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# Replacement of wybutine by hydrazines and its effect on the active conformation of yeast $tRNA^{Phe}$

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## ABSTRACT

The highly modified base wybutine (YWye)[2] next to the anticodon of yeast $tRNA^{Phe}$ has been replaced by different hydrazine derivatives. The effect of the replacement on the activity of the tRNA has been studied in the heterologous aminoacylation with synthetase from E. coli and in the poly(U) directed binding to ribosomes from both yeast and E. coli. It was found that starting from $tRNA^{Phe}_{-YWye}$ the activity increased with increasing size, aromaticity, and stacking tendency of the substituent replacing YWye. It is concluded that YWye by the size of its aromatic system and by its stacking properties is particularly well suited for stabilizing the native conformation of $tRNA^{Phe}$.

## INTRODUCTION

Among the odd bases in tRNA the highly modified ones which are located next to the 3'end of the anticodon in many tRNAs have received particular attention. For some of the bases evidence has been presented which indicates an important function in the anticodon-codon interaction on the ribosome (reviews[3,4]). Next to the anticodon of the phenylalanine specific tRNAs from several eukaryotic organisms the tricyclic base Wye or derivatives of it have been found. In $tRNA^{Phe}$ from S. cerevisiae the base YWye is further modified by a long hydrophobic side chain [5,6]. Excision of YWye[7] leads to a change of the conformation of the anticodon loop and of other parts of the $tRNA^{Phe}$ molecule as detected by changes in the CD[8] and p.m.r.[9,10] spectra as well as in the pattern of oligonucleotide binding[11,12]. The conformational change is accompanied by drastic changes of some functions of $tRNA^{Phe}$. The modified molecule is no longer aminoacylated by synthetase from E. coli and the efficiency of binding to poly(U) programmed ribosomes from both yeast and E. coli is greatly diminished[7,8,13]. Further experiments have shown that

YWye may be replaced by proflavine without much loss of activity [14-16]. As judged from p.m.r. spectra[9] the conformation of $tRNA^{Phe}_{Prf}$ is close to the native one.

Apparently the native conformation of $tRNA^{Phe}$ is dependent on the presence of a group next to the 3'side of the anticodon which may even be a synthetic dye. However, the question remained whether particular properties of the base at that position like stacking tendency or hydrophobicity are essential for its function. In order to answer this question we have prepared and assayed a number of $tRNA^{Phe}$-hydrazine derivatives. The preparative and analytical procedures, which may also be of some general interest, are described.

## MATERIALS AND METHODS

$tRNA^{Phe}$ was isolated from brewer's yeast tRNA (Boehringer Mannheim, GFR) by chromatography on BD cellulose and DEAE Sephadex A25 as described previously[17]; it accepted 1.4-1.7 nmoles Phe/$A_{260}$ unit. $tRNA^{Phe}_{-YWye}$ was prepared by excision of YWye from $tRNA^{Phe}$ and isolated by chromatography on BD cellulose[7]; the charging capacity was around 1.3 nmoles Phe/$A_{260}$ unit. Phenylalanyl tRNA synthetases from yeast[18] and E. coli K12[19] and vacant run-off ribosomes from S. cerevisiae (haploid strain X2180-1A)[13] were gifts of U. Pachmann, A. Böck, and J. Robertson, respectively; ribosomes from E. coli MRE 600 were prepared according to ref. 20 except that a high salt ( 3 M KCl ) washing step was included. Poly(U) was purchased from Boehringer Mannheim, GFR. Amines and hydrazine derivatives were purchased from Eastman-Kodak Co., Rochester, or E. Merck, Darmstadt, GFR. The other chemicals (analytical grade) were from E. Merck. [$^3$H]$NaBH_4$ (specific radioactivity 200-400 Ci/mole) was the product of New England Nuclear. The materials for polyacrylamide gel electrophoresis and for reversed phase chromatography (RPC5) were purchased from Serva, Heidelberg, GFR. Method C of Pearson et al.[21] was used to prepare polychlortrifluorethylen coated with trioctylmethylammonium bromide. Phenol and ether were distilled shortly before use from zinc dust in vacuo or from sodium, respectively.

