# REVERSIBLE INACTIVATION OF PHENYLALANINE ACCEPTOR ACTIVITY OF YEAST $tRNA^{phe}$ BY SODIUM BOROHYDRIDE

A. Danchin and M. Grunberg-Manago
Institut de Biologie Physico-chimique, rue P. Curie, Paris (France)

Received April 9, 1970

The following note describes the effect of reduction of $tRNA^{phe}$ by sodium borohydride. The rate of aminoacylation of both the reduced and unreduced species was identical in the unheated samples; however, after heating 3 minutes at 70° the rate of aminoacylation of the reduced species is markedly decreased.

Previous studies (Cerutti, 1968; Igo-Kemenes and Zachau, 1969) using sodium borohydride treatment showed no difference in the aminoacyl acceptor activity between the modified and unmodified species of yeast $tRNA^{phe}$. However, under certain conditions the acceptor activity of the modified species of yeast $tRNA^{val}$ was about 35% less than that of the unmodified.

Spin echo experiments had suggested that some structure of the ribose backbone of the tRNA was left at 100°C (Danchin and Guéron, 1970); furthermore, it was found that recombination of halves of tRNA, cut at the anticodon, showed an important contribution of negative free enthalpy (Römer et al., 1969), and it was therefore thought that a model of tRNA involving a topological property, absorbing entropy, was necessary to account for the experimental behavior observed. The NMR experiments suggested that this property was due to the ribose backbone, and the most simple explanation was that the -CCA end passed through the dihydro U loop, eventually base pairing with the GG which has been shown to be present in this loop in all known primary sequences. In order to investigate this property further we have studied the effect of slight modifications of the dihydro U loop as a function of thermal treatment. The modification technique used was the classical conversion of 5-6 dihydrouracil to ureidopropanol (Cerutti and Miller, 1967).

## Materials and Methods

Yeast $tRNA^{phe}$ was purchased from Boehringer (Mannheim,

Germany) (batch 644 9304) which contained less than 3% $tRNA^{ser}$. Phenylalanine acceptor activity was 95% under the conditions employed.

Assays for amino acid acceptor activity were performed in the following medium (final volume 100 µl) : tRNA, 0.03-0.08 $A_{260}$ units; ($^{14}C$) phenylalanine (specific activity 138 mC/mM), 15 nmoles; ATP, 1 µmole; $MgCl_2$, 1.5 µmole; Tris (pH 7.5) , 5 µmoles; aminoacyl-tRNA-synthetase, 0.2 $A_{280}$ units. The latter which was a gift from Dr. Tiebe and Dr. Zachau (prepared according to their method, Thiebe and Zachau, 1968), was a fraction precipitated between 45-67% ammonium sulfate and stored in glycerol. Aliquots of 10 or 20 µl were precipitated with 5 O.D. stripped tRNA, 2 ml 10% trichloracetic acid, and a mixture of amino acids (0.2%) at 0°, and filtered over glass filters Whatman GF/C. The filter was then washed five times with trichloracetic acid (1%) and a mixture of amino acids (0.2%), then twice with cold ethanol. Radioactivity was measured on a Packard scintillation counter.

The conditions for the reduction of tRNA with sodium borohydride were similar to those of Cerutti (1968). $NaBH_4$ was purchased from Merck (zur Synthese). One of the important factors was that the mixture, maintained at pH 9.8, contained $MgCl_2$ in order to preserve maximum structure of tRNA.

The reduction mixture contained, for 1 ml : $tRNA^{phe}$, 50 $A_{260}$ units; KCl, 0.2 mmole; $MgCl_2$, 0.01 mmole; sodium borate buffer (pH 9.8) , 0.2 mmole; and $NaBH_4$, 20 mg. This mixture was maintained at room temperature in the dark for 4 hours. Excess borohydride was destroyed by 0.1 ml of 1M acetic acid and the final mixture was dialyzed for 48 hours against Tris-HCl, pH 7.5, 0.05M (the dialysis solution was changed four times) at 4°C. Chelex 100 was included in the dialysis solution to remove traces of divalent ions. Control $tRNA^{phe}$ was submitted to exactly the same treatment except for the addition of $NaBH_4$.

