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The anticodon triplet is not sufficient to confer methionine acceptance to a transfer RNA

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ABSTRACT **Previous work suggested that the presence of the anticodon CAU alone was enough to confer methionine acceptance to a tRNA. Conversions of *Escherichia coli* non-methionine tRNAs to a methionine-accepting species were obtained by substitutions reconstructing the whole methionine anticodon loop together with preservation (or introduction) of the acceptor stem base A73. We show here that the CAU triplet alone is unable to confer methionine acceptance when transplanted into a yeast aspartic tRNA. Both non-anticodon bases of the anticodon loop of yeast $tRNA^{Met}$ and A73 are required in addition to CAU for methionine acceptance. The importance of these non-anticodon bases in other CAU-containing tRNA frameworks was also established. These specific non-anticodon base interactions make a substantial thermodynamic contribution to the methionine acceptance of a transfer RNA.**

The specificity of tRNA aminoacylation, as any catalyzed enzymic reaction, is achieved through productive binding interaction energy between the enzyme and the specific substrate (tRNA) and the unproductive binding or exclusion of the nonspecific tRNA (1–3). It is now recognized that this specificity is often manifested at the level of k_{cat} rather than on tRNA binding (4, 5). Aminoacyl-tRNA synthetases use predominantly nucleotidic sites from the acceptor stem and/or the anticodon region to achieve the specific tRNA aminoacylation (2, 3, 6, 7). Molecular genetic data have shown that the CAU anticodon in $tRNA^{Met}$ is sufficient for *Escherichia coli* methionyl-tRNA synthetase (MetRS) to discriminate cognate from a number of noncognate tRNAs (8, 9). Anticodon-mediated recognition supposes that a signal is transmitted to the active site when the correct anticodon is bound to the synthetase, leading to a shift of the catalytic groups to the correct position relative to the 3′-terminal adenosine of tRNA so that aminoacylation may take place. This raises the question of the contribution of bases from the acceptor stem to $tRNA^{Met}$ recognition. The role of the discriminator base A73 (the fourth base from the 3′ terminus; ref. 10) has been analyzed by several groups (7, 11, 12). They found that substitutions at this site decreased slightly (2- to 7-fold) the efficiency of aminoacylation. However, the best evidence that nucleotide A73 of $tRNA^{Met}$ is involved in the formation of a Michaelis–Menten complex comes from the fact that its replacement by cytidine results in loss of aminoacylation of an RNA minihelix composed of the methionine acceptor-T stems (13).

MATERIALS AND METHODS

Unmodified tRNAs were synthesized from the respective $tRNA^{Met}$, $tRNA^{Asp}$, and $tRNA^{Val}$ genes (assembled from a cassette including the T7 RNA polymerase promoter) by transcription with T7 RNA polymerase. Because the activity of T7 RNA polymerase requires that the first transcribed nucleotide be a G, we replaced A1 of initiator $tRNA^{Met}$ by a G to yield a non-Watson–Crick base pair G1-U72. All $tRNA^{Met}$ variants were constructed from the G1-U72 mutant, which shows the same activity as the transcript with the wild-type A1-U72 sequence. Variants from the $tRNA^{Asp}$ clone were constructed from the G1-C72 mutant (14). One hundred micrograms of plasmid DNA was linearized with *Bst*NI and run-off transcripts were prepared as described earlier (15) under the following conditions: 40 mM Tris·HCl (pH 8), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 4 mM (each) ribonucleoside triphosphate, 20 mM GMP, and pure T7 RNA polymerase (Pharmacia, 3000 units). The transcripts were purified by electrophoresis on 15% denaturing polyacrylamide gels. Aminoacylation reactions were carried out at 25°C in a reaction mixture of 20 μl under the following conditions: 144 mM Tris·HCl (pH 7.8), 5 mM DTT, 2 mM ATP, 10 mM $MgCl_2$, 0.1 mM [^{35}S]methionine (1 mCi/μmol; 400–1200 cpm/pmol; 1 Ci = 37 GBq), 0.1 mg of bovine serum albumin per ml, 5–60 μM transcript, and 10–300 nM pure MetRS.

