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Interactions of transfer RNA pseudouridine synthases with RNAs substituted with fluorouracil

Tore Samuelsson
Department of Medical Biochemistry, University of Göteborg, PO Box 33031, S-400 33 Göteborg, Sweden

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ABSTRACT

We have previously purified and characterized two different *S. cerevisiae* enzymes that produce pseudouridine specifically in nucleotide positions 13 and 55, respectively, in their tRNA substrates. The interactions of these enzymes with fluorinated tRNAs have now been studied. Such RNAs were produced by *in vitro* transcription using as templates synthetic genes that encode variants of a yeast glycine tRNA. RNAs substituted with fluorouracil were found to markedly inhibit pseudouridine synthase activity and the inhibitory effect of a tRNA was to a large extent dependent on the presence of fluorouracil in the nucleotide position where normally pseudouridylation occurs. Pseudouridine synthases were shown to form highly stable, non-covalent complexes with fluorinated tRNAs and we demonstrate that this interaction may be used to further characterize and purify these enzymes. The use of 5-fluorouracil as a cancer therapeutic agent is discussed in relation to our results.

INTRODUCTION

Many different fluorinated substrate analogs are known to interfere with enzymatic reactions (1,2). Some of these compounds inactivate enzymes irreversibly and they have also been thoroughly studied because of their pharmacological importance. One such example is 5-fluorouracil (FU) which has been widely used as an anticancer agent (3,4). In this compound the fluorine atom replaces hydrogen at carbon 5 and the C-F bond thus formed is highly stable.

Fluorouracil has also been used to study the catalytic mechanism of different enzymes that act on uracil. One such enzyme is thymidylate synthase which catalyzes the formation of thymidylate from deoxyuridylate (dUMP). The mechanism of this reaction involves the formation of intermediate complexes in which carbon 6 of dUMP is linked to a cysteine residue in the enzyme (5). In the presence of the 5-fluoroderivative of dUMP (FdUMP) and the cofactor 5,10-methylenetetrahydrofolate, the enzyme is inactivated by the formation of a highly stable covalent adduct composed of enzyme, FdUMP and cofactor (5). The enzyme tRNA (m^5U)methyltransferase is mechanistically related to thymidylate synthase as it also catalyzes a one-carbon transfer to carbon 5 of a uracil moiety. A tRNA substituted with fluorouracil (FUtRNA) is able to inactivate the enzyme in the presence of the methyl donor adenosylmethionine and a covalent dead-end complex is formed consisting of enzyme, FUtRNA, and the methyl group (6).

Pseudouridine synthases catalyze a reaction which involves the formation of a position isomer where the C5 instead of N1 of the uracil ring becomes attached to the ribose. In the course of this reaction the hydrogen originally present on C5 is released as a proton (7). The reaction is similar to that of thymidylate synthase and tRNA methyltransferases since it involves a reaction at C5 of uracil. The introduction of a fluorine atom at C5 would be expected to interfere with the reaction and previous studies suggest that fluorinated tRNAs effectively inhibit pseudouridine synthase activity (8,9).

We have recently described the identification and purification of yeast tRNA pseudouridine synthases (10). Making use of tRNAs produced by T7 RNA polymerase transcription *in vitro* it was possible to monitor three different activities that correspond to uridine in positions 13, 32 and 55, respectively, in the tRNA substrate. These enzyme activities were purified from a yeast extract and found to be chromatographically separable, indicating that there is one distinct enzyme for each of these positions.

In the present paper we have examined the interactions of fluorinated tRNAs with yeast pseudouridine synthases. We argued that if FUtRNAs form stable complexes with these enzymes such RNAs could be effective tools for studies of pseudouridine synthases. Thus, they could be used for identification as well as for affinity purification of enzymes responsible for pseudouridine synthesis. Furthermore, such tRNAs could be used to study the catalytic mechanism. Thus, we wanted to know whether it was possible to isolate a covalent complex composed of enzyme and FUtRNA in a similar manner as described for thymidylate synthase and tRNA-methyltransferase. Finally, 5-fluorouracil is used as an anticancer drug and although the biochemistry and pharmacology of this compound has been extensively studied (3,4) its effect on pseudouridylation when it is present in RNA has not been studied in detail so far.

