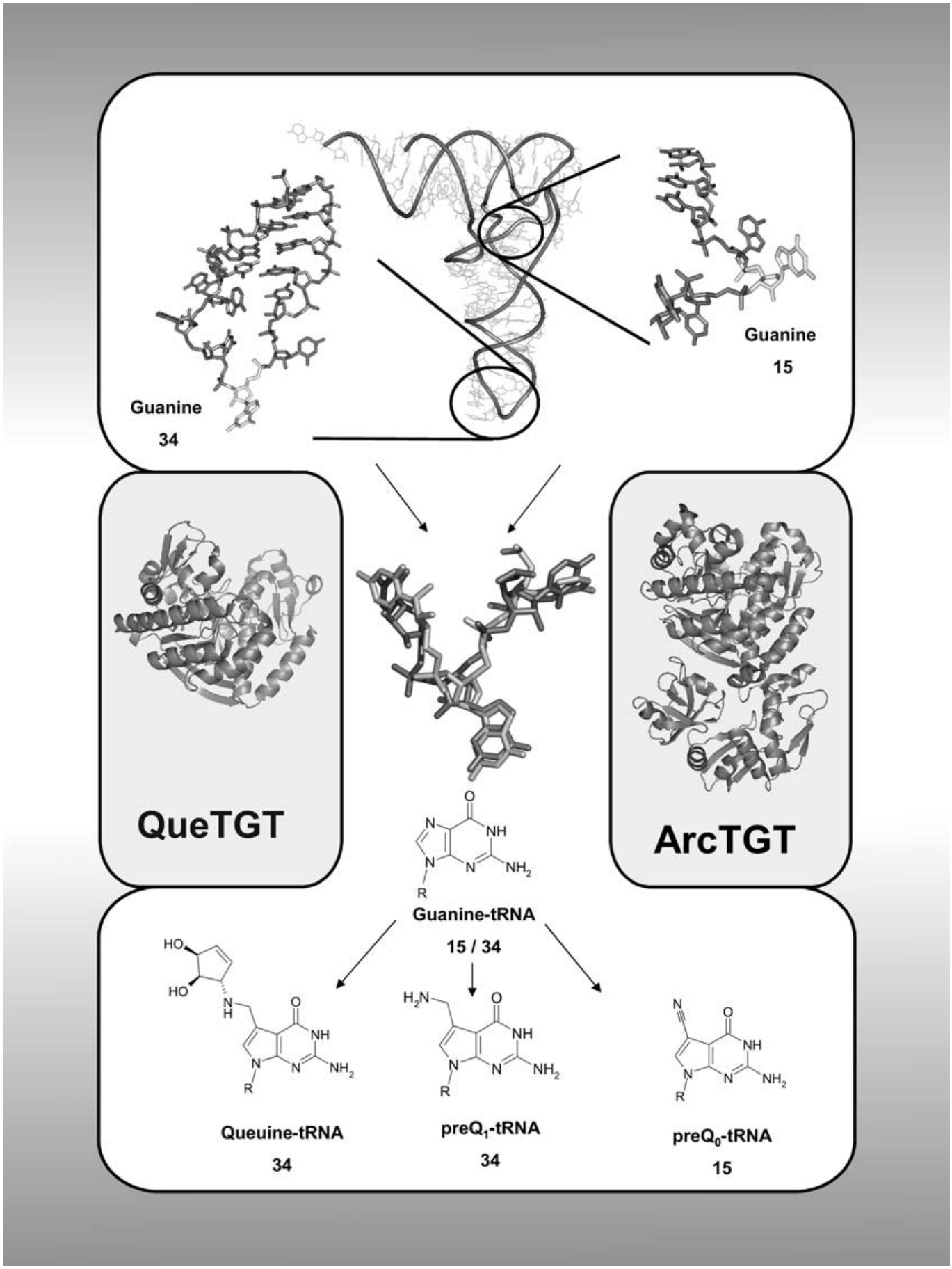

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Mechanism and Substrate Specificity of tRNA–Guanine Transglycosylases (TGTs): tRNA-Modifying Enzymes from the Three Different Kingdoms of Life Share a Common Catalytic Mechanism