The heat treatment was as follows : tRNA was heated for 3 minutes at 70° in 0.001M of Tris pH 7.5, and quickly cooled to 0°. The remainder of the assay mixture for the acceptor activity was added, samples were incubated at 25° or 37° and aliquots were collected at different times to follow the kinetics of phenylalanyl-tRNA formation.

## Results

In the absence of heat treatment no difference could be detected between the untreated $tRNA^{phe}$ (A) and the reduced $tRNA^{phe}$ (B) either after 15 min at 37° or 30 min at 25° (Fig. 1).

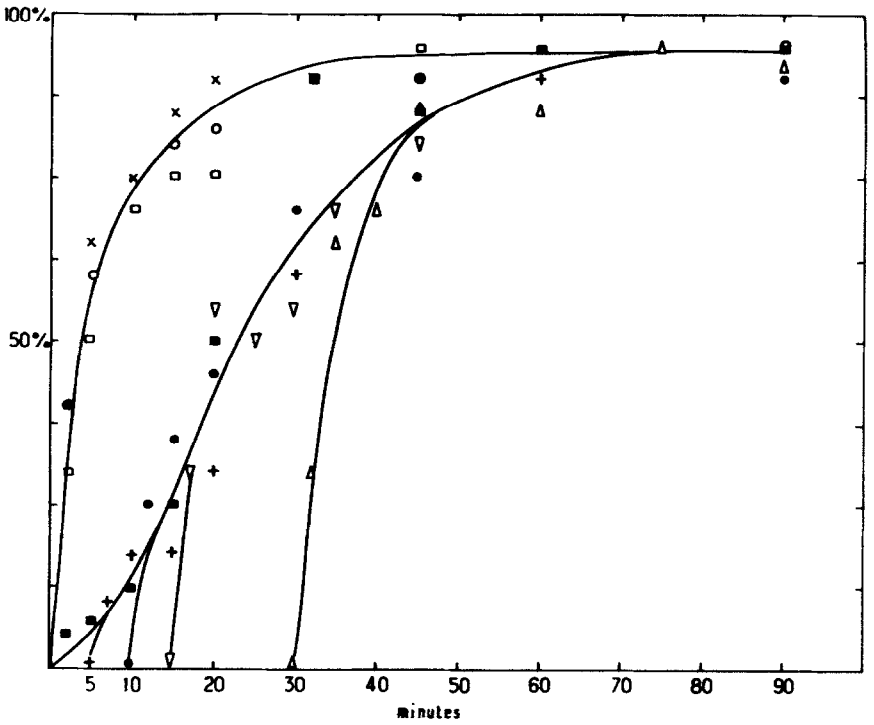


Figure 1

Incorporation of ($^{14}C$) phenylalanine

o untreated $tRNA^{phe}$ (A); x preheated (A) (3' at 70°); □ reduced $tRNA^{phe}$ (B); ■ preheated (B) (3' at 70°). The other points show heated (B) left standing at 25° in 0.015 M $Mg^{++}$ for a preselected time before the enzyme is added: + 5 min preincubation; • 10 min preincubation; ▽ 15 min preincubation; Δ 30 min preincubation. The acceptor activity is calculated assuming an absorption per μmole nucleotide of 8.3 $A_{260}$ (Cohn et al., 1969), and 76 nucleotides for $tRNA^{phe}$.

---

Both are charged at the same rate which is in accordance with the results of Igo-Kemenes and Zachau (1969). However, if the tRNA is preheated at 70°, as described in Methods, the (B) species shows a long lag before aminoacylation begins, while there is no change in the unmodified control.

