Structural analysis of the interaction of the tRNA modifying enzymes Tgt and QueA with a substrate tRNA

Stefan O. Mueller, Robert K. Slany*

Institut für Biochemie, Universität Erlangen, Fahrstr. 17, 91054 Erlangen, Germany

Received 14 December 1994; revised version received 6 February 1995

Abstract The enzymes tRNA guanine-transglycosylase (Tgt) and *S*-adenosylmethionine : tRNA ribosyltransferase-isomerase (QueA) participate in the biosynthesis of the hypermodified tRNA nucleoside queuosine (Q) in *Escherichia coli*. Here we show by HPLC analysis and gel retardation that both enzymes interact with an in vitro transcribed tRNA^{Asp} from yeast, specifically modified with a Q precursor molecule. RNase I footprinting experiments showed strong protein tRNA contacts in the anticodon stem-loop and a minor interaction with the dihydrouridine loop. This suggests that all identity elements for the recognition of Q-specific tRNAs are clustered in the anticodon region and explains earlier results that both enzymes accept a RNA microhelix with the sequence of an anticodon stem-loop as substrate.

Key words: Queuosine; tRNA modifying enzyme; Enzyme–substrate interaction; Footprinting

1. Introduction

A special feature of RNAs is their content of modified nucleosides. More than 90 modified RNA nucleosides have been found, most of them in tRNAs [1]. An extraordinary tRNA modification is represented by the deazaguanosine derivative queuosine (Q) which is present in tRNAs specific for Asp, Asn, His and Tyr. Queuosine replaces guanosine (no. 34) in the wobble (first) position of the anticodon of the respective tRNAs (review in [2]).

In contrast to eukaryotes, which obtain Q like a vitamin from the intestinal flora or from nutrients, it is synthesized de novo by several unusual reactions in prokaryotes [3]. Fig. 1 outlines the known steps of the Q biosynthesis in *Escherichia coli*. After formation of the Q precursor 7-aminomethyl-7-deazaguanosine ($preQ_1$) by unknown enzymes, the tRNA guanine transglycosylase, Tgt, catalyses the exchange of $preQ_1$ with G_{34}. Q is the only known base modification that is introduced by a base-exchange mechanism. The $preQ_1$ modified tRNA is the substrate for the *S*-adenosylmethionine:tRNA ribosyltransferase isomerase, QueA. QueA transfers the ribosyl part of AdoMet to $preQ_1$ and isomerizes it to an epoxycyclopentanediol moiety forming the next Q precursor epoxyqueuosine (oQ) [4]. In a vitamin B_{12} dependent reaction, oQ is finally reduced to Q. Interestingly a 17 nt long microhelix structure corresponding to the anticodon stem-loop of the Q-specific tRNA^{Tyr} from *E. coli* served as a substrate for Tgt as well as QueA [5,6]. In the case of the Tgt enzyme the V_{max}/K_M ratio was only 20 fold lower for the truncated 17 nt RNA compared to the whole tRNA molecule, suggesting that all necessary recognition elements are clustered around the anticodon [5]. We now investigated whether indeed the tRNA anticodon region is the sole recognition sequence for the enzymes or if extended RNA–protein interactions are required. Therefore we performed footprinting on a full-length tRNA. Since base modifications and impurities in the tRNA preparations can obscure the footprinting results, we used a T7 RNA polymerase based system to produce the required substrate tRNA. As a model tRNA^{Asp} from yeast was chosen. The three-dimensional structure of this tRNA is known and a T7 expression construct was available [7,8]. Additionally it was possible to specifically modify this tRNA with $preQ_1$ using the Tgt enzyme and synthetic $preQ_1$ base. A $preQ_1$ containing tRNA should interact with the QueA enzyme as well as with the Tgt enzyme. In the naturally occurring Q biosynthesis pathway, $preQ_1$ tRNA is the product of the Tgt reaction and the immediate substrate for QueA protein. However, at least in vitro, the Tgt reaction is reversible and therefore $preQ_1$ tRNA is simultaneously the substrate for the Tgt back-reaction.