Incorporation of hydrazine derivatives into the YWye

position of $tRNA^{Phe}_{-YWye}$. To the solution of approx. 30 $A_{260}$ units of $tRNA^{Phe}_{-YWye}$ per ml of 125 mM sodium acetate, pH 4.0, was added an equal volume of a 50 mM solution of the respective hydrazine derivative in hexamethylphosphoric triamide. The mixture was incubated for 4 hrs at 27° in the dark. After the addition of 1 M Tris-HCl, pH 8.5, to a final concentration of 0.1 M the reaction mixture was extracted four times with equal volumes of water-saturated phenol. The aqueous phase was freed from phenol by several extractions with ether. The residual ether was removed by a stream of nitrogen. After addition of 1 M potassium acetate, pH 6, to a final concentration of approx. 0.15 M the tRNA was precipitated with ethanol and purified by three consecutive precipitations.

Quantitative determination of the substitution of $tRNA^{Phe}_{-YWye}$ with hydrazine derivatives or amines by reduction with $[^3H]NaBH_4$[22]. After the reaction with a hydrazine derivative 0.2 to 0.4 $A_{260}$ units of the tRNA were reduced with 0.4 mg $[^3H]NaBH_4$ in 0.04 ml of 0.2 M Tris-HCl, pH 7.5, for 3 min at 0°. After addition of 0.2 ml 6N acetic acid the reduced tRNA was precipitated with ethanol. The dried precipitate was dissolved in 0.02 ml 0.1 M Tris-HCl, pH 7.5, and digested with 5 U T1 RNAase for 1 hr at room temperature. Disk electrophoresis in urea containing 16 % gels and staining of the gel (stains all Eastman) were performed as described previously[23]. The area around the slowest moving band, which contained the dodekanucleotide, was cut into six or seven 2 mm slices. The gel slices were oxidized (Oxymat, Intertechnique) and the radioactivity was determined in Oxysolve T scintillation mix (Koch-Light Laboratories). After subtraction of blank values (determined from gel slices before and after the dodekanucleotide band) the total radioactivity was divided by the area corresponding to the dodekanucleotide in the densitogram.

For calibration two samples of $tRNA^{Phe}_{-YWye}$ which had not been reacted with hydrazine were run in parallel in each experiment. One was treated as above and gave the specific radioactivity of the non-reacted dodekanucleotide. The second sample was reduced with non-radioactive $NaBH_4$ prior to the treatment described above. This sample served as the reference for background radio-

activity in the dodekanucleotide, i.e. total substitution by the hydrazine derivative.

The principle of this procedure can also be used for the determination of the condensation products of primary amines with $tRNA^{Phe}_{-YWye}$. However, in this case the condensation product is also reduced by [$^3$H]$NaBH_4$[14]. Therefore, the $^3$H-labeled C-1 of the ribitol is removed by treating the reduced $tRNA^{Phe}_{-YWye}$ with 25 mM periodate in acetate buffer, pH 5.3, for 1 hr at $0^o$; the secondary amine formed by reduction of a $tRNA^{Phe}_{-YWye}$-amine condensation product[14] is stable against periodate[24]. By measuring the residual radioactivity in the amine substituted dodekanucleotide the extent can be determined to which an amine has reacted with $tRNA^{Phe}_{-YWye}$. When tested with Prf the method gave accurate results[24].

## RESULTS

Reaction of $tRNA^{Phe}_{-YWye}$ with primary amines. The reaction of the free ribosylic aldehyde group in $tRNA^{Phe}_{-YWye}$ and a primary amine can lead to a condensation product which may be stable, hydrolyze back to the starting compounds, or react further by eliminating the 3'phosphoester of the substituted ribose resulting in a chain scission of the tRNA[25]. In addition to the previously described reaction of $tRNA^{Phe}_{-YWye}$ with proflavine and ethidium which led to stable condensation products[14] we have studied the reaction with three other primary amines under conditions where no (methylamine and aniline) or little (1-aminopyrene) chain scission had been observed in a screening test of a number of primary amines[24].

