TERMINAL OXIDATION-REDUCTION OF YEAST PHENYLALANINE tRNA PREVENTS DONOR AND ACCEPTOR FUNCTION AT THE PEPTIDYL TRANSFERASE CENTER

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SUMMARY: Periodate oxidation of the ribose of the 3'-terminal adenosine of yeast $tRNA^{Phe}$ followed by borohydride reduction has the net effect of splitting the C2'-C3' bond leaving two primary alcohol groups at these carbon atoms. This modified tRNA ($tRNA^{ox-red}$) could be acylated with phenylalanine but could not function as either a donor or acceptor at the peptidyl transferase center of the ribosome. Assays were performed with the phenylalanyl-pentanucleotides, $CACCA^{ox-red}$(acetylPhe) and $CACCA^{ox-red}$(Phe), which were isolated from the 3'-end of appropriately esterified $tRNA^{ox-red}$. Ado^{ox-red}(Phe) isolated from Phe-$tRNA^{ox-red}$ was also inactive as an acceptor, but synthetic Ado^{ox-red}(Phe), a mixture of the 2' and 3' phenylalanyl esters, was active with an apparent K_m of 1.16 mM compared to 0.2 mM for control Ado(Phe). These results are interpreted to mean that (1) biosynthetic aminoacylation of $tRNA^{ox-red}$ occurs specifically at the 2'-hydroxyl, (2) there is no 2':3' tautomerization in the ring-opened structure, and (3) peptidyl transferase recognizes specifically the 3'-aminoacyl esters of tRNA.

INTRODUCTION: Terminal oxidation-reduction of yeast $tRNA^{Phe}$ which results in cleavage of the 2', 3' carbon-carbon bond of the 3' terminal adenosine residue has little effect on the ability of yeast Phe-tRNA synthetase to attach phenylalanine to the modified tRNA (1). Although the V_{max} is decreased to one-half, the K_m is unaffected. Several other tRNA species can also be acylated following this cleavage reaction but this is not true for all species (2,3). On the other hand, we previously observed that Phe-$tRNA^{ox-red}$[1] was unable to form a ternary complex with the EFTu-GTP complex of E. coli and could not be bound to the ribosomal A site in an EFTu-factor mediated reaction, although it could bind to the ribosomal P site at somewhat higher Mg^{++} concentrations than control Phe-tRNA (2). Other reactions in which $tRNA^{ox-red}$ has been shown to be poorly active are pyrophosphorolysis by tRNA-nucleotidyl transferase and hydrolysis by venom phosphodiesterase (4).

In order to examine further the capabilities of oxidized-reduced tRNA in tRNA-dependent reactions of protein synthesis, we studied the function of this

[1] Phe-$tRNA^{ox-red}$, $tRNA^{Phe}$ which has been oxidized with periodate, reduced with borohydride and then acylated with phenylalanine.

modified molecule as both a donor and an acceptor at the peptidyl transferase center of the ribosome. In this report we show that the 3'-terminal fragments of Phe-tRNA, CACCA(Phe) and Ado(Phe), are inactive when isolated from Phe-$tRNA^{ox-red}$, although they are active when obtained from control Phe-tRNA. In contrast to this result, Ado^{ox-red}(Phe) synthesized chemically showed good activity as an acceptor. These experiments, and a rationale to explain these apparently contradictory findings, are presented in this communication.

EXPERIMENTAL: Phe-pentanucleotides - CACCA(Phe), $CACCA^{ox-red}$(Phe), and their N-acetylated derivatives were prepared as follows. Brewer's yeast $tRNA^{Phe}$ (1200 pmoles per A_{260}) was purchased from Boehringer. Oxidation-reduction of the terminal adenosine residue was carried out as previously described (2). Oxidation completely inactivated charging activity, and reduction under the above described conditions restored 83% of the control activity. The control tRNA was prepared as described (2) except that the phenylalanine residue was removed by treatment with 0.4 M Tris, 0.5 M KCl, pH 9.0, at 37° C for 30 min before $NaBH_4$ reduction so that the control tRNA would be as much like the oxidized one as possible. The control tRNA could be recharged to 88% of the original value. N-acetylation of both control and oxidized-reduced Phe-tRNA was carried out as described previously (5). Control experiments with [^{3}H]-acetic anhydride showed that no stable acetylation of the available primary hydroxyl at the 3' terminus of (Phe)-$tRNA^{ox-red}$ occurred under these conditions. Pentanucleotide fragments bearing the phenylalanyl residue were prepared from the four tRNA derivatives by complete digestion with T_1 RNase followed by chromatographic purification according to Pestka *et al* (6). They were characterized by their electrophoretic mobility (6).