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Transfer RNA–guanine transglycosylases (TGTs) are evolutionarily ancient enzymes, present in all kingdoms of life, catalyzing guanine exchange within their cognate tRNAs by modified 7-deazaguanine bases. Although distinct bases are incorporated into tRNA at different positions in a kingdom-specific manner, the catalytic subunits of TGTs are structurally well conserved. This review provides insight into the sequential steps along the reaction pathway, substrate specificity, and conformational adaptions of the binding pockets by comparison of TGT crystal structures in complex with RNA substrates of a eubacterial and an archaebacterial species. Substrate-binding modes indicate an evolutionarily conserved base-exchange mechanism with a conserved aspartate serving as a nucleophile through covalent binding to C1′ of the guanosine ribose moiety in an intermediate state. A second conserved aspartate seems to control the spatial rearrangement of the ribose ring along the reaction pathway and supposedly operates as a general acid/base. Water molecules inside the binding pocket accommodating interaction sites subsequently occupied by polar atoms of substrates help to elucidate substrate-recognition and substrate-specificity features. This emphasizes the role of water molecules as general probes to map binding-site properties for structure-based drug design. Additionally, substrate-bound crystal structures allow the extraction of valuable information about the classification of the TGT superfamily into a subdivision of presumably homologous superfamilies adopting the triosephosphate isomerase type barrel fold with a standard phosphate-binding motif.

1. Introduction

Transfer RNA–guanine transglycosylases (TGTs, EC 2.4.2.29) are tRNA-modifying enzymes present in eubacteria and eukaryotes, as well as in archaebacteria; these enzymes incorporate modified 7-substituted 7-deazaguanine bases into tRNAs in a kingdom-specific manner (Scheme 1).[1] Despite those differences, all of the enzymes adopt the highly populated triose-phosphate isomerase (TIM) like $(\beta\alpha)_8$-barrel fold, with specific insertions involved in tRNA recognition and binding, the most prominent of which is a zinc-binding site close to the C terminus (Figure 1a and b).[2–4] Due to these insertions, the overall shape of TGTs is sufficiently unique to form a homologous superfamily within the TIM/$(\beta\alpha)_8$-barrel fold (SCOP Version 1.65,[5,6] CATH Version 2.5.1,[7,8]). TGTs are involved in the hypermodification of tRNAs.[1] Modified bases are very common to tRNAs.[9,10] Although their ultimate functions are still unknown in most cases, they most likely influence the translational properties or support the stability of tRNAs depending on the site of occurrence.[11–14] In particular, TGTs from eubacteria and archaebacteria have been well characterized, thereby unraveling some important differences.[1]

Eubacterial TGTs catalyze the exchange of guanine by the premodified base 7-(aminomethyl)-7-deazaguanine ($preQ_1$) in the anticodon position 34 (the "wobble position") of tRNAs specific for the amino acids Asn, Asp, His, and Tyr and characterized by the anticodon sequence $G^{34}U^{35}N^{36}$ (N = A, U, C, or G) (Figure 1e).[15] These tRNAs share a common $U^{33}G^{34}U^{35}$ sequence, which serves as a recognition sequence for TGT (Figure 1g).[16,17] $PreQ_1$ is supposedly synthesized from guanosine triphosphate (GTP).[18] Recently, four genes referred to as *queC*, *queD*, *queE*, and *queF*, whose products are thought to be involved in this process, have been identified.[19] Once incorporated, $preQ_1$ is further modified into 7-(((4,5-*cis*-dihydroxy-2-cyclopenten-1-yl)amino)methyl)-7-deazaguanine (queuine) in two subsequent steps, catalyzed by *S*-adenosylmethionine:tRNA ribosyltransferase-isomerase (the QueA enzyme) and a still unknown cofactor B_{12} dependent enzyme (Scheme 2).[1,20] To date, the only available crystal structure of a eubacterial TGT comes from *Zymomonas mobilis* (Figure 2b; PDB code: 1PUD; for further details of the presented structure, see Table 2).[2]