RESULTS AND DISCUSSION

To further assess the role of bases from the anticodon region and the discriminatory site we analyzed the k_{cat}/K_m parameter of yeast $tRNA^{Met}$ transcripts from mutants of the entire anticodon loop and of base A73. Indeed, a composite structure of sequences common to initiator and elongator $tRNA^{Met}$ species from *E. coli* and yeast shows that the anticodon loop and the discriminator base A73 are conserved in all four $tRNA^{Met}$ (Fig. 1*a*). Their functional importance is underlined by the occurrence of an efficient cross-aminoacylation, at least in the unmodified forms of *E. coli* $tRNA^{Met}$ by the yeast MetRS (17) or the reciprocal yeast $tRNA^{Met}$ cross-aminoacylation by the *E. coli* enzyme (unpublished results). *In vivo* genetic data implying the CAU anticodon of yeast $tRNA^{Met}$ in recognition by the yeast MetRS have been reported (18). Table 1 shows the effects of anticodon substitutions on the catalytic efficiency k_{cat}/K_m measured *in vitro* with synthetic T7 RNA variants of yeast initiator $tRNA^{Met}$. Previous work has shown that specific recognition of the yeast $tRNA^{Met}$ by the cognate MetRS enzyme involves the interaction with the CAU anticodon sequence (17). The analysis of all nine possible base substitutions of the CAU anticodon (Table 1, experiments 4–12) shows that only those tRNAs with the anticodon change CCU (experiment 7) and CAG (experiment 11) retained detectable methionylation activity, probably because of conservation of chemical groups upon mutation of A35 into C35 and U36 into G36. These groups (the 6-exocyclic NH_2 group and the N-1 nitrogen ring of A35 on one hand; the N-3 hydrogen and the 4-keto group of U36 on the other hand) are likely to be the functional subsites that engage

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Abbreviation: MetRS, methionyl-tRNA synthetase.
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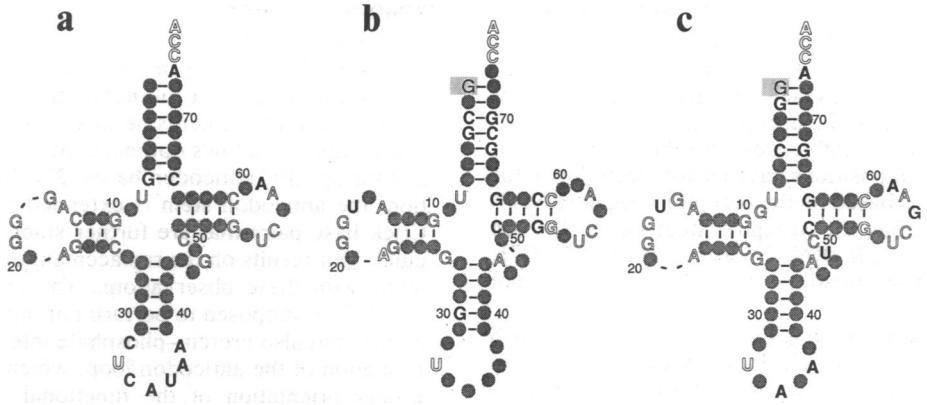

FIG. 1. (*a*) Sequence comparison between initiator and elongator $tRNA^{Met}$ species from yeast and *E. coli*. (*b*) Sequence comparison between transcripts of yeast initiator $tRNA^{Met}$ and yeast $tRNA^{Asp}$. A1 nucleotide of initiator $tRNA^{Met}$ has been replaced by G1 to yield the base pair G1-U72. The $tRNA^{Asp}$ transcript has the U1-A72 pair replaced by G1-C72 (14). (*c*) Sequence comparison between transcripts of yeast initiator $tRNA^{Met}$ and yeast $tRNA^{Val}$. The sequence of the $tRNA^{Val}$ isoacceptor is that having an UAC anticodon. Sequences of tRNAs were from Sprinzl *et al.* (16). Nucleotides conserved or present at 90% in all tRNAs are represented by white (open) letters. Nucleotides different from those of yeast initiator $tRNA^{Met}$ are represented by shaded circles. Nucleotides common to yeast initiator $tRNA^{Met}$ are shown in boldface letters. In *b* and *c*, the G1 nucleotide is represented by a black shaded letter because of its presence in the transcript only. Gaps (–) are introduced in the D and variable loops to take into account variations in size.

direct hydrogen bonding with hydrogen donor/receptor amino acids of the protein, as also suggested by Schulman and Pelka (19) in the case of *E. coli* MetRS. All other anticodon changes severely decrease the catalytic efficiency by at least 3 orders of magnitude compared to that of the wild-type transcript. Despite conservation of the exocyclic NH_2 group and the N-1 nitrogen ring, the A34 mutant (experiment 6) is inefficient in MetRS catalysis. This may be due to steric exclusion of the purine base from a narrow pyrimidine binding pocket. The fact that no substitution of C34 can be tolerated indicates that this base plays a dominant role in the selection of methionine anticodons as is the case in *E. coli* (8, 19, 20). Substituting A73 for G73 resulted in a 40-fold decrease of k_{cat}/K_m (Table 1, experiment 3).