Phenylalanyl-adenosines - Ado(Phe) and Ado^{ox-red}(Phe) were obtained from their respective Phe-tRNAs by complete hydrolysis with pancreatic RNase followed by chromographic purification on DEAE-Sephadex (6) and CM-cellulose (7). They were characterized by thin-layer chromatography as previously

described (2). Synthetic Ado(Phe) and Ado^{ox-red}(Phe) were prepared from 5'-trityl-Ado (Schwarz-Mann) and 5'-trityl-Ado^{ox-red} (prepared by periodate oxidation and $NaBH_4$ reduction of 5'-trityl-Ado) by condensation with tBOC-phenylalanine (Schwarz-Mann) in the presence of DCC (8). The protecting groups were removed by treatment with anhydrous trifluoroacetic acid followed by ether extraction (8). Although not analyzed, the product is probably a mixture of the 3' and 2' isomers of phenylalanyl-adenosine. Details of the preparation and characterization of these and the above pentanucleotide fragments will be published elsewhere.

Other methods - 70S ribosomes and polysomes were prepared as described (5) except that washing of the polysome preparation was omitted. Activation of 70S particles was carried out by the procedure of Miskin et al (9).

RESULTS: Donor-site activity - Earlier experiments had shown that Phe-tRNA^{ox-red} could bind to the ribosomal P site, although with a lower affinity, as judged by the increased Mg^{++} requirement (2). Despite this, peptide bond formation does not occur with oxidized-reduced Phe-tRNA (10). In order to test this effect more rigorously, we used CACCA (AcPhe) and CACCA^{ox-red}(AcPhe) (obtained by hydrolysis of their respective AcPhe-tRNAs) in the "fragment reaction" described by Monro and Marcker (11). This assay is thought to be the most direct way of investigating the catalytic center of peptidyl transferase itself, uncomplicated by any side-effects of ribosome binding sites (11,12). Fig. 1 shows that in confirmation of our earlier results, oxidation-reduction completely inactivated the tRNA fragment for reaction as a peptidyl donor using puromycin as an acceptor. Since only 5 nucleotides are involved, the effect cannot be due to any alteration at some distant point in the molecule, nor is it likely to be due to some distortion of tertiary structure due to opening of the terminal ribose ring. Note that the control fragment (described in Experimental) has been exposed to the same chemical procedures.

Acceptor-Site Activity: A similar experiment was performed to test for activity as an acceptor by using unacetylated CACCA(Phe) and N-acetyl-Phe-tRNA

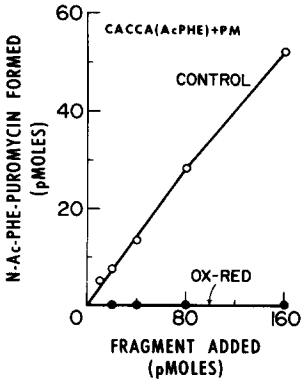

Fig. 1.

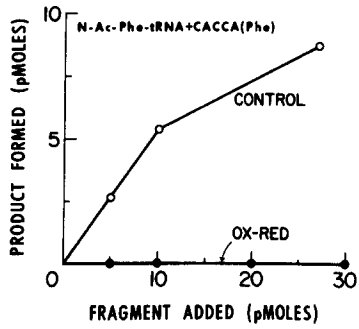

Fig. 2.