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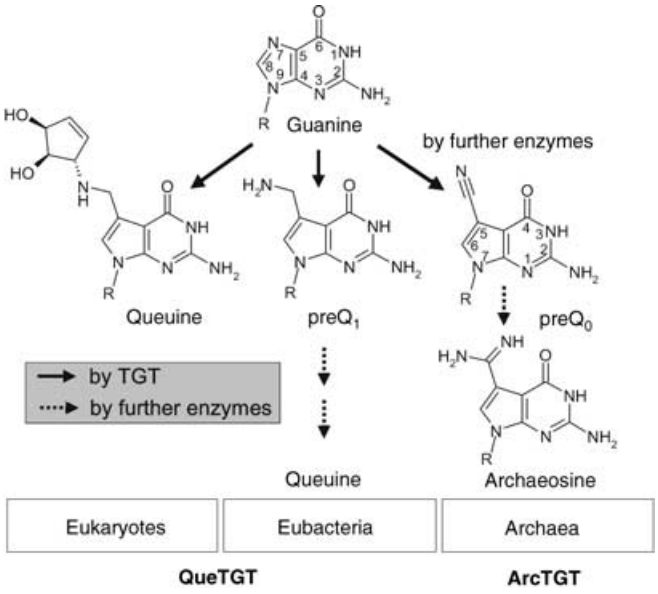
Bernhard Stengl, born in 1973, studied biology and chemistry for the teacher's profession at the University of Erlangen, Germany. In 2000 he stayed as a Cusanuswerk scholar at the University of Florence, Italy, to investigate cuticular hydrocarbon profiles and signaling in an ant species for his state examination thesis in the laboratory of Prof. J. Heinze, University of Erlangen. Since 2002 he has been working in the laboratory of Prof. G. Klebe, where he is developing new lead structures against tRNA-guanine transglycosylases

Klaus Reuter, born in 1962, studied biology at the University of Erlangen, Germany, and obtained his doctorate in biochemistry in 1992. In 1997 he moved, after a postdoctoral position at the Institute of Biochemistry in Erlangen and a three-month stay at the University of Michigan in Ann Arbor, USA, to the Institute for Molecular Biology and Tumor Research in Marburg, Germany. In 2001 he joined the group of Prof. G. Klebe at the Institute of Pharmaceutical Chemistry in Marburg. In the same year he obtained his Habilitation in microbiology and biochemistry. His research concentrates on the biochemical and structural investigation of bacterial- and protozoan-specific proteins that are not present in humans.

Gerhard Klebe, born in 1954, studied chemistry at the University of Frankfurt, Germany, and obtained his doctorate in physical chemistry. As a Studienstiftung scholar he spent a year in Grenoble, France, at the CNRS and ILL. After postdoctoral positions in crystallography (working with Prof. H. Fuess and Prof. H. B. Bürgi), he joined BASF-AG in Ludwigshafen, Germany, where he was responsible for molecular modeling and crystallography in drug research. In parallel he obtained his Habilitation in pharmaceutical and structural chemistry at the University of Heidelberg, Germany, in 1992 and was appointed full Professor for Pharmaceutical Chemistry at the University of Marburg, Germany, in 1996. The focus of his research is directed towards various aspects of drug design, including areas from chemistry, microcalorimetry, and crystallography to bioinformatics and software development for the elucidation of details of the interactions of small-molecule ligands with proteins.



Scheme 1. Chemical structures of guanine and 7-substituted-7-deazaguanine bases. TGTs catalyze the exchange of specific guanine residues in tRNAs towards modified bases in a kingdom-specific manner. $PreQ_1$ = 7-(aminomethyl)-7-deazaguanine, $preQ_0$ = 7-cyano-7-deazaguanine, ArcTGT = archaeosine TGT, QueTGT = queuosine TGT.

Eukaryotic TGTs show high sequence identity to eubacterial TGTs—for example, 43% between *Homo sapiens* and *Z. mobilis*[21]—but exhibit a different substrate specificity (Scheme 1).[22] They directly incorporate queuine at the wobble position of tRNAs showing the same specificity as those recognized by the eubacterial TGTs. Since eukaryotes are unable to synthesize queuine de novo, it has to be taken up from food.[23] Until now, no crystal structures could be determined of any eukaryotic TGT, but homology models based on eubacterial TGT indicate a very high similarity concerning the structure of the active site.[3] Eubacterial and eukaryotic TGTs are also referred to as queuosine TGTs (QueTGTs) since they both are involved in the modification of tRNA anticodons with queuine (Scheme 1). Queuine modification is thought to affect the speed and accuracy of the translational process and was reported to exhibit pleiotropic effects on cellular metabolism.[1,24]

In contrast, archaebacterial TGTs, showing only about 20–25% sequence identity to eubacterial TGTs,[3] exhibit some exceptional differences. Most remarkably, they address guanine at a completely different site, namely position 15 of the D-arm in the majority of archaeal tRNAs (Figure 1e).[25] Usually G^{15} is involved in the formation of tertiary interactions to stabilize the canonical L shape and is thus buried in the tRNA core.[1] To make G^{15} accessible, the tRNA has to undergo a pronounced conformational rearrangement. Crystal structure analysis of the archaebacterial TGT from *P. horikoshii* in complex with $tRNA^{Val}$ (PDB: 1J2B) showed that this rearrangement results in the so-called λ-shaped tRNA, which exhibits a conformation that has never been observed before (Figure 1e).[26] Crucial for the enzymatic stabilization of this unusual conformation are three C-terminal domains (referred to as C1, C2, and C3), which are