In view of the difficulty in obtaining radioactive amines in sufficiently pure state we have developed a procedure for the quantitative determination of the condensation products of $tRNA^{Phe}_{-YWye}$ with unlabeled amines lacking suitable optical properties for detection. The procedure, which is detailed in Methods, involves the introduction of a $^3$H-label by reduction of the condensation product with [$^3$H]$NaBH_4$[22].

According to the analytical procedure the reaction of $tRNA^{Phe}_{-YWye}$ with methylamine or aniline under conditions similar to those previously described[14] did not lead to condensation products which were sufficiently stable to reach the reduction

step (time for work-up approx. 30 min at pH 8 and room temperature). The reaction with aminopyrene led to a condensation product in moderate yield, which in this case was determined spectrophotometrically. However, the rather low stability against hydrolysis of the $tRNA^{Phe}$-aminopyrene compound (t1/2 90 min in Tris buffer, pH 7.5, 37°) precluded further experiments with this compound.

Apparently the use of primary amines for the incorporation into the positions of excised bases in tRNA is limited to amines such as proflavine, ethidium, and related compounds. The amino groups of these compounds exhibit rather low pK values; this may be important for the formation of condensation products which are stable at least at neutral pH.

Preparation and characterization of $tRNA^{Phe}$ hydrazine derivatives. Reactions of hydrazines or hydrazides with tRNAs from which single bases had been selectively removed or the 3'terminal adenosine of which had been oxidized with periodate have been described[26-29]. The stability of the hydrazones makes them attractive candidates for a semi-systematic variation of the substituent replacing YWye in $tRNA^{Phe}$. Under the conditions specified in Methods hydrazine incorporation was observed only with $tRNA^{Phe}_{-YWye}$ whereas $tRNA^{Phe}$ did not accept any dye (Fig. 1). The specificity of the reaction was established unambigously by the chromatographic analysis of the T1 RNAase digestion products of $tRNA^{Phe}_{Dnph}$ (Fig. 2)(abbreviations in ref. 2 and Table 1). Absorbance due to incorporated Dnph was found only in the dodekanucleotide from the anticodon region which after excision of YWye contained the ribosylic aldehyde group. The same result was obtained when $tRNA^{Phe}_{Hac}$ was analyzed (not shown).

In order to be able to detect and quantitatively determine the incorporation of hydrazines which cannot be measured spectrophotometrically the $[^3H]NaBH_4$ reduction procedure was adapted to hydrazines. The free ribosylic aldehyde group in $tRNA^{Phe}_{-YWye}$ is reduced by $NaBH_4$ very rapidly whereas the hydrazone is not reduced. The extent of hydrazone formation therefore can be monitored directly by the decreasing amount of $^3H$ incorporated into the C-1 of the ribose. Because of the difficulty in removing traces of radioactive impurities from tRNA by washing

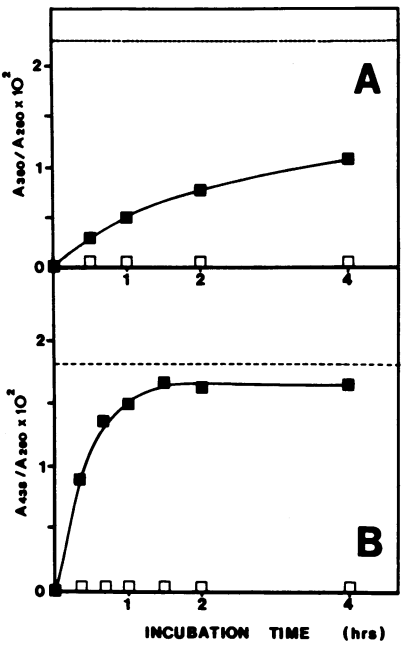


Figure 1. Reaction of Dnph (A) or Hac (B) with $tRNA^{Phe}_{-YWye}$. The reaction of the hydrazines with $tRNA^{Phe}_{-YWye}$ (closed symbols) or with $tRNA^{Phe}$ (open symbols) was performed as described in Methods. The dotted line indicates 100 % substitution as calculated on the basis of the extinction coefficients given in Table 1.

procedures the radioactivity was determined in the electrophoretically separated dodekanucleotide (Fig. 3). The precision of the method is rather high as shown by a comparison with the results of absorbance measurements (Fig. 4). The reduction procedure specifically monitors hydrazone formation at the YWye position; non-specific reactions of hydrazines with tRNA cannot be excluded. However, this possibility is considered very unlikely in view of the analytical results shown in Fig. 2.