To measure the influence of the CAU anticodon and base A73 on methionylation of a noncognate yeast tRNA, these nucleotides were transplanted into a transcript of yeast $tRNA^{Asp}$ having sufficiently distinguishing features in the primary and tertiary structures (21). Neither the anticodon change from GUC to CAU (Table 1, experiment 16) nor the single replacement of base G73 by A73 (Table 1, experiment 17) conferred methionine acceptance to $tRNA^{Asp}$. The failure

Table 1. Methionylation of yeast mutant tRNA transcripts by wild-type MetRS

Exp.	Transcript	Anticodon loop sequence (bases 32–38)	Base 73	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, $s^{-1}\cdot\mu M^{-1}$	Relative loss of efficiency
1	$tRNA^{Met}$	C U C A U A A	A	10	1.3	0.13	—
2	$tRNA^{Met}$ (G1)	C U C A U A A	A	13	1.3	0.10	1
3			G	15	0.040	0.0027	40
4		G34	A	NM	NM	$<0.6 \times 10^{-4}$	>1700
5		U34	A	NM	NM	$<2 \times 10^{-4}$	>500
6		A34	A	NM	NM	$<0.6 \times 10^{-4}$	>1700
7		C35	A	9	0.0060	0.0007	140
8		G35	A	NM	NM	$<2 \times 10^{-4}$	>500
9		U35	A	NM	NM	$<0.6 \times 10^{-4}$	>1700
10		C36	A	NM	NM	$<0.6 \times 10^{-4}$	>1700
11		G36	A	200	0.36	0.0018	55
12		A36	A	NM	NM	$<0.6 \times 10^{-4}$	>1700
13		U U C A U A C	A	25	0.015	0.00060	170
14		U U C A U A C	G	NM	NM	$<0.6 \times 10^{-4}$	>1700
15	$tRNA^{Asp}$ (G1-C72)	U U G U C G C	G	NM	NM	$<0.6 \times 10^{-4}$	>1700
16		U U C A U G C	G	NM	NM	$<0.6 \times 10^{-4}$	>1700
17		U U G U C G C	A	NM	NM	$<0.6 \times 10^{-4}$	>1700
18		U U C A U G C	A	36	0.026	0.00072	140
19		C U C A U A A	A	17	0.57	0.034	3
20		C U C A U A A	G	15	0.018	0.0012	80
21	$tRNA^{Val}$	U U U A C A C	A	NM	NM	$<0.6 \times 10^{-4}$	>1700
22		U U C A U A C	A	25	0.049	0.0020	50
23		U U C A U A C	G	NM	NM	$<0.6 \times 10^{-4}$	>1700
24		C U C A U A A	G	15	0.17	0.011	10
25		C U C A U A A	A	4	1.6	0.40	0.25

k_{cat} and K_m values of the various transcripts were measured from material obtained from two independent transcriptions. Values of the kinetic parameters for duplicate experiments were within ±15%. NM, not measurable; in this case, values of k_{cat}/K_m were estimated from the lower limit of detectable aminoacylation activity and ranged from 0.6 to 2×10^{-4} $s^{-1}\cdot M^{-1}$. Boldface letters indicate the nature of the substitution introduced. Values of experiments 4, 7, and 10 have been published (17).

of $tRNA^{Asp/CAU}$ ($tRNA^{Asp}$ with a CAU anticodon) to be aminoacylated by the yeast MetRS (Table 1, experiment 16) may be due to the lack of a positive determinant (i.e., A73) and additionally to the presence of negative elements at nonconserved positions in the acceptor stem (Fig. 1*b*). Evidence for such negative influences introduced by certain bases at nonconserved positions have clearly been shown for a minisubstrate that is based on the acceptor stem of $tRNA^{Ile}$ (13). This substrate has the acceptor stem bases that are conserved among *E. coli* $tRNA^{Met}$ isoacceptors (G2-C71, C3-G70) and the discriminatory base A73, but it is not charged by MetRS.

However, the lack of charging of $tRNA^{Asp/CAU}$ by MetRS is mainly due to the lack of A73 base: indeed, a significant methionine charging activity was seen when the CAU anticodon and base A73 were introduced into $tRNA^{Asp}$ (Table 1, experiment 18). The k_{cat}/K_m for methionylation of this mutant is reduced 140-fold with respect to that of the wild-type $tRNA^{Met}$ transcript, suggesting the lack of additional determinants elsewhere in the molecule. The introduction of bases from the entire anticodon loop of $tRNA^{Met}$ (bases 32–38; see Table 1) and A73 transformed $tRNA^{Asp}$ into a quasi-$tRNA^{Met}$ substrate (Table 1, experiment 19). The contribution of bases from the methionine anticodon loop alone to MetRS catalysis is emphasized in experiment 20: the resulting mutant shows a decrease in k_{cat}/K_m of 80-fold as compared to $tRNA^{Met}$, establishing the predominant role of bases from the $tRNA^{Met}$ anticodon loop in methionine identity.