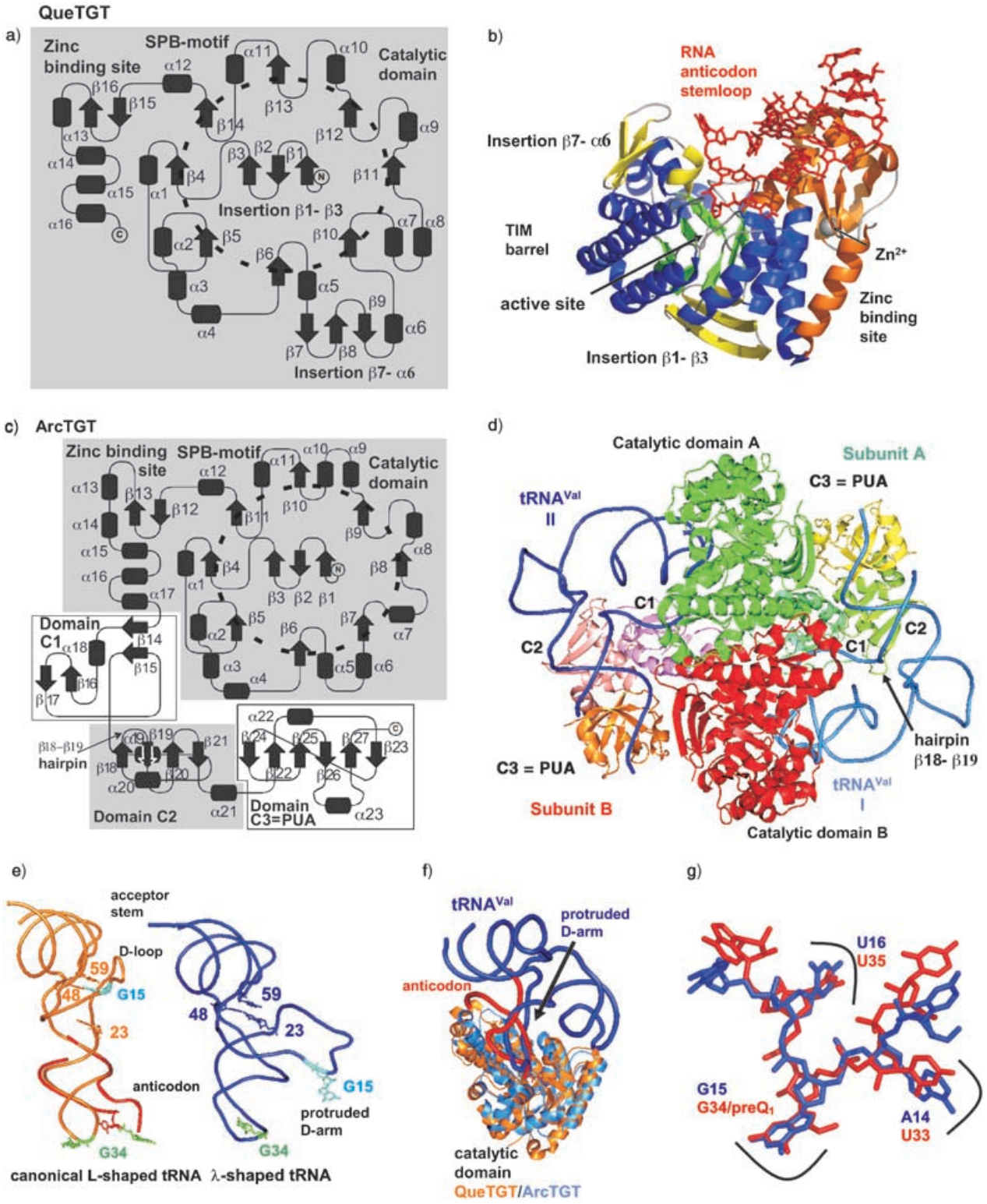
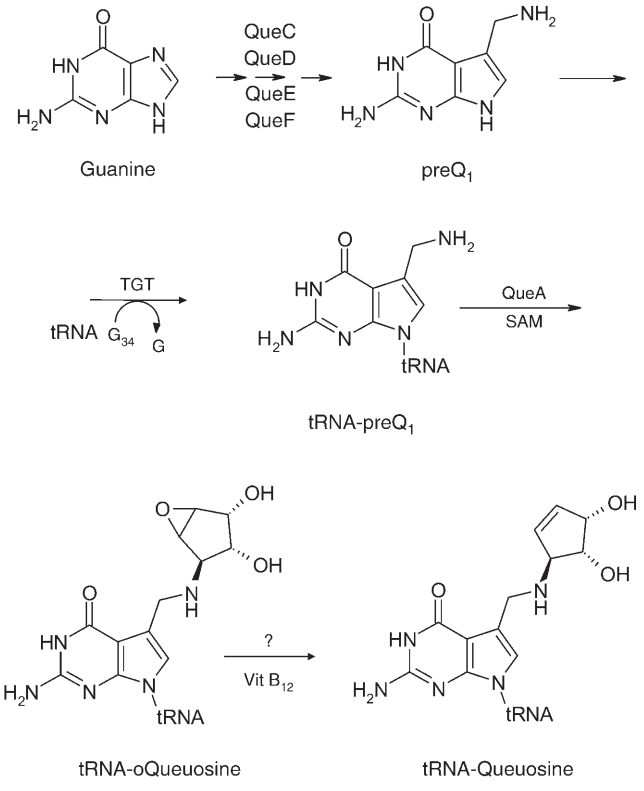