A variety of hydrazine derivatives was incorporated into the YWye position of $tRNA^{Phe}_{-YWye}$ (Table 1). The $tRNA^{Phe}$-derivatives containing Hac, Hqn, Dnph, and Dmh, respectively, were isolated by reversed phase chromatography as shown by the two examples in Fig. 5.

Aminoacylation with phenylalanyl tRNA synthetase from Yeast or E. coli. In our standard aminoacylation assay with phenylalanyl tRNA synthetase from yeast[7] the $tRNA^{Phe}$-hydrazine compounds listed in Table 1 accepted 1.1-1.3 nmol Phe/$A_{260}$ unit. After RPC 5 chromatography the same extent of aminoacylation was obtained when the tRNAs had been isolated from the eluate by

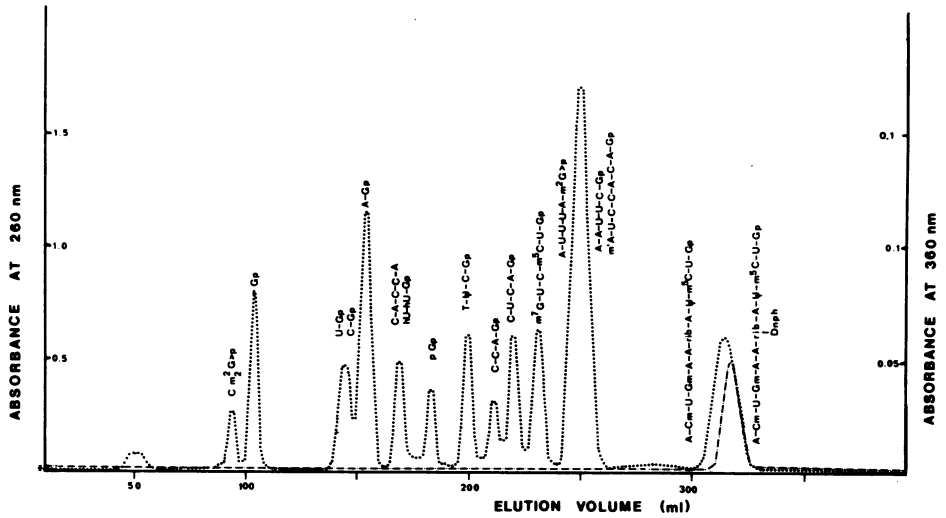


Figure 2. Oligonucleotide analysis of $tRNA^{Phe}_{Dnph}$. 40 $A_{260}$ units of a mixture of $tRNA^{Phe}_{Dnph}$ and $tRNA^{Phe}_{-YWye}$ were digested with T1 RNAase and chromatographed on a DE 52 column (0.3x50 cm) at 15 bar pressure and room temperature. Elution was performed with a linear gradient (500 ml) of 0.01 M to 0.35 M NaCl in 0.01 M Tris-HCl, pH 7.4, 7 M urea. The free ribose in the dodekanucleotide (-YWye) is designated rib. (...) $A_{260}$; (-.-.) $A_{360}$. The pattern of the T1 RNAase oligonucleotides of $tRNA^{Phe}$ chromatographed under similar conditions has been published[30].

ethanol precipitation followed by three reprecipitations. Other isolation procedures occasionally yielded less active tRNA preparations.