MATERIALS AND METHODS

Materials

5-fluorouridine 5′ triphosphate (FUTP) was purchased from Sierra Bioresearch (Tucson, AZ) or produced by phosphorylating 5-fluorouridine 5′ monophosphate (Calbiochem) with nucleoside monophosphate kinase and nucleoside diphosphate kinase (both

enzymes from Boehringer). 5-bromouridine triphosphate (BrUTP), ribonuclease T2 and Micrococcus nuclease were obtained from Sigma. Agarose adipic acid hydrazide was purchased from Pharmacia LKB Biotechnology.

Pseudouridine synthases

Pseudouridine synthases were partially purified from *S. cerevisiae* using DEAE-Sepharose and Heparin-Sepharose as described previously (10). The preparations of PS 13 and PS 55 were not significantly cross-contaminated with each other.

Transcription *in vitro* and analysis of products

Transcription *in vitro* was carried out as described (10) using, instead of UTP, 0.5mM FUTP or, for the production of RNA containing 5-bromouracil, 0.5mM BrUTP. The template DNAs used were pYGLY-3, -4, -5, and -6 (10) and the corresponding fluorinated tRNAs were denoted FUtRNA3, 4, 5 and 6, respectively. T7 RNA polymerase was purified as described from the overproducing strain HMS12/pGP1-5/pGP1-1, which was generously provided by Dr. S. Tabor (11). For the synthesis of ^{32}P-labeled RNA 12.5 μM cytidine 5'-[α-^{32}P]triphosphate at a radioactive concentration of 0.4 μCi/μl was used. In order to verify that 5-fluorouracil was effectively incorporated into RNA the tRNA was produced as described above and labeled using cytidine 5'-[α-^{32}P]triphosphate and after hydrolysis with ribonuclease T2 the nucleotides were separated with thin layer chromatography on cellulose using in the first dimension isobutyric acid: 0.5 M NH_4OH (5:3) and in the second dimension isopropanol:HCl:H_2O (75:15:15) (12). The same TLC system was used to test whether pseudouridine or any other products were produced when the FUtRNA-6 was incubated with PS-55.

Inhibition of pseudouridine synthases by FUtRNAs

Enzyme was incubated in 50 mM TrisHCl pH 7.5, 10 mM $MgCl_2$, 20 mM DTT, BSA 0.1 mg/ml with ^{3}H-labeled RNA (0.64 nM RNA-4 or 0.93 nM RNA-6) in a final volume of 100 μl. Incubation was for 20 min at 30°C. The amount of tritium released was measured as previously described (7,10).

Micrococcal nuclease digestion

The enzyme PS55 was incubated with 2.5 μM FUtRNA-6, micrococcus nuclease (5 U/μl) and 1 mM $CaCl_2$ in the incubation buffer used for the enzyme assay. The final volume was 100 μl and incubation was for 10 min at 37°C. Aliquots were monitored for enzyme activity after the addition of EGTA to a final concentration of 10 mM.

Complexes between FUtRNA and pseudouridine synthase

The enzyme PS 13 was added to a mixture containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.2 mg BSA/ml, 0.1 mM CTP, 6 nN RNA-3 (10), 2.5 μM FUtRNA-6 and 30 nM ^{32}P-labeled FUtRNA-4 in a total volume of 10 μl. In the case of PS 55 the incubation mixture contained 30 nM ^{32}P-labeled FUtRNA-6 and non-radioactive 2.5 μM FUtRNA-4. Similar conditions were used for experiments where tRNAs containing bromouracil or normal uracil were used instead of FUtRNAs. The mixtures were briefly heated to 37°C. Glycerol was then added to a concentration of 5% and the samples were subjected to electrophoresis in a 2% agarose gel at +2°C using as running buffer 40 mM Tris-acetate pH 8.0. UV-crosslinking was carried out with a 300 nm UV lamp (Fotodyne model 300-1, New Berlin, WI, U.S.A.) and the irradiated samples were then analyzed by SDS-PAGE (13).