Figure 1. a) Schematic folding patterns of QueTGT. b) Overall structure of *Z. mobilis* QueTGT in complex with an RNA anticodon stemloop. Insertions (yellow, orange) flanking the TIM-barrel core (green/blue) guarantee proper tRNA recognition. c) Schematic folding pattern of ArcTGT. In contrast to the situation in QueTGT, in ArcTGT the catalytic domain is extended by three supplementary C-terminal domains (C1, C2, C3 = PUA). d) Overall structure of functional *P. horikoshii* ArcTGT dimer in complex with two $tRNA^{Val}$ substrates. Both TGT subunits are involved in the binding of one tRNA substrate. e) Overall structures of yeast $tRNA^{Phe}$ in the canonical L shape (orange) with superimposition of the RNA anticodon stemloop (red) from the QueTGT complex and $tRNA^{Val}$ from *P. horikoshii* in λ shape (blue) as found in the ArcTGT complex. QueTGTs address G^{34} in the anticodon (green). To make G^{15} (blue) addressable for ArcTGT, the D-arm is protruded. f) Structural superimposition of the highly homologous catalytic domains from QueTGT (orange) and ArcTGT (blue) with bound RNA substrates in remarkably different overall binding geometry (blue: $tRNA^{Val}$, red: RNA anticodon stemloop). g) ArcTGTs and QueTGTs bind RNA trinucleotide sequences with G^{34} or G^{15}, respectively, in the central position in similar conformations through three conserved substrate binding subpockets. (a) and (c) were produced by using the TopDraw program.[49] All other figures were produced by using the PYMOL program.[50]