That this lag phase is du to the change in the tRNA and not in the enzyme is shown by the fact that when enzyme is added at

different times to the heated (B) species, preincubated at 25° in the presence of 0.015M $Mg^{++}$, a certain number of molecules have undergone a change and are charged at the same rate as the unheated species (Fig. 1). After 30 minutes preincubation without enzyme in 0.015M $Mg^{++}$, followed by the addition of synthetase, the incorporation of ($^{14}C$) phenylalanine with the heated (B) species rises to the same rate and level as with the unheated. Apparently the (B) preheated tRNA has to undergo a slow change before it is charged.

In order to find the influence of magnesium on the conversion, the preheated (B) species was incubated at 25° without $Mg^{++}$ in Tris-HCl (pH 7.5), 0.001M, and with a small amount of $Mg^{++}$ (0.0005M $MgCl_2$) in the same buffer.

In the absence of $Mg^{++}$ only 30% of the molecules are changed back after 30 minutes to the high rate of aminoacylation, while the sample with $Mg^{++}$ (0.0005M) shows an aminoacyl incorporation very similar to that of the unheated tRNA (Fig. 2).

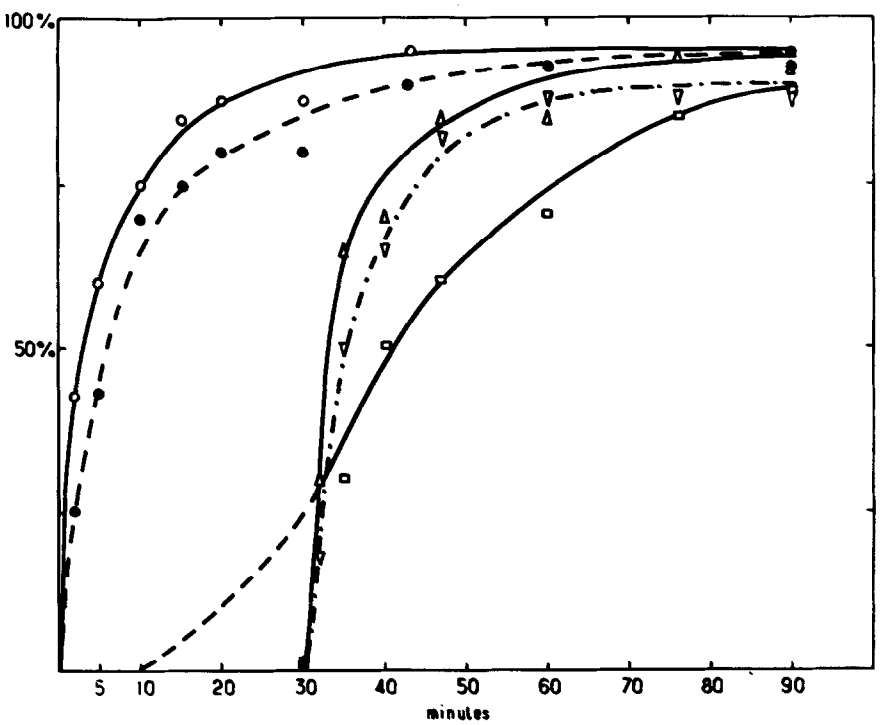


Figure 2

o unheated $tRNA^{phe}$; • reduced $tRNA^{phe}$, preheated for 10 min at 50° in Tris pH 7.5.
Assays with reduced $tRNA^{phe}$ (heated at 70°), left at 25° in preincubation mixture devoid of enzyme, enzyme added after 30 min. Δ preincubation in 0.015M $Mg^{++}$; ▽ preincubation in 0.0005M $Mg^{++}$; □ preincubation without $Mg^{++}$.

The influence of the temperature on the reduced sample has been tested and an incubation of 10 minutes at 50° in 0.001M Tris (pH 7.5) was not enough to destabilize the structure of reduced $tRNA^{phe}$ and only gives a slightly slower rate of incorporation than that of the unheated species (Fig. 2).