In the heterologous aminoacylation assay with phenylalanyl tRNA synthetase from E. coli K12 the various $tRNA^{Phe}$-hydrazine compounds exhibited very different activities. Fig. 6 shows the data obtained for the chromatographically pure compounds in comparison with the ones for $tRNA^{Phe}$, $tRNA^{Phe}_{-YWye}$, and $tRNA^{Phe}_{Prf}$ (prepared as in ref. 14). While the incorporation of Dmh did not alter the very low activity of $tRNA^{Phe}_{-YWye}$ the rate of aminoacylation increased from Hqn to Dnph, Hac, Prf, and finally YWye as substituents. The $tRNA^{Phe}$ derivatives in which YWye was substituted by Inh, Ptsc, Tsc, or Agd (Table 1) have been assayed without prior purification by column chromatography. After a one hour incubation under the same assay conditions the extent of aminoacylation was found intermediate between $tRNA^{Phe}_{-YWye}$ and $tRNA^{Phe}_{Hqn}$, i.e. 10-15 % of the aminoacylation level

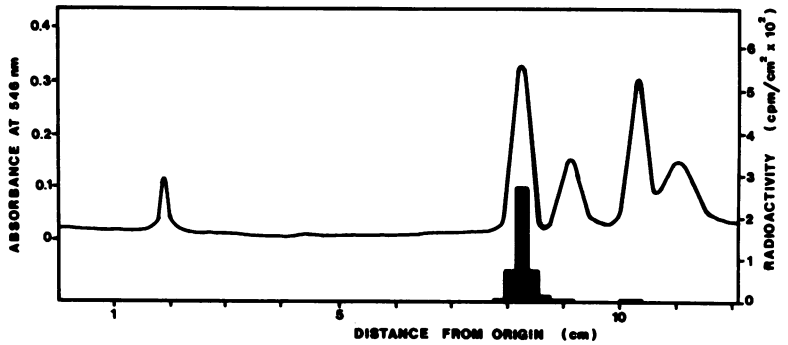


Figure 3. Densitogram of an electrophoretic separation of the T1 RNAase digestion products of [$^{3}$H]$NaBH_4$ reduced $tRNA^{Phe}_{-YWye}$.

obtained with yeast synthetase.

Poly(U) dependent binding to ribosomes from yeast and E. coli. The extent to which the $tRNA^{Phe}$-hydrazine compounds were bound non-enzymatically to poly(U) programmed yeast ribosomes was found to be dependent on the substituent at the YWye position (Fig. 7) in a qualitatively similar manner as in the heterologous aminoacylation assays. Again the Dmh-substituted $tRNA^{Phe}$ was as inactive as $tRNA^{Phe}_{-YWye}$ whereas the aromatic substituents led to intermediate or high binding efficiency. $tRNA^{Phe}_{Prf}$ would bind with an efficiency of 85 % in this assay (not shown). The $tRNA^{Phe}$-derivatives carrying Agd, Tsc, Ptsc, or Inh at the YWye position (Table 1) bound with efficiencies of approximately 30 %. The amounts of ribosomes used in these experiments were just sufficient to bind unmodified [$^{14}$C]Phe-$tRNA^{Phe}$ completely.

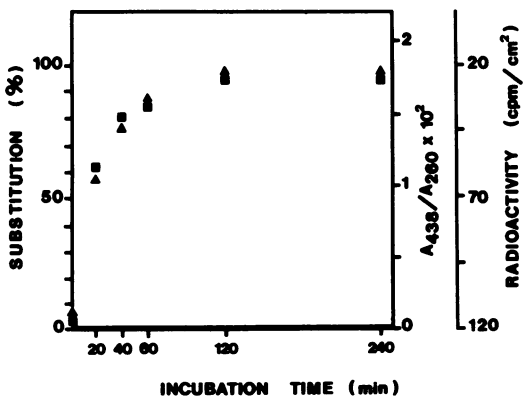


Figure 4. Incorporation of Hac into $tRNA^{Phe}_{-YWye}$. The reaction was monitored by measuring dye absorbance (■) or the specific radioactivity of the dodekanucleotide (▲) (Methods).