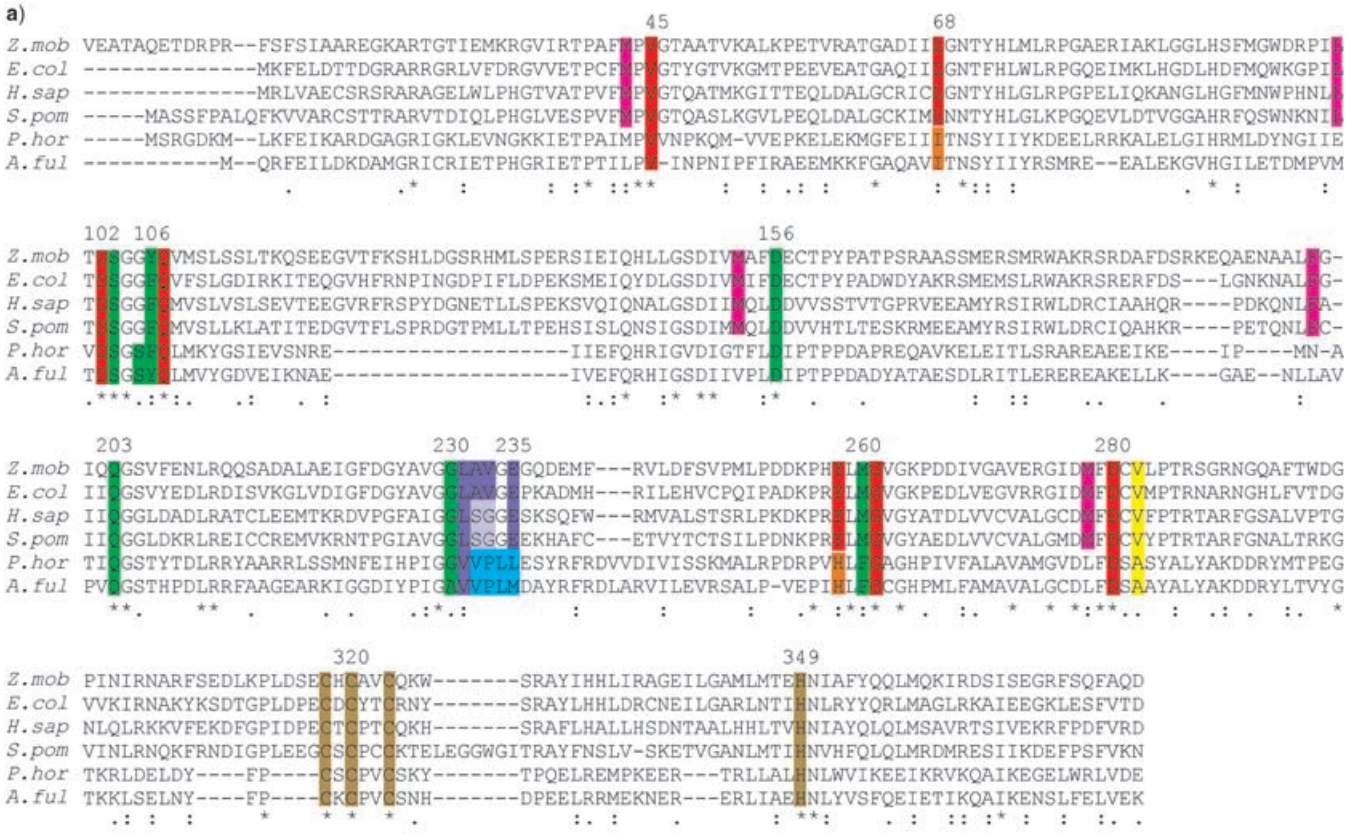


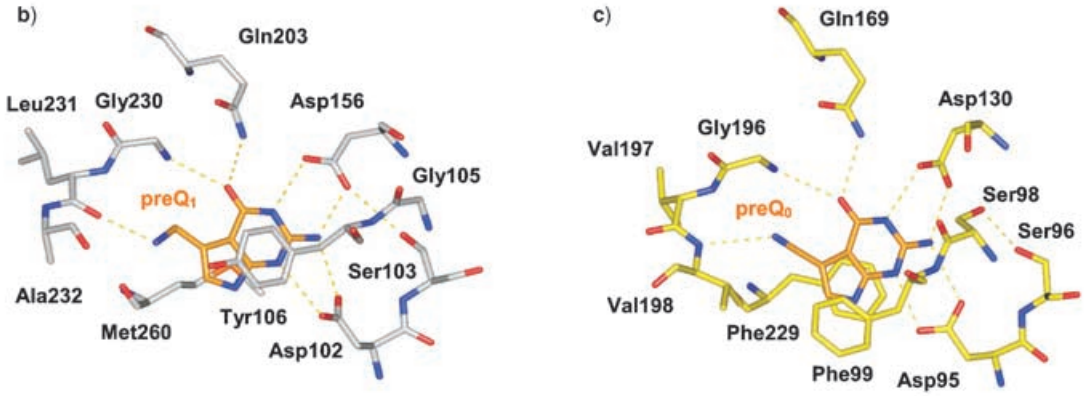
Scheme 2. Assumed steps of queuosine synthesis in eubacteria. Eukaryotic TGT directly incorporates queuine into tRNAs. It is unable to synthesize queuine de novo. In archaea the steps of $preQ_0$ synthesis and the modification steps towards archaeosine are still unknown. QueA = *S*-adenosylmethionine:tRNA ribosyltransferase-isomerase, SAM = *S*-adenosylmethionine, QueC–QueF have not yet been characterized.