A confirmatory experiment (Fig. 3) was done using NMR. In the presence of paramagnetic ions the relaxation time of the water protons is very sensitive to fine details of the tRNA structure (Cohn et al., 1969), and upon addition of a small concentration of $MnCl_2$ one can follow the conformational changes of the (B) species heated as previously described. Although magnesium has been replaced by manganese for the measurement of the relaxation rate, and while the concentrations are different (0.0001M $MnCl_2$, 14.8 $A_{260}$/ml $tRNA^{phe}$) the kinetics of the relaxation rate corresponding to the structural changes of tRNA are similar.

## Discussion

In yeast $tRNA^{phe}$ Igo-Kemenes and Zachau (1969) showed that only the dihydro U and the still unkown Y base near the anti-codon were modified by sodium borohydride under the assay conditions used here. At pH 9.8, however, N-7-methylguanosine may be partially converted to 2-amino-6-hydroxy-5-methyl-formamide-4-ribosyl-amino-pyrimidine (Lowley and Brookes, 1963).

Since three modifications occurred in the sodium borohydride treated $tRNA^{phe}$, we shall discuss each of them to see which might be the most likely to have induced a kinetic change in the aminoacyl acceptor activity. We must emphasize that with the heat treatment following the $NaBH_4$ treatment one induces a reversible transformation on the aminoacylation rate, since the presence of divalent ions for a period of time is enoug to reverse the observed effect. The alkaline product of N-7-methylguanosine cannot be responsible for the effect on the kinetics since the (A) and the (B) species have both been submitted to the same alkaline tratement, and heating, which could have transformed the final product, gives no rate difference for (A) but a significant difference for (B).

To test the importance of the reduction on the Y base, a binding experiment (Leder and Nirenberg, 1964) was performed with both (A) and (E) species preheated and unheated. No significant difference was found, indicating that the heat treatment did not excise the Y (reduced and unreduced) base. Thiebe and

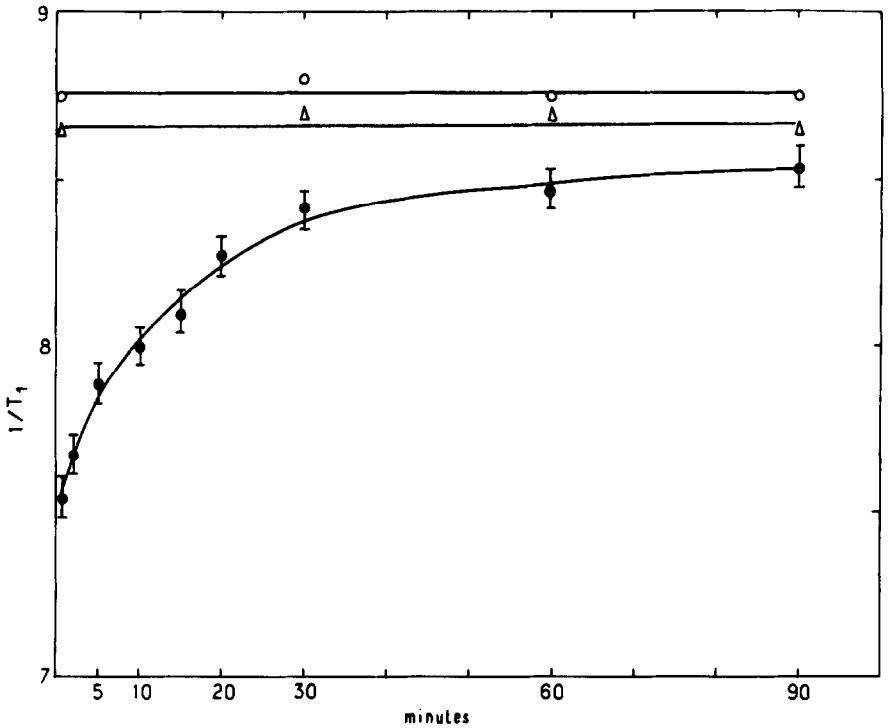


Figure 3

Relaxation measurements of water protons in the $tRNA^{phe}$-$Mn^{++}$ complex at 15.42 Mc/s in 0.0001M $MnCl_2$. These were done in conditions similar to those of Cohn et al. (1969) in collaboration with Dr. M. Guéron.
o untreated $tRNA^{phe}$ preheated; Δ reduced $tRNA^{phe}$;
● reduced and preheated $tRNA^{phe}$.