2. Materials and methods

2.1. Generation of a synthetic substrate tRNA

For large scale preparations of tRNA^{Asp} we used a modified T7 RNA polymerase in vitro transcription protocol according to [9]. The pUC18 derivative plasmid pTFMa (a generous gift of R. Giegé) contains the gene for the tRNA^{Asp} downstream of a T7 RNA polymerase promoter. A double mutation G_1-C_{72} was introduced to ensure efficient initiation by the T7 RNA polymerase [7]. The linearization of the DNA with *Bst*NI (New England Biolabs) creates the 3′-CCA terminal of the final RNA transcript. The nascent tRNA^{Asp} transcript was modified concomitantly with transcription using synthetic $preQ_1$ base (a generous gift of S. Nishimura) and purified Tgt enzyme (a generous gift of K. Reuter). 50 μg linearized pTFMa were dissolved in 100 μl of 5× transcription buffer (400 mM HEPES-KOH, pH 7.5, 60 mM $MgCl_2$, 10 mM spermidin, 200 mM DTT), 60 μl of rNTP mixture (25 mM each), 500 U RNasin (Promega), 3 U of inorganic pyrophosphatase (Sigma), 900 U of T7 RNA polymerase (New England Biolabs), 100 μg synthetic $preQ_1$ base, 30 μg Tgt enzyme and water up to 500 μl. After incubation at 37°C for 2 h, an additional 900 U of T7 RNA polymerase were added and the mixture was kept at 37°C for another 3 h. Finally the DNA template was degraded by 50 U RNase-free DNase (Promega) at 37°C for 15 min. Typical yields were approximately 400 μg of RNA transcript in a single reaction.

2.2. Purification of the T7 transcribed $preQ_1$ modified tRNA^{Asp}

The raw transcript was purified by anion-exchange chromatography on a FPLC MonoQ 5/5 column (Pharmacia) equilibrated with buffer A (0.14 M NaAc, pH 4.5). Elution was performed by a linear gradient

*Corresponding author. Fax: (49) (9131) 854 605.
E-mail: mfb102@cd4680fs.rrze.uni-erlangen.de

This publication is dedicated to Helga and Walter Kersten.

Abbreviations: Q, queuosine; $preQ_1$, 7-aminomethyl-7-deazaguanosine; oQ, epoxyqueuosine; Tgt, tRNA guanine-transglycosylase; QueA, *S*-adenosylmethionine:tRNA ribosyltransferase-isomerase; AdoMet, *S*-adenosylmethionine.

SSDI 0014-5793(95)00169-7

of buffer B (buffer A+1 M NaCl; flow 1 ml/min) as follows: 0–5 min, hold at 0% B; 5–25 min, linear increase from 0% to 100% B; 25–27 min hold at 100% B; 27–28 min, decrease from 100% to 0% B; 28–32 min, hold at 0% B. The $preQ_1$ modified $tRNA^{Asp}$ was eluted at a concentration of about 570 mM NaCl.

2.3. Reversed-phase HPLC analysis of tRNA nucleosides

The purified tRNA transcript was analysed as described [6,10]. In short, the tRNA was hydrolysed with nuclease P1 (Boehringer Mannheim) and dephosphorylated with alkaline phosphatase from *E. coli* (Boehringer Mannheim). The nucleosides were analysed by reverse-phase HPLC (Supelcosil LC-18S column 4.6 × 250 mm; Supelco, Bellefonte, PA) equilibrated in buffer C (10 mM $NH_4(H_2PO_4)$, 2.5% MeOH, pH 5.3). The nucleosides were eluted with a gradient of buffer D (10 mM $NH_4(H_2PO_4)$, 20% MeOH, pH 5.1) at a flow rate of 1 ml/min. The following gradient was used: 0–12 min, hold at 100% C; 12–20 min, linear increase to 25% D, 20–25 min, linear increase to 60% D; 25–32 min, linear increase to 62% D; 32–36 min, linear increase to 100% D; 36–45 min hold at 100% D; 45–60 min linear decrease to 0% D. The peaks were identified by comparison of retention times with standard substances.

2.4. 5'end-labelling of tRNA

The tRNA transcript was labelled at the 5' end with [γ-^{32}P]ATP (10 μCi/μl, 3000 Ci/mmol; Amersham, UK) and T4 polynucleotide kinase (Promega). 1 μg $preQ_1$ modified $tRNA^{Asp}$ was dephosphorylated with shrimp alkaline phosphatase (USB) and labelled after phenol extraction with 4 U T4 polynucleotide kinase and 100 μCi of [γ-^{32}P]ATP in a reaction volume of 15 μl. The labelled tRNA was purified on a 10% polyacrylamide, 8 M urea gel. Bands corresponding to the full length transcript were eluted overnight at 4°C in 200 μl elutiuon buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% phenol), ethanol precipitated and redissolved in water to 20,000 cpm/μl (measured by Cherenkov radiation, approximately 2–10 ng/μl). For subsequent experiments the correct tertiary structure was renatured by heating the tRNA for 2 min to 70°C, followed by cooling down to room temperature.