Fig. 1. Formation of N-acetyl-Phe-puromycin from oxidized-reduced N-acetyl-Phe-pentanucleotide and puromycin. 0.05 ml reaction mixtures contained (prior to methanol addition), 0.06 M Tris-HCl pH 7.4, 0.4 M KCl, 0.02 M Mg $(OAc)_2$, 5 A_{260} of activated 70S ribosomes (9) and 1 mM puromycin. The [^{14}C]Phe-containing donor fragments at the concentrations indicated were added last and the reaction initiated by adding 25 µl of methanol. After incubation at 35° C for 40 min, 5 µl of 10 N NaOH was added and the mixture heated for 5 min at 37° C (9). 0.5 ml of 1.0 M potassium phosphate pH 7.0, was then added and the mixture extracted with 2 ml of ethyl acetate; 2 ml of the upper ethyl acetate phase was mixed with 15 ml of Bray's solution and counted. Results were corrected back to 3 ml of ethyl acetate extract, and the blank (puromycin omitted) was subtracted for each donor concentration. ○, CACCA (Ac[^{14}C]Phe); ●, CACCA^{ox-red} (Ac[^{14}C]Phe).

Fig. 2. Formation of N-acetyl-Phe-Phe- from N-acetyl-Phe-tRNA and oxidized-reduced CACCA(Phe). The assay takes advantage of the fact that both CACCA([^{14}C]Phe) (ref. 6) and the product CACCA(Ac[^{3}H]Phe[^{14}C]Phe) are soluble in cold 5% TCA. This was verified by the failure to find any TCA-precipitable [^{14}C]Phe radioactivity at the end of the reaction. The extent of product formation can thus be measured by the decrease in TCA-precipitable N-acetyl-[^{3}H]Phe-tRNA radioactivity after incubation with CACCA([^{14}C]Phe).

50 µl of reaction mixture contained (before methanol addition), 0.006 M Tris-HCl, pH 7.4, 0.4 M KCl, 0.02 M $Mg(OAc)_2$, 4.0 A_{260} of activated 70S ribosomes, and 40 pmole of N-acetyl[^{3}H]Phe-tRNA. Acceptor fragments at the indicated concentrations were added last and the reaction initiated by the addition of 25 µl of methanol. After incubation at 35° C for 15 min, 3 ml of cold 5% TCA was added to stop the reaction and the mixture kept at 0° C for 10 min. The precipitated RNA was collected on a Millipore filter, washed 3 times with 3 ml portions of cold 5% TCA, dissolved in 10 ml of Bray's solution (13) and counted. The amount of peptide synthesized was determined as the difference between precipitable [^{3}H] radioactivity after incubation with and without added acceptor. ○, CACCA (Phe); ●, CACCA^{ox-red}(Phe).

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as the donor. The results (Fig. 2) show equally clearly that the oxidized-reduced compound was inactive.

SUMMARY OF KINETIC CONSTANTS OF ACCEPTOR SUBSTRATES FOR PEPTIDE BOND FORMATION ON RIBOSOMES AND POLYSOMES

Acceptor Substrate	Ribosome Assay[a]		Polysome Assay[b]
	K_m	V_{max}	K_m
	μM	pmole/min/A_{260}	μM
Puromycin	170	4.5	3.0
Ado(Phe)-[synthetic]	200	5.3	1.9[c]
Ado^{ox-red}(Phe)-[synthetic]	1160	5.0	37[c]

[a] Kinetic constants for the ribosome assays were obtained by double reciprocal plots of the rate data shown in Fig. 4. The constants for the polysome assay were obtained in other experiments (10).

[b] The polysome assay was as described in the legend to Fig. 3 except that initial rates were measured.

[c] In these two cases, the synthetic substrates were not of a sufficiently high radioactivity to allow direct measurement of peptide bond formation with unlabelled peptidyl-tRNA on the polysomes. The indicated values were therefore obtained by Dixon (17) plots of the competitive inhibition by the indicated substrates of the rate of peptidyl-[^{3}H]puromycin formation (10).

In order to simplify the chemical nature of the acceptor further, we wished to test the activity of Ado^{ox-red}(Phe) derived from $Phe\text{-}tRNA^{ox-red}$. However, because of the limited amounts of biologically synthesized material available and the high K_m expected for such substrates (14) (see also Table I) it was not possible to do so in the same system as used above. Consequently, for this assay we used the polysome system recently shown by Pestka (15) to have a 75-fold lower apparent K_m for acceptor substrates of this type. In this system also (Fig. 3) Ado^{ox-red}(Phe) was totally inactive, both at 35° C and at 25° C.