Zachau (1969) have shown that excised phen-$tRNA^{phe}$ does not bind to ribosomes.

The most important modification, then, is the one which occurred at the dihydro U, and the opening of the dihydro uridine cycle may have induced a steric hindrance of the movement of some part of the molecule which had been destabilized by heating.

In Fig. 1 it can clearly be seen that the rate of incorporation of the heated (E) species is limited by the rate of conversion of an inactive (B) species into the active form, since after incubation for 30 min at 25° in the presence of $Mg^{++}$, the rate of incorporation becomes the same as that of the unheated species. This is confirmed by NMR experiments which show that in the absence of enzyme the preheated(B) molecules change from one conformation to another. It is known that manganese is bound to the phosphate

and the conversion visible at the manganese site shows a change in the ribose backbone of the tRNA.

The aspect of the kinetics shown in this work might account for the differences observed by Cerutti (1968), and Igo-Kemenes and Zachau (1969), on the acceptor activity and the acceptance of adenosine 5' phosphate by yeast $tRNA^{val}$. One of the interpretations favored by these last authors is that the inhibition is due to the presence of an impurity. Another explanation might be that the borohydride reduction, in the case of $tRNA^{val}$ (which has three dihydro U instead of two for $tRNA^{phe}$) has had a destabilizing effect on the tertiary structure, even in the absence of heating, so that there must be a slow structural change before the reduced $tRNA^{val}$ can be acylated. The passage over a DEAE-cellulose column might well have produced the transition towards the active form, which is then charged at the same rate as the unreduced one.

The conversion of 5-6 dihydrouridine to ureidopropanol has changed a rather rigid chemical group to a more dynamic and extended one so that the space inside the dihydro U loop is smaller in the modified species. In a model where the -CCA goes through the dihydro U loop the observed phenomenon can be easily understood since the movement of the -CCA terminal end inside the loop must be more difficult in the case of the reduced species. Furthermore, if the heat treatment has displaced the pG-CCA end from an active conformation, the return to the correct conformation would be slower for the reduced species than for the unreduced one. Such a model (without base pairing of -CCA with the GG of the dihydro loop) recently appeared in the literature (Connors et al. 1969) and the experiments described here are much in favor of this model, at least as regards its conformation at the -CCA end.

This work was supported by the following grants : C-04580 of United States Institutes of Health; Convention N° 66 00 020 of D.G.R.S.T. (Commission de Biologie Moléculaire); French National Research Council (G.R.N°5); Comité de la Seine de la L.N.F.C.C.; and French Atomic Energy Commission.

## References

Cerutti, P., Miller, N., J. Mol. Biol., 26, 55 (1967)

Cerutti, P., Biochem. Biophys. Res. Comm., 30, 434 (1968)

Cohn, M., Danchin, A., Grunberg-Manago, M., J. Mol. Biol., 39, 199 (1969)

Connors, P.G., Labanauskas, M., Beeman, W.W., Science, 166, 1528 (1969)

Danchin, A., Guéron, M., Submitted to Oxford Conf. on Nuclear Magnetic Resonance in Biology (1970)

Igo-Kemenes, T., Zachau, H.G., Europ. J. Biochem., 10, 549 (1969)

Leder, P., Nirenberg, M., Proc. Natl. Acad. Sci., 52, 620, 1521 (1964)

Lowley, D., Brookes, P., Biochem. J., 89, 127 (1963)

Römer, R., Riesner, D., Maas, G., Wintermeyer, W., Thiebe, R., and Zachau, H.G., F.E.B.S. Letters, 5, 14 (1969)

Thiebe, R., Zachau, H.G., Europ. J. Biochem., 5, 546 (1968)

Wintermeyer, W., Thiebe, R., Zachau, H.G., Riesner, D., Römer, R., and Maas, G., F.E.B.S. Letters, 5, 23 (1969)