain scission at the m^7G-position as in a previously described reaction [5]. In order to prove this assumption we have isolated the smaller fragment (see Materials and methods) and analyzed the oligonucleotides obtained by digestion with T_1 RNAase. We obtained the same pattern as for the previously analyzed fragment Phe 47–76 [5] except that the full amount of m^1A was found in the octanucleotide. We conclude that m^7G in $tRNA^{Phe}$ had reacted with $NaBH_4$. The modification of m^7G by $NaBH_4$ as the first step in the preparation of the fragments of $tRNA^{Phe}$ is much superior to the one by alkali [5] because it gives higher yields and makes unnecessary the careful removal of divalent cations by dialysis which was critical in order to avoid unspecific chain breaks during the treatment with alkali.

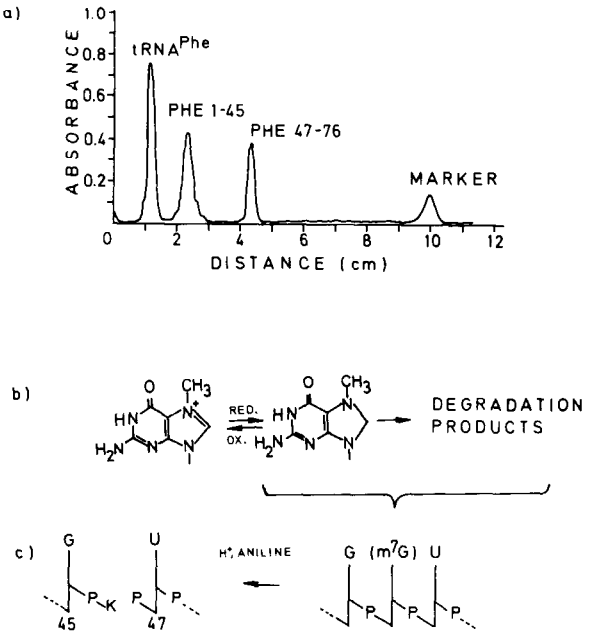

Fig.1. Chain scission at the m^7G position of $tRNA^{Phe}$. (a) Disc electrophoresis of the fragments formed by aniline treatment of reduced $tRNA^{Phe}$; (b) hypothetical reaction scheme for m^7G conversion during $NaBH_4$ treatment; (c) chain scission by aniline catalyzed β-elimination at reduced or further degraded m^7G. K denotes the elimination product of the ribose moiety of m^7G [8]. The numbers refer to the position of nucleotides in the $tRNA^{Phe}$ sequence [13].

Pertaining to the mechanism of reduction and chain scission, we tentatively conclude as outlined in the scheme of fig.1b that both reduced m^7G and its degradation products are susceptible to hydrolysis of the *N*-glycoside bond with subsequent chain scission by β-elimination [5,8]. This assumption is based on the observation that the extent of chain scission obtained in different experiments is critically dependent on the manner in which the reaction is performed and upon the conditions of isolation and further treatment of the reduced tRNA. A varying extent of reoxidation of reduced m^7G [7,14] readily explains this observation. However, a certain proportion of the modified m^7G appears to be stable, since we found reduced tRNA susceptible to chain scission by aniline in appreciable amounts even after prolonged storage. Irreversible degradation of reduced m^7G, probably by cleavage of the five-membered ring, has been described [7,14].

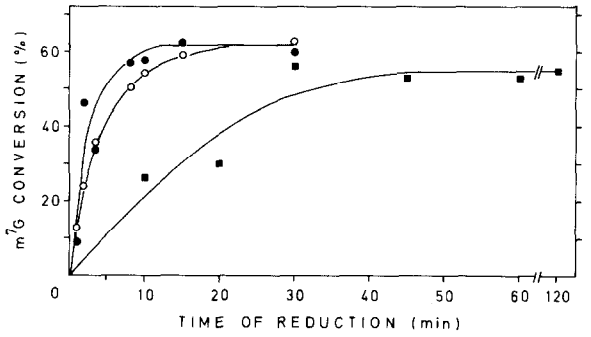

Fig.2. Kinetics of the conversion of m^7G in $tRNA^{Phe}$. $tRNA^{Phe}$ was reduced according to procedure A (Materials and Methods) in 0.2 M Tris–HCl, pH 7.5, 0.01 M $MgCl_2$ (○), in this buffer plus 0.2 M KCl (●), and in 0.2 M borate, pH 9.8, 0.01 M $MgCl_2$, 0.2 M KCl (■) (6). 'm^7G conversion' includes the $NaBH_4$ reduction and the subsequent reactions; it was calculated from the amount of aniline splitting products on disc electrophoresis (Materials and methods).