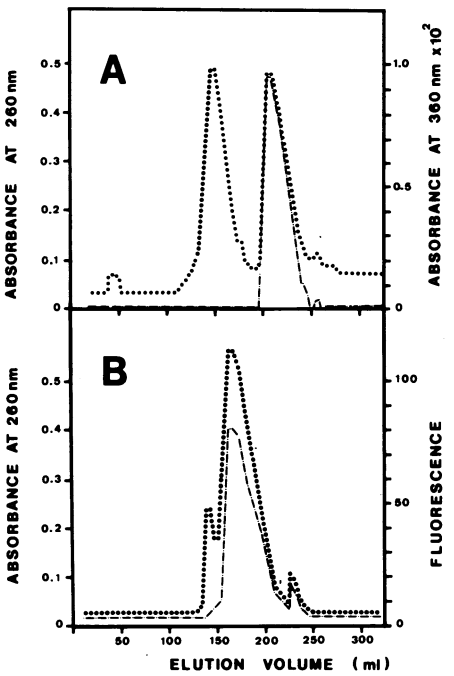


Figure 5. Chromatography of $tRNA^{Phe}_{Dnph}$ (A) and $tRNA^{Phe}_{Hac}$ (B) on RPC 5 columns. 100 $A_{260}$ units (A) or 50 $A_{260}$ units (B) of hydrazine reacted $tRNA^{Phe}$-YWye were chromatographed on RPC 5 columns[25] (0.3x50 cm) at 30-40 bar and 24°. Elution was performed with a linear gradient of 200 ml each of 0.35 M and 0.7 M NaCl in 0.01 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$. (....) $A_{260}$; (-.-.) $A_{360}$ (A) or fluorescence at 470 nm upon excitation at 435 nm (B).

Table 1. Incorporation of hydrazine derivatives into the YWye position of $tRNA^{Phe}$-YWye. The condensation reaction was performed as described in Methods. In some cases yields were determined by absorption measurements using the extinction coefficients for the bound dyes which were determined from the chromatographically pure $tRNA^{Phe}$ derivatives [$mM^{-1}$ $cm^{-1}$ ( max)]: Hac, 11.4 (438 nm); Hqn, 2.8 (325 nm); Dnph, 14.0 (360 nm). For all derivatives, except Dnph, yields were determined by the [$^3$H]$NaBH_4$ reduction procedure (Methods). The $tRNA^{Phe}$ derivatives with Inh, Ptsc, Tsc, and Agd were used directly in the assay systems of Figs. 6 and 7; the results with the former two derivatives have to be corrected for the amounts of $tRNA^{Phe}$-YWye in the preparations.

| Hydrazine Derivative | Yield of Condensation (%) |
|---|---|
| 9-Hydrazinoacridine (Hac) | 90 |
| 3-Hydrazinoquinoline (Hqn) | 95 |
| 2,4-Dinitrophenylhydrazine (Dnph) | 40 |
| Isonicotinic acid hydrazide (Inh) | 40 |
| 4-Phenyl-3-thiosemicarbazide (Ptsc) | 80 |
| Thiosemicarbazide (Tsc) | 100 |
| Aminoguanidine (Agd) | 100 |
| 1,1-Dimethylhydrazine (Dmh) | 100 |

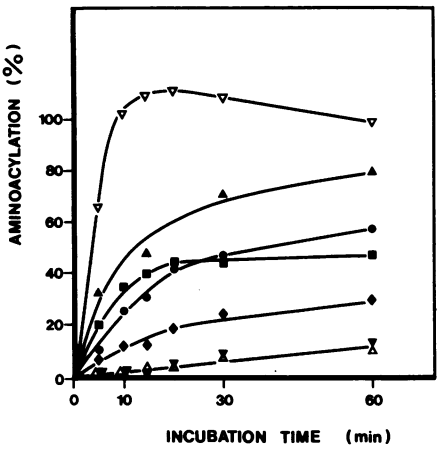


Figure 6. Aminoacylation with phenylalanyl tRNA synthetase from E. coli K12. The assay was performed in the presence of 1.2 M ammonium sulfate [10]. The aminoacylation levels are given relative to the maximal charging capacity of the respective tRNA derivative with yeast synthetase. Due to a higher efficiency of the heterologous assay the relative charging capacity of $tRNA^{Phe}$ was found slightly higher than 100 % in the present case. (▽) $tRNA^{Phe}$; (▲) Prf, (■) Hac, (●) Dnph, (◆) Hqn, or (▼) Dmh as substituents; (△) $tRNA^{Phe}$-YWye.

Lower plateau values therefore indicate a lower stability of the complex of modified [$^{14}$C]Phe-$tRNA^{Phe}$, ribosomes, and poly(U).