2.5. Band shift experiments

For band shift experiments 20,000 cpm of radiolabelled $tRNA^{Asp}$ modified with $preQ_1$ was incubated with QueA or Tgt enzyme for 15 min at room temperature in 20 μl 1× reaction buffer (20 mM glutamic acid, 10 mM spermidin, adjusted to pH 7.5). The reaction mixtures were analysed by native 8% PAGE in 1× TBE and autoradiographed.

2.6. Footprinting experiments

20,000 cpm radiolabelled $tRNA^{Asp}$ was incubated in a final volume of 20 μl 1× reaction buffer (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM DTT) with 3 U RNasin (RNase inhibitor; Promega), and different amounts of Tgt or QueA enzyme for 15 min on ice, to allow the formation of the enzyme substrate complexes. After addition of 0.06 U RNase I (RNaseONE; Promega; diluted in 1× reaction buffer supplemented with 0.1 μg BSA/μl) the reaction mixture was incubated at 37°C for 10 min and the digestion was terminated by extraction with phenol/chloroform. Nucleic acids were precipitated, separated on a 12.5% PAA, 8 M urea gel and autoradiographed. As controls, reactions without Tgt, QueA and RNase I were included. The positions of protein–RNA interactions were assigned by comparison with an alkaline total hydrolysis ladder and a RNaseT1 digest. RNaseT1 cuts specifically after single stranded guanosine residues. For alkaline hydrolysis the tRNA was incubated with 50 μl 50 mM Na_2CO_3, pH 9.0, 1 mM EDTA for 5 min at 95°C. RNaseT1 cleavage was achieved by digestion with 0.1 U RNaseT1 (diluted in 1× reaction buffer, 0.1 μg BSA/μl) for 10 min at 37°C.

3. Results

For the production of the specific tRNA substrate the transcription and modification with $preQ_1$ was performed simultaneously. To drive the equilibrium of the Tgt reaction during the modification of the nascent tRNA transcript in the direction of the $preQ_1$ incorporation, a large excess of $preQ_1$ base was added. To assess the amount of $preQ_1$ incorporation achieved, a 20 μg sample of the modified tRNA was analyzed by HPLC. As Fig. 2A shows, the tRNA contained, except for the four major bases A, C, G, and U, a significant amount of $preQ_1$. Additionally a few unidentified minor impurities were present in the preparation. Calculated by the peak areas (data not shown) approximately 80% of the tRNA was modified with $preQ_1$.

After incubation of this monomodified tRNA with QueA enzyme and AdoMet almost all $preQ_1$ was converted to epoxyqueuosine, as shown by HPLC (Fig. 2B). This proves that the synthetic tRNA is a substrate for the QueA enzyme. The detection of the Tgt backreaction by HPLC is difficult, since $preQ_1$ is re-exchanged to G which is already present in large amounts (24 G residues in $preQ_1$ containing $tRNA^{Asp}$ vs. 25 G residues in unmodified $tRNA^{Asp}$). Therefore gel retardation experiments with both enzymes and radioactively labelled tRNA were performed.

Fig. 3 demonstrates the formation of strong protein–RNA complexes with the Tgt enzyme. With increasing amounts of Tgt, multiple retarded bands are visible, indicating a multimeric structure of the bound complex at higher protein concentrations. Surprisingly no retardation of the tRNA was observed after addition of QueA protein. A QueA/tRNA complex could not be observed independent of addition of AdoMet, in differ-