Chromatography of pseudouridine synthases on immobilized FUtRNA

FUtRNA, 0.5 mg, was produced by *in vitro* transcription as described above. It was coupled to 1.5 ml agarose adipic acid hydrazide as previously described (14,15). The protein sample (partially purified pseudouridine synthase) was mixed with the resin (1 ml) by stirring overnight and the column was then washed with a buffer containing 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 3 mM DTT, 1 mM EDTA, 5% glycerol and 50 mM NaCl. Elution was carried out with the same buffer using a gradient of 15 ml from 50 mM NaCl to 1 M NaCl.

RESULTS

Synthesis of tRNAs substituted with 5-fluorouracil

We have previously studied yeast enzymes that produce pseudouridine in transfer RNA. To measure the activity of these enzymes we have made use of specific substrate RNAs (10) which were all derived from an RNA denoted RNA-1. This substrate is an *in vitro* transcript which corresponds exactly to the major glycine tRNA of *S. cerevisae* and it has uridine in four different positions that can all be modified to pseudouridine by a yeast extract. To produce this RNA we made use of a template DNA which has the T7 RNA polymerase promotor immediately upstream of the tRNA gene and a *Bst*NI restriction site is located a the 3' end of the tRNA gene. The transcript was obtained by cleavage of the DNA with *Bst*NI followed by T7 RNA polymerase transcription *in vitro* (10,16,17). We also produced

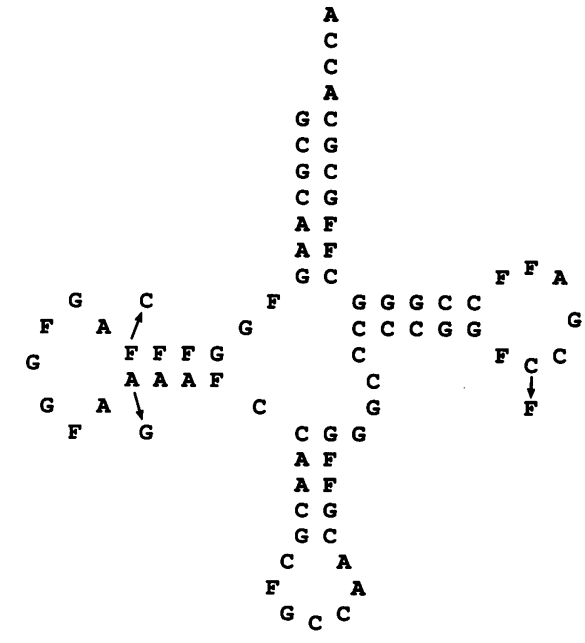

Fig. 1. Cloverleaf structure of fluorinated tRNAs obtained by in vitro transcription. The letter F indicates 5-fluorouridine nucleotides. The primary structure of the tRNA specifically interacting with pseudouridine synthase 13 (PS 13) is shown and the structure of FUtRNA-6, specific for PS 55, has the changes as indicated by arrows. As controls we also made use of FUtRNA-3 (featuring nucleotides C13, G22, C32, A38 and C55) and FUtRNA-5 (with nucleotides C13, G22, F32, A38 and C55).

variants of RNA-1. In RNA-4, -5 and -6 uridines have been eliminated so that these RNAs contain pseudouridylation sites only in nucleotide positions 13, 32 and 55, respectively.

In the present investigation we studied the interactions of pseudouridine synthases specific for the tRNA nucleotide positions 13 and 55, respectively, with fluorinated RNAs. Fluorouracil is readily incorporated into RNA *in vivo* (4) as well as *in vitro* using T7 RNA polymerase (18–20). To produce tRNAs substituted with 5-fluorouracil we used the same template DNAs as described above (10) with 5-fluorouridine triphosphate as one of the substrates. The RNAs obtained by transcribing pYGLY-4 and pYGLY-6, for instance, were denoted FUtRNA-4 and FUtRNA-6, respectively. The cloverleaf structure of these two fluorinated RNAs are indicated in Fig. 1. As controls we also made use of FUtRNA-3 (featuring nucleotides C13, G22, C32, A38 and C55) and FUtRNA-5 (with nucleotides C13, G22, F32, A38 and C55). The efficient introduction of 5-fluorouracil into RNA was confirmed by TLC analysis of a ribonuclease T2 hydrolysate of RNA labeled using $\alpha[^{32}P]$CTP (data not shown).