When the analogous assay was performed with ribosomes from E. coli MRE 600 qualitatively the same results were obtained

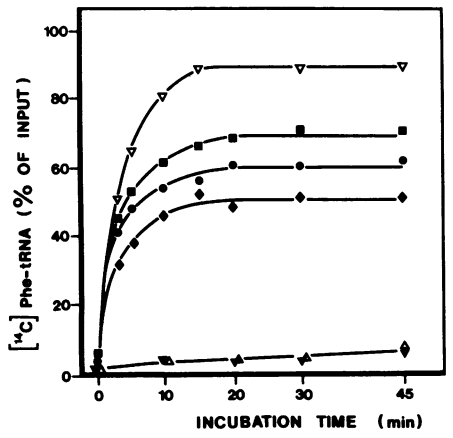


Figure 7. Poly(U) dependent binding to ribosomes from yeast. The previously described modification (procedure B in ref. 13) of the assay of Nirenberg and Leder[20] was followed. Approximately 70 pmoles of ribosomes per assay were used. Binding efficiencies are given relative to the input of [$^{14}$C]Phe-tRNA (10 pmoles). Blank values were determined without poly(U) and are subtracted. The symbols of Fig. 6 are used.

although the differences between the binding efficiencies of the various $tRNA^{Phe}$-derivatives were less pronounced (Table 2).

Table 2. Poly(U) dependent binding of [$^{14}$C]Phe-$tRNA^{Phe}$ derivatives to ribosomes from E. coli MRE 600. Chromatographically pure $tRNA^{Phe}$ derivatives have been used. Abbreviations as in ref. 2 and Table 1. The assay was performed according to ref.20. Blank values (5-7 % of the input) were determined without poly(U) and are subtracted throughout.

| Substituent at the YWye Position | Relative Amount of [$^{14}$C]Phe-tRNA Bound (% of Input) |
|---|---|
| YWye | 91 |
| none | 15 |
| Prf | 70 |
| Hac | 64 |
| Dnph | 65 |
| Hqn | 59 |
| Dmh | 19 |

## DISCUSSION

The present results strongly suggest an important function of YWye in the stabilization of the native conformation of yeast $tRNA^{Phe}$. This conclusion is drawn from the observation that the activity of the $tRNA^{Phe}$ in assays of the reactions of protein biosynthesis is strongly dependent on the nature of the substituent located at the YWye position. Ordering these substituents according to the extent to which they lead to an increase of the activity of the substituted $tRNA^{Phe}$ in comparison to $tRNA^{Phe}_{-YWye}$ one obtains the following sequence: none = Dmh $<$ Agd $\approx$ Tsc $<$ Ptsc $\approx$ Inh $<$ Hqn $<$ Dnph $<$ Hac $<$ Prf $<$ YWye. The lack of stimulation by Dmh shows that the hydrazone formation at the free ribosylic aldehyde group of $tRNA^{Phe}_{-YWye}$ itself has no effect. Clearly aromatic groups lead to higher activity than aliphatic ones, and there is a correlation between the size of the aromatic system and the extent to which the activity of the tRNA is stimulated. Accordingly the rather high activity of $tRNA^{Phe}_{Dnph}$ is probably due to the extension of the conjugated system of the phenyl ring by the two nitro groups. $tRNA^{Phe}_{Prf}$ exhibited the highest activity of all $tRNA^{Phe}$ deriva-

tives tested. In view of the strong stacking tendency of Prf this result suggests that stacking interactions of the base at the YWye position are of particular importance for its role in maintaining the native conformation of $tRNA^{Phe}$. Evidence for a stacked arrangement of the five purine bases of the anticodon loop of $tRNA^{Phe}$ has been obtained from the crystallographic analyses[31,32] as well as from physicochemical studies of the molecule in solution[10-12,33,34].

The present results suggest that YWye by its particular stacking properties plays an important role in preserving the functional conformation of the anticodon loop. There is evidence suggesting that the conformation of the anticodon loop [13,33,34] and of other parts of the tRNA molecule[13,35] changes upon binding of the cognate codon. It appears that YWye may be optimally designed to mediate a conformational switch of the anticodon loop by stabilizing alternative conformations.

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