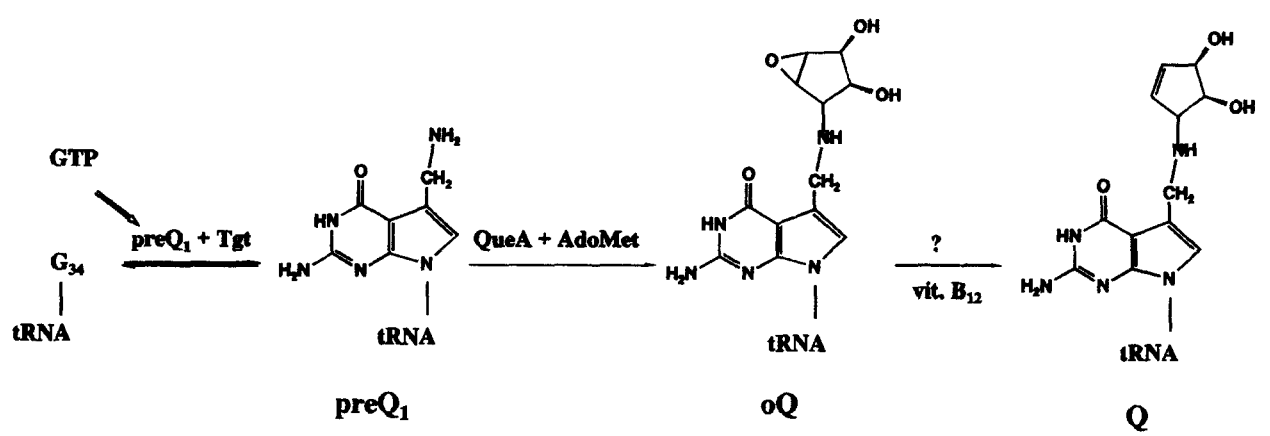

Fig. 1. tRNA dependent steps of queuosine biosynthesis (Tgt, tRNA-guanine transglycosylase; QueA, *S*-adenosylmethionine:tRNA ribosyltransferase-isomerase; $preQ_1$, 7-(aminomethyl)-7-deazaguanine; AdoMet, *S*-adenosylmethionine; vit B_{12}, adenosylcobalmine; oQ, epoxyqueuosine; Q, queuosine).

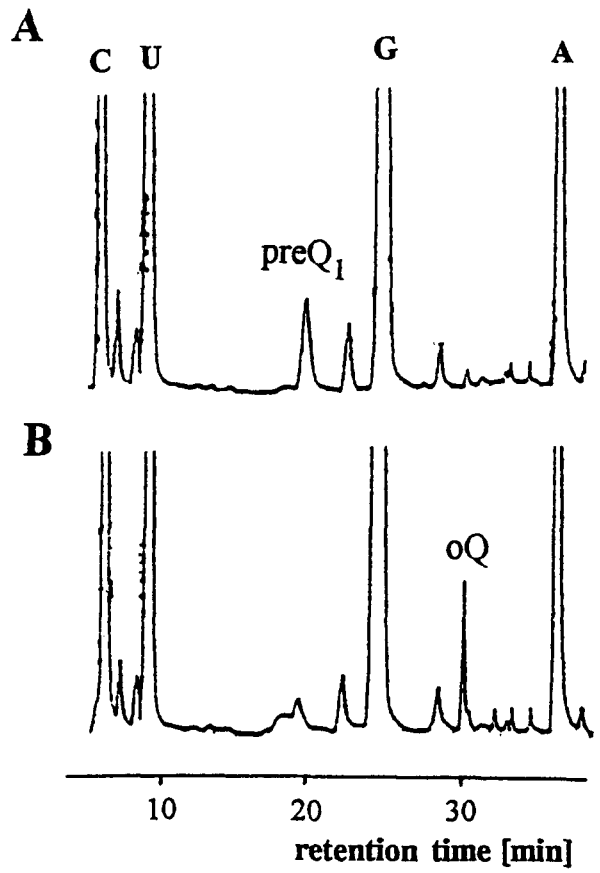

Fig. 2. Reverse-phase HPLC analysis of tRNA nucleosides; detection by UV absorbance at 254 nm; for technical details see section 2. (A) T7 transcribed, $preQ_1$ modified $tRNA^{Asp}$. (B) $preQ_1$ modified $tRNA^{Asp}$ after incubation with QueA enzyme and AdoMet. Peaks corresponding to $preQ_1$ and oQ are labelled.

ent buffer systems or at different temperatures. Although the tRNA is a substrate for QueA, as revealed by HPLC, the intermediary protein–RNA complexes are obviously too labile to yield retarded bands during gel electrophoresis.

When DNA–protein complexes are probed by footprinting, nicks in the DNA backbone normally do not interfere with further protein binding because the requirement for the DNA recognition, mostly the DNA sequence, is still intact. In contrast, a primary nick in the tRNA will probably affect the integrity of its three-dimensional structure [11]. This can interfere with substrate recognition and shift the equilibrium between protein-bound and free tRNA towards free tRNA. Any following secondary nuclease cut will reflect the artificially altered and not the native structure of the tRNA. Therefore we used a large molar excess (up to 32 fold in the case of the QueA enzyme, and up to 176 fold with the Tgt enzyme) of binding protein with a limiting amount of nuclease under empirically determined conditions. In our hands the single strand specific RNase I from *E. coli* gave the best footprinting results. tRNA fragments generated by a digestion with the G-specific RNaseT1 and an unspecific alkaline hydrolysis reaction served as size markers to assign the nucleotide positions to the footprinting reactions.