The above analysis of nucleotides important for methionine identity has revealed that one or several bases in the anticodon loop of $tRNA^{Asp}$ (U32, G37, C38) interfere with MetRS recognition (Table 1, experiments 16 and 20). Nucleotides U32 and C38 are also present in the anticodon loop of $tRNA^{Val}$ (Table 1). A combinatory analysis of anticodon loop and discriminatory base substitutions in a transcript of yeast $tRNA^{Val}$ revealed that U32 and C38 exert a strong negative influence on aminoacylation of this tRNA by MetRS (experiments 22–24). Their presence in $tRNA^{Val}$ is partly compensated by that of A73 (Table 1, experiment 22) and a substitution giving to this $tRNA^{Val}$ the complete CUCAUAA sequence of $tRNA^{Met}$ anticodon loop yields a substrate that is 4-fold better aminoacylated by MetRS than the wild-type $tRNA^{Met}$ transcript (Table 1, experiment 25). Therefore, the nonconserved positions in the contact area between $tRNA^{Val}$ and MetRS are less detrimental to the formation of an active Michaelis–Menten complex than in the case of the cognate $tRNA^{Met}$.

We also verified that the methionine acceptance in $tRNA^{Met}$ depends critically on the presence of C32 and A38 nucleotides in the anticodon loop. Their respective replacement by U32 and C38 caused a nearly 170-fold decrease in k_{cat}/K_m (Table 1, experiment 13) and an additional substitution of A73 for G73 completely abolished the methionine charging activity (Table 1, experiment 14). These results unambiguously identify bases 32, 34, 35, 36, and 38 of the methionine anticodon loop and the discriminatory A73 base as the set of determinants important for specific recognition by the yeast MetRS and not only the CAU triplet. Additivity of the individual contributions to transition state formation is probably responsible for the substrate specificity conversion of $tRNA^{Asp}$ or $tRNA^{Val}$ to an efficient $tRNA^{Met}$ variant. Consistent data from Table 1 (experiments 3, 13, 20, 22, and 24) indicate that substitution of the C32-A38 and A73 identity elements yields a loss of specificity of about 170- and 40-fold, respectively. A double mutant will have k_{cat}/K_m reduced 6800-fold if the individual elements contribute independently to MetRS recognition. The results of experiments 16 and 23 are consistent with such a hypothesis.

How can one explain the effect of substitution of nucleotides C32 and A38? Based on sequence and crystallographic homologies between *E. coli* glutaminyl-tRNA synthetase (GlnRS) and MetRS, a similar overall orientation of $tRNA^{Met}$ and $tRNA^{Gln}$ binding to their respective synthetase was suggested (22). The crystal structure of the $tRNA^{Gln}$-GlnRS complex revealed that the non-anticodon bases in the loop serve as identity elements because of their ability to build up a structure that allows optimal contacts between the protein and the specific anticodon bases (23). In the new conformation, the anticodon stem is extended by two non-Watson–Crick base pairs that are further stabilized by water molecules. Our results on the replacement of the C32-A38 pair go along with these observations. The incorrect "base pair" U32-C38 is supposed to perturb not only protein–base interactions but also protein–phosphate interactions and the conformation of the anticodon loop, which all contribute to the proper orientation of the functional groups of the CAU anticodon bases.

The observation that yeast MetRS achieves stringent discrimination by contacts with the entire anticodon loop CUCAUAA and base A73 seems to be a property that is shared by its *E. coli* counterpart. (*i*) A significant negative effect of A73 substitution has already been noted in a minihelix of *E. coli* $tRNA^{Met}$ (13), and a corollary experiment showed a 10-fold increase of methionine acceptance when G73 of *E. coli* $tRNA^{Trp/CAU}$ is replaced by A73 (7). (*ii*) Successful conversion of *E. coli* $tRNA^{Val}$, $tRNA^{Trp}$, and $tRNA^{Ile}$ to methionine identity by changing their anticodon sequences (8, 9) also reconstruct a methionine CUCAUAA anticodon loop. (*iii*) MetRS from *E. coli* is able to bind a minisubstrate made up of the anticodon stem and loop (24) in accordance with our observation that bases from this loop are the major determinants for methionine acceptance. (*iv*) Conservation of the anticodon identity element in yeast and *E. coli* $tRNA^{Met}$ parallels conservation of important catalytic residues in the anticodon binding region of the cognate MetRS (17, 25). This illustrates the importance of conserved functional groups on the tRNA and the synthetase in establishing the specificity of tRNA aminoacylation. Concerning base A73, this site may indirectly contribute to $tRNA^{Met}$ identity. Indeed, in the case of the *E. coli* enzyme, the available data show that A73 and U73 (which share no functional groups) are fully active, suggesting that there is no positive interaction at this site and that the reduced activity seen with G73 and C73 is due to negative interactions with MetRS (7).

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