FUtRNA is not a substrate for pseudouridine synthase

To determine whether FUtRNA-6 could be a substrate for pseudouridine synthase-55 this RNA, labeled using $\alpha[^{32}P]$CTP, was incubated with enzyme under standard assay conditions. The tRNA was then hydrolyzed with ribonuclease T2 and the resulting nucleotide mixture was analyzed with TLC. In the RNA6 substrate the uridine-55 is followed by a cytidine and hydrolysis with ribonuclease T2 will transfer the radioactive phosphate from C-56 to U-55. No radioactive compounds other than those originally present in the FUtRNA could be detected, indicating that no covalent bonds (such as the glycosidic bond between ribose and the uracil ring) were broken in the FUtRNA as it interacted with the enzyme. Although we cannot exclude that some reaction took place which could not be detected by the particular method used it seems reasonable to assume that the fluorouridine moiety, just as the rest of the tRNA molecule, is unaffected by the pseudouridine synthase.

Inhibition of pseudouridine synthase activity by FUtRNA

In the course of the reaction catalyzed by pseudouridine synthases the hydrogen at C5 of uracil is released as a proton (7). The release of radioactivity from a tritium-labeled substrate RNA can therefore be used as a measure of enzyme activity. We examined the effect of FUtRNAs on tritium release activity and the results are shown in Fig. 2. In the case of PS13 it is most effectively inhibited by FUtRNA-4, i.e. the RNA with 5-fluorouracil in position 13, the position normally modified by the enzyme (Fig 2, upper panel). Thus, a reduction of tritium release to 50% is observed at 0.3 nM FUtRNA-4 whereas the same amount of inhibition with FUtRNA-6 is noted at a much higher concentration, 10 nM. In the same vein, PS 55 is inhibited most effectively by FUtRNA-6 (Fig. 2, lower panel). These results strongly indicate that both FUtRNAs interact in a highly specific manner with the corresponding enzymes, PS 13 and PS 55.

In order to test whether the enzymes were irreversibly inactivated by FUtRNAs (for instance by the formation of a dead-end covalent complex between RNA and enzyme), PS 13 and PS 55 were incubated together with FUtRNA-4 and FUtRNA-6, respectively. The incubation mixture was then treated with micrococcal nuclease and after inactivation of the nuclease with EGTA the tritium release activity was determined. The results (not shown) demonstrate that all of the enzyme activity was restored after the removal of FUtRNA, indicating that the enzymes were not irreversibly inactivated by FUtRNA. Therefore, it seems unlikely that a stable covalent product was formed between enzyme and some portion of the RNA. This conclusion is further supported by the fact that no such complexes were detected by SDS-PAGE analysis of enzyme that had been incubated with radioactively labeled RNA (see results below on UV-crosslinking).

Stable complex between FUtRNA and pseudouridine synthase

The fact that FUtRNAs were inhibitory at very low concentrations together with the fact that the inhibition was reversible suggested that stable and non-covalent complexes were formed between RNA and enzyme. In an attempt to identify such complexes the enzymes PS 13 and PS 55 were incubated with ^{32}P-labeled FUtRNAs in the presence of non-radioactive non-specific FUtRNA. The incubation mixtures were subsequently subjected to agarose gel electrophoresis followed by blotting onto DEAE