As Fig. 4A (lane 7) and B (lane 9) show, the free tRNA without addition of binding proteins is cleaved not only in the single stranded regions of the dihydrouridine loop (positions 14–21) and the anticodon loop (positions 32–38), but degradation can also be seen in the regions from position 29 to 31 and from 10 to 13. Normally these bases are base-paired and should not be cleaved by RNase I. As stated above these cleavages most probably reflect secondary cuts of the RNase I that occurred after a first nick in a loop of the RNA destabilized the stem structure. These minor secondary cuts are especially obvious after longer exposures of the autoradiography films, as in Fig. 4A.

The lanes 4–6 in Fig. 4A demonstrate that an addition of increasing amounts of Tgt enzyme protected the bases 29–36 from RNase I attack, indicating a strong interaction of the protein with the anticodon loop. Enhanced RNase accessibility could be detected for bases 15–17 in the dihydrouridine loop. This is characteristic for a distortion of the natural conformation. No further cleavage protections or enhancements could be detected. In Fig. 4B, lanes 5–9, a similar footprinting pattern for the QueA protein is depicted. The footprinting reactions were performed either with or without the addition of AdoMet. The inclusion of the QueA cofactor yielded a qualitatively similar, but weaker footprint, indicative of a shorter half-life of the intermediary enzyme substrate complex. Again the bases 31–37 in the anticodon loop were protected from digestion, whereas in the dihydrouridine loop an enhancement at bases 15 and 16 was observed. Longer exposures (Fig. 4C) revealed additional protections at bases 19 and 20.

4. Discussion

The specific recognition of tRNAs is a challenging task in nature. Because of the structural constraints imposed by their interaction with the ribosome during translation, all tRNAs must possess more or less the same overall three-dimensional structure. However, for the accuracy of protein biosynthesis it is absolutely crucial to differentiate the at least 20 different tRNA species in every cell (there are 45 different tRNA species in *E. coli*) [12]. In the case of the aminoacyl tRNA synthetases a lot is known about the identity elements of the tRNAs, and the first structures of protein–tRNA complexes have been solved by X-ray crystallography (for a review, see [13]). In contrast very little is understood about the interaction of

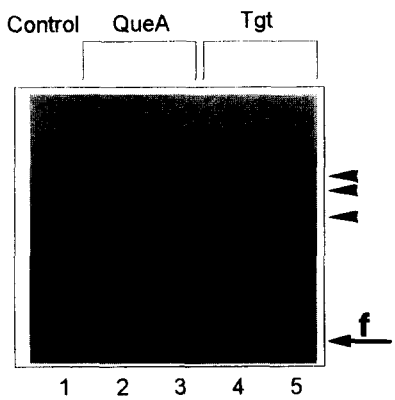

Fig. 3. Bandshift experiment with Tgt, QueA and substrate tRNA. Lane 1, control, without protein. Lane 2, 0.5 μg QueA. Lane 3, 1 μg QueA. Lane 4, 0.5 μg Tgt. Lane 5, 1 μg Tgt. Retarded complexes are labelled by arrows. f denotes unbound $tRNA^{Asp}$.