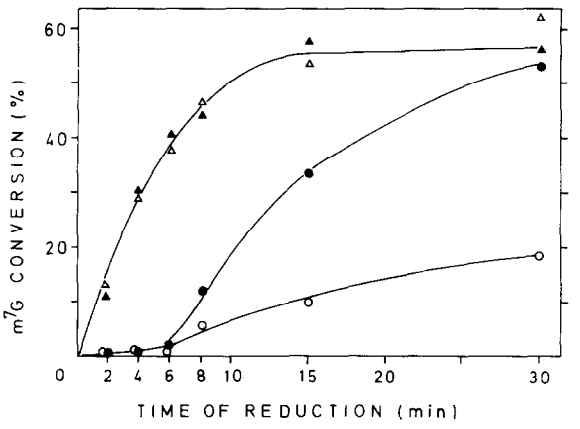

Fig.3. Effect of KCl on the reduction of m^7G. $tRNA^{Phe}$ (○,●) and Phe 38–76 (△,▲) were reduced according to procedure B (Materials and methods) in 0.01 M Tris–HCl, pH 7.5, 0.01 M $MgCl_2$ (open symbols) and in this buffer plus 0.1 M KCl (closed symbols).

As shown in fig.2, m^7G is modified to about the same extent under the two conditions, which have been used previously for the reduction of $tRNA^{Phe}$ (pH 9.8, [6,7]) and of dye-substituted tRNA (pH 7.5, [15]). The reaction is substantially slower at the alkaline pH, even though it is conducted at a higher temperature.

3.2. *Accessibility of m^7G in $tRNA^{Phe}$*

In order to study the influence of the tertiary structure of $tRNA^{Phe}$ on the reaction of m^7G with $NaBH_4$ we compared the rates of m^7G modification in $tRNA^{Phe}$ and in the half molecule Phe 38–76. The experiments were done in a Mg^{++}-containing buffer of low ionic strength and in the same buffer containing 0.1 M KCl. There is a large difference between $tRNA^{Phe}$ and Phe 38–76 in the respective rates of m^7G reduction at low ionic strength (fig.3). In the presence of KCl, however, the rate of m^7G conversion is increased in the case of $tRNA^{Phe}$ and, after a short lag, approaches that of Phe 38–76. Close examination of fig.2 shows the effect of KCl on $tRNA^{Phe}$ reduction also at high $NaBH_4$ concentration.

The extent of m^7G conversion under the conditions of fig.3 varied between 50 and 70% in different experiments. Low plateau values probably are caused by a varying extent of reoxidation of reduced m^7G and/or concomitant hydrolysis of $NaBH_4$, which is rather fast at the pH used for the reaction [16]. The lag phase, which presently cannot be explained, was always observed when $tRNA^{Phe}$ was reduced with low concentrations of $NaBH_4$ at 0°C. Under this condition no modification of dihydrouracil or only a very slow one takes place. This was established by the determination of the dihydrouracil content [17] of $tRNA^{Phe}$ which had been reduced under the conditions of fig.3. The Y-base was not affected either as judged from its chromatographic behavior.

3.3. *Activity of reduced $tRNA^{Phe}$*

We have shown previously that the fragment combination Phe 1–45 + Phe 47–76 which lacks m^7G could be aminoacylated to 15% [18] and the combination Phe 1–36 + Phe 47–76 lacking a few additional nucleotides to 10% [5]. It was not surprising therefore, to find that the acceptor activity of $tRNA^{Phe}$ is not changed (within ± 8%) by $NaBH_4$ reduction under conditions which lead to about 60% conversion of m^7G (Materials and methods, procedure A). The same result was obtained when phenylalanyl tRNA synthetase from *E. coli* was used. The latter finding is noteworthy because it has been shown that the heterologous assay is sensitive to small perturbations

of the tRNA structure [19]. In a polyphenylalanine synthesizing system from *E. coli* [20], reduced and unreduced $tRNA^{Phe}$ were equally active.

4. Discussion

Reduced m^7G is relatively unstable and at least part of it is degraded under most conditions. Under the previously used conditions [7], the further conversion was probably rather extensive and this prevented the detection of reduced m^7G in $tRNA^{Phe}$ by spectrophotometry [7]. The chain scission by aniline, however, which was used in the present study, allows the detection of both reduced and further degraded m^7G.

The slow rate of m^7G modification in $tRNA^{Phe}$ as compared to Phe 38–76 observed at low ionic strength strongly suggests an involvement of m^7G in tertiary structure interactions under these conditions. This is in accordance with the results of the crystallographic analyses, which are obtained from crystals grown in buffers of low ionic strength [1,2]. The finding of full activity of reduced $tRNA^{Phe}$ in a number of assay systems demonstrates that the integrity of m^7G is not required for proper functioning of the tRNA at least in the in vitro systems.

The almost complete loss of protection against $NaBH_4$ reduction of m^7G in $tRNA^{Phe}$ upon addition of salt is attributed to the loosening of an electrostatic interaction. The effect seems to be specific since in general the conformation of tRNA is stabilized rather than weakened by higher ionic strength. The increase of the rate of aminacylation of $tRNA^{Phe}$ which is observed upon addition of up to 0.15 M KCl [21], may be related to the effect of salt on the mobility of m^7G. The location of m^7G in the 'hinge region' of the tRNA molecule and its sensitivity to changes in ionic strength suggest it may trigger conformational transitions of the tRNA in response to variation of the ionic environment during protein synthesis.

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