Acceptor Site Activity of Chemically Synthesized Substrates: At the same time as the polysome assay was being developed, the chemical synthesis of Ado^{ox-red}(Phe) was initiated, in order to produce enough material for testing with washed ribosomes using N-Ac-Phe-tRNA as donor. Although the chemically synthesized product was expected to behave similarly to the biosynthetic

material tested in Fig. 3, it surprisingly showed good activity in the 70S ribosome assay (Fig. 4). The initial velocity of peptide bond formation as a function of acceptor concentration is shown in this figure for chemically synthesized Ado^{ox-red}(Phe), its chemically synthesized control, and for puromycin

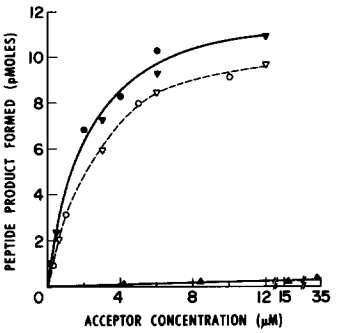

Fig. 3.

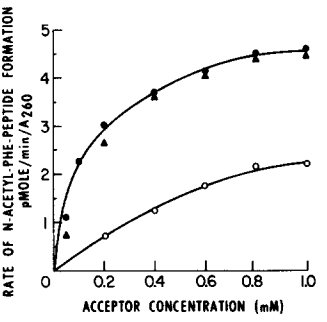

Fig. 4.

Fig. 3. Extent of peptide bond formation between peptidyl-tRNA on polysomes and Ado^{ox-red}(Phe) isolated from Phe-tRNA^{ox-red}. Each 50 μl reaction mixture contained 50 mM Tris-acetate, pH 7.2; 10 mM NH_4Cl, 100 mM KCl, 5 mM $MgCl_2$, 2.8 A_{260} of unwashed polysomes and acceptor substrates as specified in the Figure. The reaction mixture was prewarmed for 1 min at the incubation temperature and the acceptors were added last to start the reaction. After incubation for 5 min at the desired temperature, the reaction was stopped by addition of 3 ml of cold 10% TCA. After 10 min at 0° C, the mixtures were filtered through a BDWP-Millipore filter and washed with 5% TCA and with absolute ethanol as described by Pestka (15) After drying, the radioactivity was counted under 10 ml of toluene scintillation fluid. ○, ● ; [^{3}H]Puromycin; △, ▲ ; Ado^{ox-red}([^{3}H]Phe), derived from [^{3}H]Phe-tRNA^{ox-red}; ▽, ▼ ; Ado ([^{14}C]Phe), derived from [^{14}C]Phe-tRNA; solid symbols, solid line, reaction at 35° C; open symbols, dashed line, reaction at 25° C.

Fig. 4. Rate of formation of N-acetyl-Phe peptide from N-acetyl-Phe-tRNA and synthetic Ado^{ox-red}(Phe) on washed ribosomes. Each 0.05 ml reaction mixture contained 0.05 M Tris-acetate pH 7.4, 0.1 M NH_4Cl, 0.08 M KCl, 0.01 M DTT, 0.02 M ($MG(OAc)_2$, 2 A_{260} units of activated ribosomes (9), 0.1 mg/ml Poly U, and 50 pmoles of N-acetyl-[^{3}H]Phe-tRNA. Reaction was started by adding acceptor substrates at concentrations shown in the figure. The mixture was incubated at 35° C for the times indicated below. Reaction was terminated by the addition of 0.05 ml saturated $MgSO_4$ in 0.2 M potassium acetate pH 5.0 (16) and the mixture extracted with 3 ml of ethyl acetate. Two ml of the upper ethyl acetate phase was mixed with 15 ml of Bray's solution and the radioactivity counted. The radioactivity of a blank incubation in absence of acceptor was subtracted. After correction for sample size, the results were expressed as pmole product formed per minute per A_{260} of ribosomes. ● , Puromycin, incubation time was 1.5 min; reaction was linear up to 2 min at 600 μM acceptor. ▲ , Ado(Phe), incubation time was 3 min; reaction was linear up to 4 min at 200 μM acceptor. ○ , Ado^{ox-red}(Phe), incubation time was 3 min; reaction was linear up to 6 min at 600 μM acceptor.

as a reference standard. It is clear that while oxidation-reduction has reduced the affinity of Ado(Phe) for peptidyl transferase, strong activity is still present, in marked contrast to the complete inactivity of biosynthetic Ado^{ox-red}(Phe). K_m values were calculated from the double reciprocal plots of this data, and are tabulated in Table I. Note that while the V_{max} is not affected, the K_m has been increased 6 times by the oxidation-reduction cleavage.