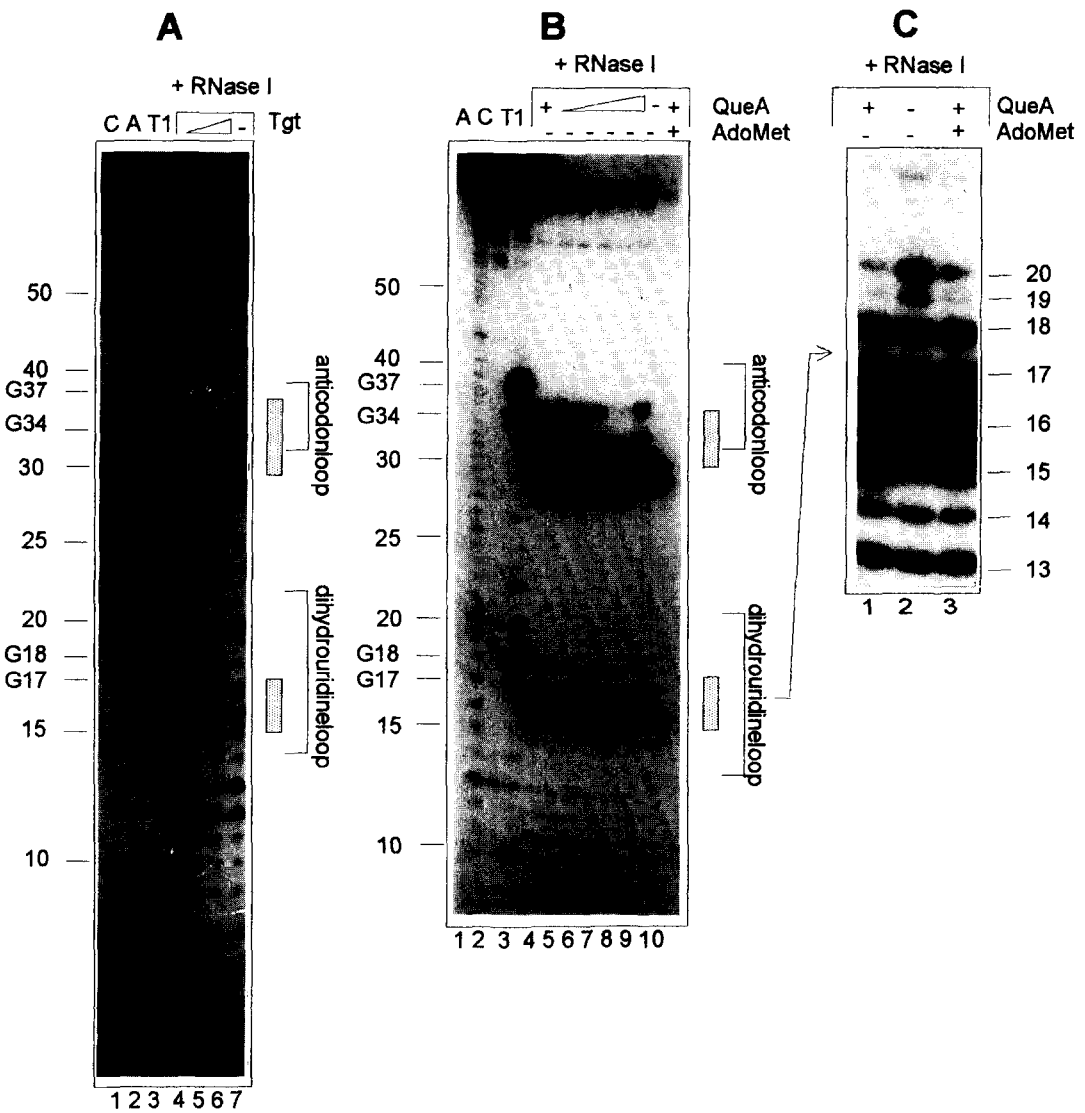

Fig. 4. Autoradiographs of footprints generated with Tgt and QueA enzyme on 5′ labelled, preQ$_1$ containing tRNA^{Asp}. The bands corresponding to the dihydrouridineloop and the anticodonloop are marked by brackets. Postions of protein–RNA interactions are indicated by shaded boxes. The numbers on the left side indicate the respective nucleoside positions. 20,000 cpm labeled tRNA^{Asp}, corresponding to approximately 10 ng, was used per lane. (A) Footprinting with Tgt protein. The lanes are denoted as follows: C, control, without RNase; A, total hydrolysis in alkaline buffer, bands are visible after longer exposures only; T1, digestion with RNase T1, the major bands representing single-stranded guanosines are labelled with arrows and numbered according to their position in the tRNA, minor bands correspond to normally base-paired guanosines which were exposed to RNase cleavage in areas with melted secondary structure. Lanes 4–6, samples with Tgt protein added; lane 4, 0.75 μg (corresponding to a 44 fold molar excess compared to the amount of tRNA); lane 5, 1.5 μg (88 fold excess); lane 6, 3 μg (176 fold excess). Lane 7, no protein. (B) Footprinting with QueA protein. The lanes A, C, and T1 are as in A; lanes 4–8 samples with QueA protein; lane 4, 0.25 μg (corresponding to a 16 fold molar excess compared to the amount of tRNA); lane 5, 0.01 μg (0.6 fold excess); lane 6, 0.05 μg (3 fold excess); lane 7, 0.1 μg (6 fold excess); lane 8, 0.5 μg (32 fold excess); lane 9, no protein added; lane 10, 0.5 μg QueA and 2 mM AdoMet. (C) Enlarged portion of a footprint showing the interactions of QueA protein with the dihydrouridine loop; the tRNA positions are indicated on the right. Lane 1, 0.5 μg QueA protein; lane 2, no protein added; lane 3, 0.5 μg QueA protein and 2 mM AdoMet.