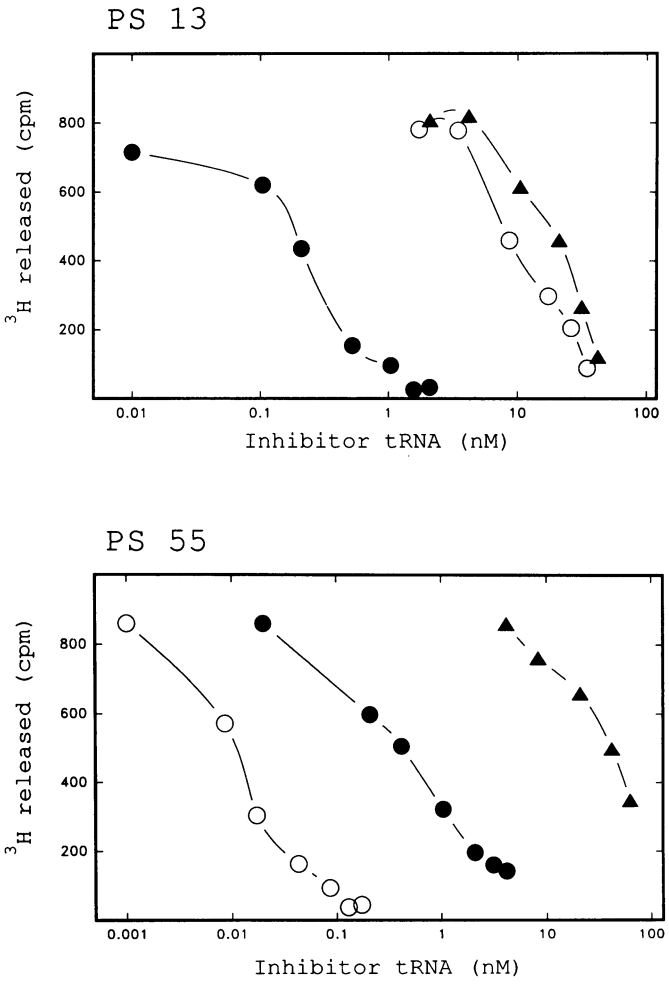

Fig. 2. Inhibition by fluorinated tRNAs of tritium release catalyzed by pseudouridine synthases. Substrate RNAs labeled with tritium were incubated with inhibitory RNAs and the amount of tritium released was measured as described under 'Materials and methods'. The enzyme activities measured were pseudouridine synthases specific for tRNA nucleotide positions 13 (upper panel) or 55 (lower panel). The inhibitory RNAs used were unfractionated *E. coli* tRNA (triangles), fluorinated RNA-4 which is specific for PS 13 (closed circles), and fluorinated RNA-6 which is specific for PS 55 (open circles).

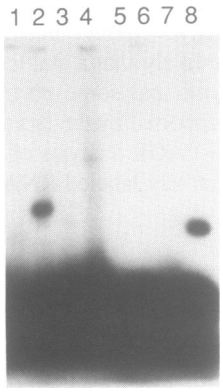

Fig. 3. Agarose gel electrophoresis of complexes between pseudouridine synthases and fluorinated tRNAs. Pseudouridine synthases 13 (PS 13, lanes 1–4) and 55 (PS 55, lanes 5–8) were incubated together with radiolabeled FUtRNAs using the conditions described under 'Materials and methods'. The tRNAs used were FUtRNA-3 (lane 1 and 5), FUtRNA-4 (lane 2 and 6), FUtRNA-5 (lanes 3 and 7) and FUtRNA-6 (lanes 4 and 8). The FUtRNA-4 is specific for PS 13 and FUtRNA-6 is specific for PS 55. Samples were applied at the top of the gel and the radioactivity present in all lanes at the bottom of the gel corresponds to unbound tRNA.