In view of this difference between chemically synthesized and biosynthetic acceptors, the chemically prepared Ado^{ox-red}(Phe) was also tested in the polysome system. Unfortunately, the specific radioactivity of the available Ado^{ox-red}(Phe) was not sufficient to allow direct determination of the K_m values in this system. Since the donor peptidyl-tRNA is unlabelled, the acceptor must be labelled in order to obtain a measurable product. Instead, the K_m was determined indirectly by a Dixon (17) plot of the competitive inhibition of Ado^{ox-red}(Phe) and Ado(Phe) on the rate of peptidyl-[^{3}H]puromycin formation. These data (10) are summarized also in Table I. Since by this method the control Ado(Phe) gave a K_m value (1.9 μM) which is close to that of puromycin (3.0 μM) and similar to the directly determined value for Ado(Tyr) of 4.5 μM (5), the technique seemed valid. In this system also, chemically prepared Ado^{ox-red}(Phe) was active, although the decrease in K_m was somewhat larger than was seen in the 70S ribosome assay system.

DISCUSSION: The findings reported here show clearly that cleavage of the C2'-C3' bond of the 3'-terminal ribose renders the aminoacyl tRNA (or fragment) totally inactive as either a donor or acceptor at the peptidyl transferase catalytic center of the ribosome. Several hypotheses could be advanced as explanation for this effect. For example, the ribose structure is destroyed by this cleavage, and it has been shown (4) that the original planar structure becomes distorted by rotation about the C1'-O-C4' acetal linkage. The adenine base probably also occupies a different spatial position with respect to the rest of the structure, and other effects could no doubt be proposed. However, the fact that chemically synthesized Ado^{ox-red}(Phe) was active rules out all

such hypotheses which are consequential to opening of the ribose ring.

We propose that the sole difference, and explanation, for the contradictory results obtained with the chemically synthesized and biosynthetic Ado^{ox-red}(Phe), lies in the fact that the chemically synthesized product is almost certainly a mixture of the 3' and 2' isomers, while the biosynthetic product is probably a single isomer. We must further assume that cleavage of the C3'-C2' bond eliminates the tautomerization of the phenylalanyl residue between the 3' and 2' hydroxyls which is known to be extremely rapid in aminoacyl adenosines and similar compounds (18). This point follows automatically since the biosynthetic product was completely inactive and has never shown any sign of a slow conversion to an active form even when used at very high concentrations.

In view of the fact that puromycin, a 3' derivative, is a functional acceptor, and the claim that the 2'-isomer of puromycin is not (19), we suggest that the same is true for the Ado^{ox-red}(Phe) compounds. Therefore, it follows that the product synthesized by Phe-tRNA synthetase must be the 2'-isomer of Phe-tRNA^{ox-red}. Consequently, if the EFTu-GTP complex and peptidyl transferase were 3'-isomer specific, this hypothesis would neatly explain the unreactivity of Phe-tRNA^{ox-red} in these two unrelated enzyme systems.

One is tempted to predict further that the esterification of the 2'-hydroxyl of tRNA^{ox-red} is not an abberation of this particular synthetase due to the structural modification of its substrate tRNA, but that this position is the primary site of esterification for all AA-tRNA synthetases. The 2'-hydroxyl group is the more reactive one from a chemical standpoint (20), and further support for this view comes from the fact that the K_m for esterification of the "wrong" hydroxyl is the same as for the normal reaction (1). Normally, at physiological pH, tautomerization would almost instantaneously produce a mixture of 3' and 2' isomers even if the original biosynthetic process were 2'-specific, so that specificity for the 3'-isomer by the other enzymes involved in protein synthesis would not be a problem. We are currently engaged in

chemical synthesis of isomer-specific Ado^{ox-red}(Phe) and CCA^{ox-red}(Phe) in order to test some of the foregoing predictions.

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