tRNAs with a second major class of mostly neglected proteins, the tRNA modifying enzymes. Especially interesting in this regard are the enzymes involved in the biosynthesis of the hypermodified nucleoside Queuosine (Q). (i) Four different tRNAs (tRNA^{Asp,Asn,His,Tyr}) with vastly varying primary structure are modified with Q by the same enzymes. (ii) The Q modification is conserved throughout evolution and the isolated enzymes from *E. coli* are still able to recognize tRNAs from higher and lower eukaryotes, including the yeast tRNAs, although yeast is one of the very rare exceptions where Q is not naturally present. To learn more about this unusual interaction we analysed the structure of complexes between the tRNA modifying enzymes Tgt and QueA and a specific substrate by footprinting.

As shown by HPLC and band shift analysis, an in vitro transcribed tRNA serves as a substrate for both enzymes. Therefore no modifications are necessary for recognition. This is congruent with the finding of Curnow et al. [5] that a T7 transcript of an *E. coli* tRNA^{Tyr} is accepted by the Tgt enzyme with essentially the same kinetic parameters as a purified wild-

Table 1
Compilation of the anticodon stem-loop sequences of Q specific tRNAs in *E. coli* and yeast

tRNA specific for		Anticodon stem-loop sequence			
Asn from	*E. coli*	G^{27}CGGAC	U **preQ$_1$** U U	t6A	AFCCGU[43]
	yeast	U^{27}GCGAC	U **preQ$_1$** U U	t6A	AFCGCA[43]
Asp from	*E. coli*	C^{27}CUGCC	U **preQ$_1$** U C	m2A	CGCAGG[43]
	yeast	G^{27}GCGCF	U **preQ$_1$** U C	m1G	CGUGCC[43]
His from	*E. coli*	C^{27}UGGAU	U **preQ$_1$** U G	m2A	FFCCAG[43]
	yeast	F^{27}ACGCF	U **preQ$_1$** U G	m1G	FGCGUU[43]
Tyr from	*E. coli*	G^{27}CAGAC	U **preQ$_1$** U A	ms2i6A	AFCUGC[43]
	yeast	F^{27}UGAGC	U **preQ$_1$** U A	i6A	ACUCAA[43]

Conserved nucleosides are indicated in bold letters. preQ$_1$, 7-aminomethyl-7-deazaguanosine; t6A, *N*-((9-ribofuranosylpurin-6-yl)carbamoyl) threonine; F, pseudouridine; m2A, 2-methyladenosine; m1G, 1-methylguanosine; ms2i6A, 2-methylthio-*N*-6-isopentenyladenosine; i6A, *N*-6-isopentenyladenosine.
Data taken from [15].

type $tRNA^{Tyr}$. Interestingly three different Tgt tRNA complexes are visible in band shift analysis depending on the protein concentration, which is consistent with the reported existence of Tgt trimers in concentrated solutions [14].

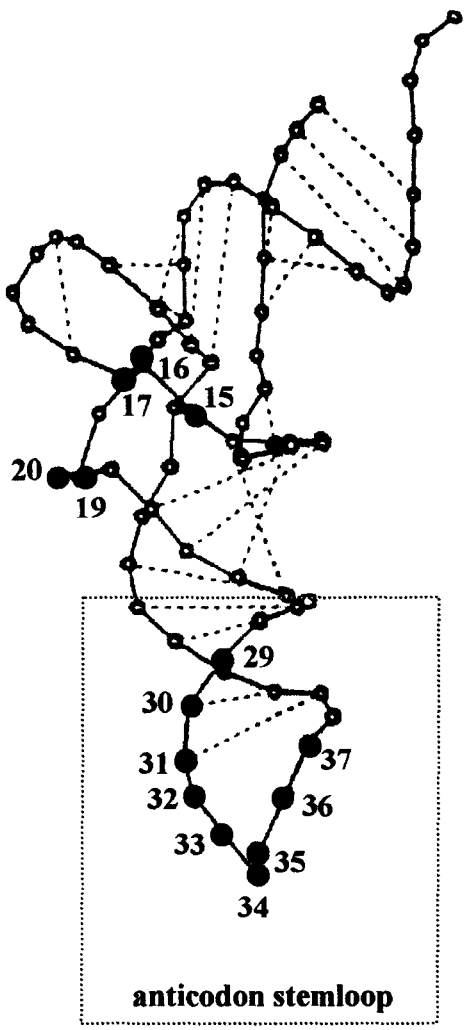

Fig. 5. Schematic drawing of the proposed protein-tRNA interaction sites, according to the footprinting results, superimposed on the structure of $tRNA^{Asp}$. (The three-dimensional structure of wild-type $tRNA^{Asp}$ is modified from Edqvist et al. [8].) Nucleotides that are protected or more accessible for RNase I after protein binding are indicated by filled circles and labeled by the corresponding position numbers. The anticodon stem-loop structure equivalent to the RNA microhelix that served as a substrate for the Tgt and QueA proteins is boxed.