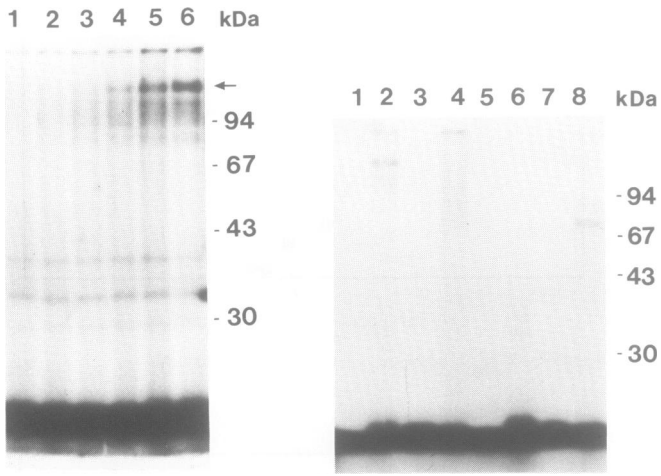

Fig. 4. UV-crosslinking of pseudouridine synthases to fluorinated tRNAs. Pseudouridine synthases were incubated together with radiolabeled FUtRNAs, irradiated with UV-light and subjected to SDS-PAGE as described under 'Materials and methods'. Left panel: The enzyme PS 13 was incubated with radioactively labeled FUtRNA-4 and irradiated at the time intervals 0 min (lane 1), 0.25 min (lane 2), 0.5 min (lane 3), 1 min (lane 4), 2 min (lane 5) or 5 min (lane 6). A radioactive band corresponding to a size of 130 kDa appears in the lanes 4–6 and is indicated by an arrow. Bands on top of the gel in lanes 4–6 are located at the site of sample application. Right panel: The enzymes used were PS13 (lanes 1–4) or PS 55 (lanes 5–8) and the RNAs were FUtRNA-3 (lane 1 and 5), FUtRNA-4 (lane 2 and 6), FUtRNA-5 (lanes 3 and 7) and FUtRNA-6 (lanes 4 and 8). Radioactive bands are found in lanes 2 and 8 and correspond to molecular weights of 130 kDa and 75 kDa, respectively. The band on top of the gel in lane 4 is located at the site of sample application.

paper. In one experiment a partially purified PS-13, free of activity corresponding to PS-32 and PS-55, was incubated with ^{32}P-labeled FUtRNA-3, 4, 5 and 6, respectively and the results are shown in Fig. 3, lanes 1–4. A complex was detected only in the case of FUtRNA-4 (lane 2). In a similar manner a partially purified PS-55 (free of PS-13 and PS-32) was incubated with the same FUtRNAs and found to bind selectively the FUtRNA-6

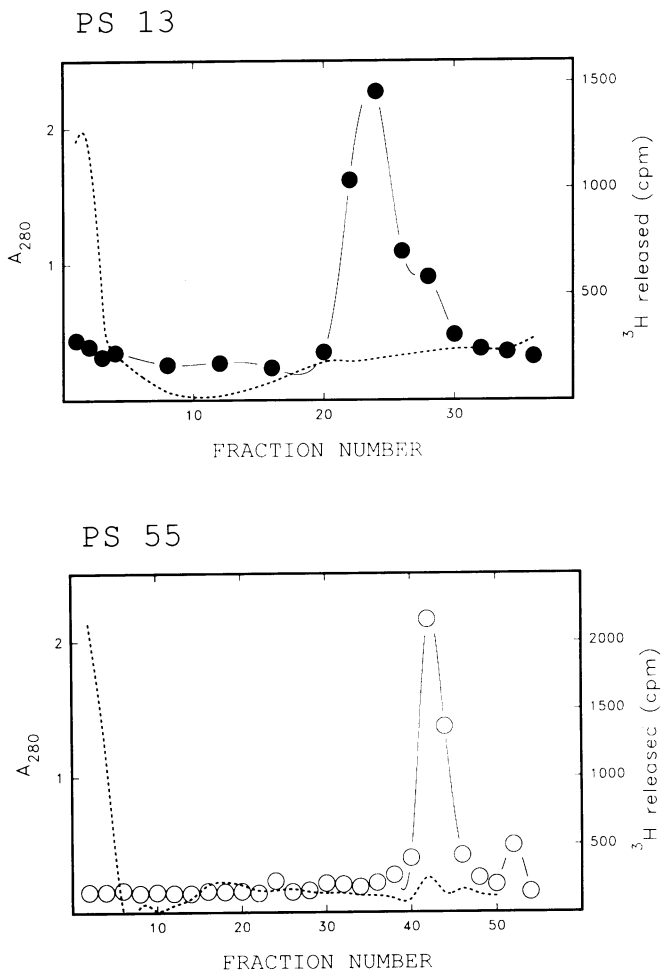

Fig. 5. Chromatography of pseudouridine synthases on immobilized fluorinated tRNA. Partially purified PS 13 (upper panel) and PS 55 (lower panel) was chromatographed on columns of agarose with immobilized FUtRNA4 (upper panel) and FUtRNA6 (lower panel). Elution was carried out using a gradient of 15 ml from 50 mM NaCl to 1 M NaCl in a buffer containing 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 3 mM DTT, 1 mM EDTA, and 5% glycerol. Fractions were monitored for A_{280} (dotted lines), PS 13 activity (filled circles) and PS 55 activity (open circles).