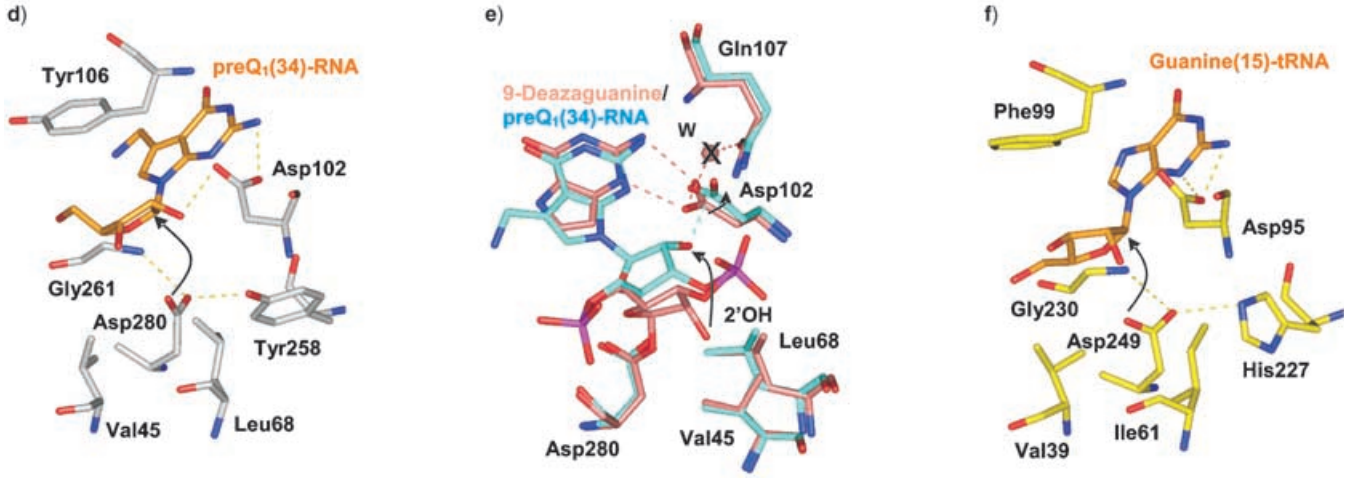
specific for archaebacterial TGTs and are completely missing in QueTGTs (Figure 1c).[27] Among these domains, the most C-terminal one (C3) represents a pseudouridine synthase and archaeosine TGT (PUA) domain, widespread among RNA-binding proteins.[28] Furthermore, full functionality of archaebacterial TGTs requires dimer formation (Figure 1d). Both TGT subunits forming the dimer are involved in the modification of an attached tRNA substrate molecule. While one TGT subunit recognizes the tRNA and stabilizes the λ shape through its three C-terminal domains, the other subunit catalyzes the base exchange after accommodation of G^{15} in the active site of its catalytic domain. By contrast, QueTGT is probably active as a monomer. With respect to substrate specificity, a further significant difference has to be noted. Archaebacterial TGTs will recognize neither $preQ_1$ nor queuine. Their physiological function is to incorporate 7-cyano-7-deazaguanine ($preQ_0$), a base structurally related to $preQ_1$, into tRNA (Scheme 1).[29,30] Finally, this base is converted by further, as yet unknown, steps into 7-formamidino-7-deazaguanine (archaeosine). Consequently, archaebacterial TGTs, in analogy to QueTGTs, are referred to as archaeosine TGTs (ArcTGT). Archaeosine modification is thought to support tRNA stability under the predominantly high temperatures most archaea are exposed to, due to supplementary salt bridges formed between the positively charged formamidino group of archaeosine and the RNA phosphate backbone groups.[1,29]