For both enzymes the only strong protection of the tRNA from digestion with RNase I in the footprinting studies occurs in the anticodon loop. Further interactions are visible in the dihydrouridine loop, mainly by enhancement of the RNase accessibility, indicating a distortion of its natural conformation. This interaction pattern could be explained by an approach of the protein from the 'backside' of the L-shaped tRNA molecule. The protein–tRNA interaction sites, as detected by footprinting, are imposed on a three-dimensional picture of the $tRNA^{Asp}$ in Fig. 5. Previous results showed that under laboratory conditions Tgt and QueA recognise a microhelix structure equivalent to the anticodon stem-loop of a Q specific tRNA. Additionally it was found that for Tgt this microhelix is almost as efficient as a whole tRNA in kinetic terms [5]. Our findings strongly suggest that even under physiological conditions the only important part of the tRNA for the recognition by Tgt and QueA is indeed the anticodon stem-loop.

Since the 5 base-paired stem and the 7 base anticodon loop is common to all tRNAs the ultimate specificity for Q tRNAs must depend on special sequences therein. Comparison of the sequences of the anticodon stem-loops of the Q specific tRNAs in *E. coli* and yeast, as shown in Table 1, reveals that there are no conserved nucleosides except U^{33}, G/preQ$_1^{34}$, and U^{35}. Furthermore U^{33} is conserved among all tRNAs. Therefore only the two bases at position 34 and 35 within a general stem-loop structure seem to be the true identity elements specifying Q tRNAs.

Acknowledgements: We thank H. Kersten for excellent advice and critical discussion, R. Giegé for providing us with the pTFMa plasmid, S. Nishimura for the synthetic preQ$_1$ base, and K. Reuter for the Tgt enzyme. This work was supported by the Deutsche Forschungsgemeinschaft Grant Ke 98/20-1.

References

[1] Limbach, P.A., Crain, P.F. and McCloskey, J.A. (1994) Nucleic Acids Res. 22, 2183–2196.
[2] Kersten, H. and Kersten, W. (1990) in: Chromatography and Modification of Nucleosides, Part B. Biological Roles and Function of Modification (Gehrke, W. and Kuo, K.C.T. eds.) Elsevier, Amsterdam.
[3] Slany, R.K. and Kersten, H. (1994) Biochimie (in press).
[4] Slany, R.K., Bösl, M. and Kersten, H. (1994) Biochimie 76, 389–393.

[5] Curnow, A.W., Kung, F.-L., Koch, K.A. and Garcia, G.A. (1993) Biochemistry 32, 5239–5246.
[6] Slany, R.K., Bösl, M., Crain, P.F. and Kersten, H. (1993) Biochemistry 32, 7811–7818.
[7] Perret, V., Garcia, A., Grosjean, H., Ebel, J.P., Florentz, C. and Giegé, R. (1990) Nature 344, 787–789.
[8] Edqvist, J., Grosjean, H. and Stråby, K.B. (1992) Nucleic Acids Res. 20, 6575–6581.
[9] Milligan, J.F. and Uhlenbeck, O.C. (1989) Methods Enzymol. 180, 51–62.
[10] Frey, B., McCloskey, J.A., Kersten, W. and Kersten, H. (1988) J. Bacteriol. 170, 2078–2082.
[11] Knapp, G. (1989) Methods Enzymol. 180, 192–212.
[12] Komine, Y., Adachi, T., Inokuchi, H. and Ozeki, H. (1990) J. Mol. Biol. 212, 579–598.
[13] Cavarelli, J. and Moras, D. (1993) FASEB J. 7, 79–86.
[14] Garcia, G.A., Koch, K.A. and Chong, S. (1993) J. Mol. Biol. 231, 489–497.
[15] Steinberg, S., Misch, A. and Sprinzl, M. (1993) Nucleic Acids Res. 21, 3011–3015.