(Fig. 3, lane 8). These results give further support to the notion that complexes formed between pseudouridine synthases and fluorinated RNAs are both stable and highly specific.

The same type of experiment was carried out using tRNAs that instead of FU contained 5-bromouracil or normal uracil, respectively. In neither case was any radioactive complex between tRNA and enzyme detected under the particular conditions used. It should be noted that pseudouridine is rapidly formed when synthase is incubated together with a tRNA with unmodified uridines. Therefore, what is actually monitored in this latter experiment is the affinity between enzyme and the product of the enzymatic reaction.

UV-crosslinking of FUtRNA to enzyme

It was possible to join FUtRNA to pseudouridine synthase by UV irradiation and in Fig. 4 (left panel) is shown the time-dependent formation of a such a covalent complex between PS 13 and radiolabeled FUtRNA-4. The size of the complexes detected by agarose gel electrophoresis could thus be estimated

by UV irradiation followed by SDS-PAGE analysis (Fig. 4, right panel). Only one major radioactive band was detected for each of the enzymes (Fig 4, right panel, lanes 2 and 8, respectively). The PS-13 and the PS-55 complexes were estimated to be 130 kDa and 75 kDa, respectively. These values would correspond to sizes of the individual free polypeptides of approximately 110 kDa and 55 kDa, respectively, assuming that a 1:1 complex between enzyme and tRNA is formed. In the case of PS13 additional minor bands were detected in the range 100−120 kDa and could possibly represent proteolytic degradation of the larger complex.

When analyzing samples that were not irradiated radioactive bands were visible using SDS-PAGE (for instance the 30−40 kDa bands of Fig. 4 left panel, lane 1) but they corresponded to only a very small fraction of the total amount of FUtRNA that was complexed with enzyme. Furthermore, these bands were not reproducible and they did not correlate with the amount of enzyme activity contained in the particular protein fraction analyzed. We therefore believe them to be artifacts not related to specific pseudouridine synthases. Consequently, we have failed to obtain any evidence of a stable covalent complex being formed between FUtRNA and enzyme.

Chromatography of pseudouridine synthases on immobilized FUtRNA

The fact that stable, specific complexes were formed between pseudouridine synthases 13 and 55 and their corresponding fluorinated RNAs suggested that these enzymes could be retained on a column with immobilized FUtRNA. Therefore, FUtRNAs-4 and FUtRNA-6, approximately 0.5 mg each, were synthesized by *in vitro* transcription as described above. They were subsequently coupled to agarose adipic acid hydrazide (14,15) and pseudouridine synthases PS 13 and PS 55 were chromatographed on the resins thus obtained (Fig 5). The majority of the protein material in the samples applied was not retained by the columns. Both enzymes, however, bound very firmly to their respective FUtRNA column and could only be eluted using a very high salt concentration, in both cases approximately 1 M NaCl.

DISCUSSION

The potential of FUtRNAs for the identification and purification of pseudouridine synthases

We have used *in vitro* transcription to produce tRNAs substituted with 5-fluorouracil and have demonstrated that they are powerful inhibitors of pseudouridine synthase activity and that they form specific and stable complexes with these enzymes. The affinity of a particular tRNA for a pseudouridine synthase is to a large extent dependent on the presence of fluorouracil in the nucleotide position that is normally occupied by the uridine which is modified by the enzyme. For instance, PS 13 is more effectively inhibited by FUtRNA-4 (which contains FU in position 13) than by FUtRNA-6 (which contains cytosine in the same position). In the same way, the affinity of PS 13 for FUtRNA-4 (as estimated from a gel retardation experiment) is much higher than for FUtRNA-6.

We have made use of the strong interaction of FUtRNAs with pseudouridine synthases PS 13 and PS 55 to identify polypeptides related to enzyme activity. Thus, it was possible to radioactively label enzymes by UV-crosslinking complexes that contained labeled RNA and the size of the relevant polypeptides could be estimated from SDS-PAGE analysis. These findings indicate that fluorinated tRNAs will be useful for the study of pseudouridine synthases in general.

In order to improve the purification procedure for pseudouridine synthases we have sought to develop methods based on the affinity between RNA and enzyme. We first attempted to make use of columns with immobilized bulk tRNA from *S. cerevisiae*. However, the pseudouridine synthases studied by us were not retained by such a chromatographic medium, presumably because of a comparatively low affinity between enzyme and mature, fully modified RNAs. As an alternative it may be possible to use FUtRNAs in an affinity purification. We have demonstrated in the present work that pseudouridine synthases are firmly bound to immobilized FUtRNA. It should be possible to achieve a high degree of selectivity in such an affinity chromotography by allowing a specific FUtRNA to compete with a non-specific FUtRNA.

Mechanism of pseudouridine synthesis

Our observation that pseudouridine synthases interact in a highly specific fashion with FUtRNAs and that stable complexes are formed indicates that the fluorouridine moiety interacts intimately with the active site of the enzyme and that this interaction contributes significantly to the overall binding strength. A corresponding tRNA substituted with bromine does not form such a stable and specific complex. Therefore, it seems likely that the small size and/or high electronegativity of the fluorine atom is important for the strength of the interaction. We have not been able to detect a stable covalent complex between enzyme and FUtRNA of the type which has been described for thymidylate synthase (5) and tRNA (m^5U)methyltransferase (6). It should be noted, however, that these enzymes are mechanistically different from pseudouridine synthases as a methyl group is required in the stable covalent complex.

We have not been able to demonstrate that any covalent bonds in the FUtRNA are broken by the enzyme. It is conceivable that the C-F bond present in fluorouracil is resistant to cleavage and that it therefore precludes any of the normal catalytic actions of the enzyme. In particular, the glycosidic bond is intact, indicating either that this step occurs at a stage after the cleavage of the C5-hydrogen bond or that the presence of the fluorine atom in some other way prevents the cleavage of the glycosidic bond.

Physiological effects of 5-fluorouracil

Fluorinated tRNAs are potent inhibitors of the yeast pseudouridine synthases 13 and 55 studied in the present paper. The work of Kammen et al (8) suggests that also the *E. coli* pseudouridine synthase I, which is specific for the tRNA nucleotide positions 38−40, is inhibited in a similar way by bulk fluorinated tRNA prepared from cells of *E. coli* grown in medium containing 5-fluorouracil. It is therefore tempting to assume that pseudouridine synthases in general are inhibited by fluorinated tRNAs in the fashion described in the present paper.

When cells are allowed to grow in an environment containing 5-fluorouracil this nucleoside is effectively incorporated into RNA (1). It is conceivable that such RNAs *in vivo* effectively inhibit pseudouridine synthase activity so that also tRNA nucleotide positions occupied by normal uridine will remain unmodified. Therefore, an individual tRNA molecule will contain, instead of pseudouridine, unmodified uridines as well as 5-fluorouridines. In the same way it has been demonstrated that a tRNA methyltransferase is inhibited by fluorouracil-substituted tRNA,

and uridines that are normally methylated are left unmodified. This may be a property distinctive for all methyltransferases, and, consequently, few methylations are allowed to proceed in cells that are subjected to fluorouracil. Indeed, such cells have been shown to contain a limited number of nucleoside modifications (21) and a plausible mechanism is the strong inhibitory effect of fluorinated RNAs on pseudouridine synthases as well as methyltransferases.

Fluorouracil and derivatives of this compound have been widely used to treat certain types of cancer. It has generally been believed that the primary target of 5-fluorouracil is thymidylate synthase (1). FU is readily metabolized *in vivo* into 5-fluoro-dUMP (FdUMP) which inactivates the enzyme by forming a covalent complex together with the methyl donor tetrahydrofolate and DNA replication is consequently shut off. However, there are also implications that the incorporation of fluorouracil into RNA plays a role here (22). For instance, it has recently been shown that cells which lack thymidylate synthase are also severely retarded in their growth by the presence of fluorouracil (23). It is clear that although the biochemistry and pharmacology of fluorouracil has been extensively studied since the 1950s many questions still remain regarding its effect on both normal and tumor cells. Many nucleotide positions in tRNA, ribosomal RNA and snRNA are posttranscriptionally modified from uridine to pseudouridine but the physiological role of these modifications in eukaryotic cells is almost compeletely unknown (24). In particular, we lack information on the biological consequences of the aberrant modification patterns that result from a misincorporation of fluorouracil into RNA.

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