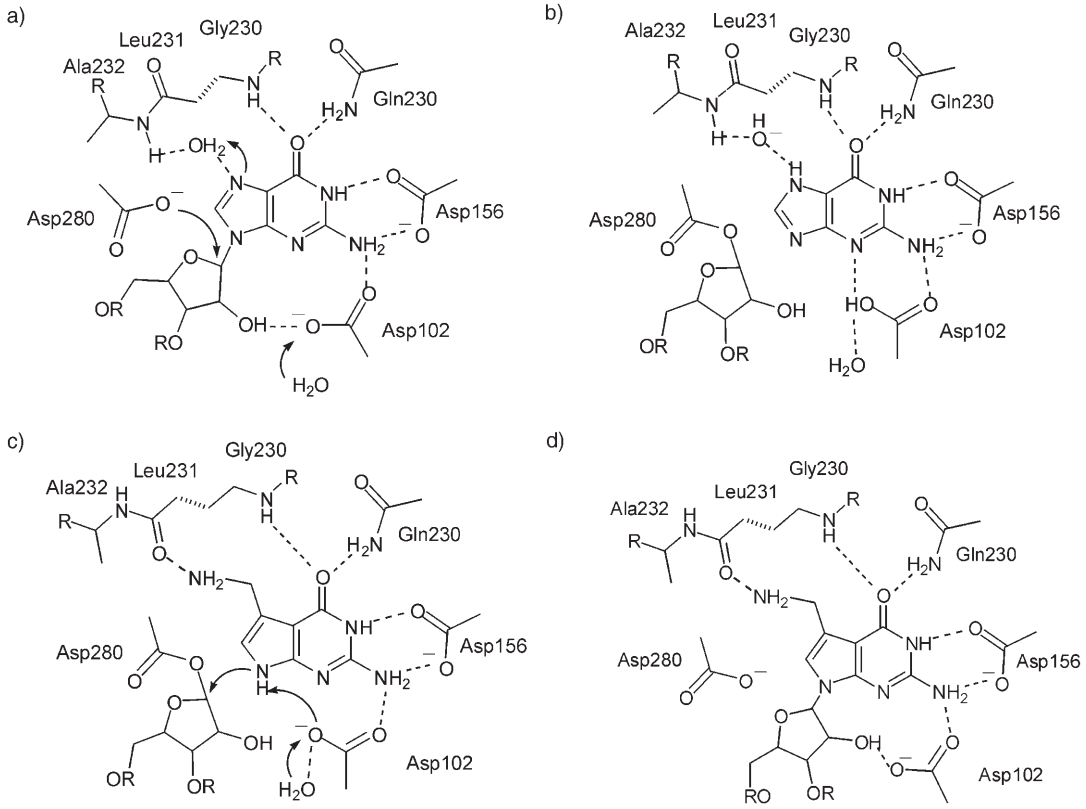
The base-exchange mechanism catalyzed by TGTs was thoroughly examined by means of mutational, kinetic, and further biochemical studies. An associative, ping-pong mechanism was proposed to be effective for the base exchange (Scheme 3).[31] In a first step, an aspartic acid in the binding pocket nucleophilically attacks C1′ of the G^{34} ribose sugar, therby resulting in cleavage of the glycosidic C–N bond and in the formation of a covalent intermediate between the enzyme and the depurinated tRNA (Scheme 3a and b). After the cleaved guanine is displaced by $preQ_1$ within the substrate-binding pocket, the covalent TGT·tRNA complex is disintegrated and an *N*-glycosidic bond is formed between the $preQ_1$ N7 atom and C1′ of the ribose (Scheme 3c and d). The crystal structure of the reaction intermediate of eubacterial *Z. mobilis* TGT covalently bound to an RNA substrate (Figure 1b; PDB code: 1Q2R) clearly indicates Asp280 to be the catalytic nucleophile,[32] although Asp102 had been assigned this role previously due to misinterpretation of the results from structural, mutational, and biochemical studies.[33–35] Chemical trapping of a covalent TGT·RNA intermediate in the solid state was achieved by using inactive 9-deazaguanine as a substrate analogue to block the active site after covalent complex formation (Figure 2e). In a further experiment, 9-deazaguanine was replaced by the natural substrate $preQ_1$ in the crystalline state. As a result, $preQ_1$, incorporated in the tRNA, remained trapped in the crystal; structure determination of the altered complex was performed (Figure 2e; PDB code: 1Q2S). 9-Deazaguanine and $preQ_1$ occupy the binding pocket in a similar fashion to that observed in binary TGT·$preQ_1$/$preQ_0$/inhibitor complex structures published elsewhere.[36,37]

Figure 2. a) T-COFFEE alignment[51] of the TIM/$(\beta\alpha)_8$ barrel and zinc-binding site of TGT protein sequences from the three kingdoms of life (sequences from SWISS-PROT[52]): QueTGTs from *Z. mobilis* and *Escherichia coli* (eubacterial), *Homo sapiens*, and *Schizosaccharomyces pombe* (eukaryotic), with ArcTGTs from *Pyrococcus horikoshii* and *Archaeoglobus fulgidus*. Colors: red/orange: residues involved in base exchange; pink: conserved residues involved in stabilization of Tyr258 in QueTGT; green: residues involved in recognition of common substrate functionalities; blue, slate blue, grey: residues responsible for substrate specificity; yellow: conserved tRNA-phosphate-binding position in the SPB motif; brown: conserved zinc-binding residues from the zinc-binding site. Residue numbers correspond to *Z. mobilis* TGT. b) Binding mode of $preQ_1$ complexed with QueTGT from *Z. mobilis*. The amino methyl group of the ligand is hydrogen bonded to the carbonyl group of Leu231. c) Binding mode of $preQ_0$ complexed with ArcTGT from *P. horikoshii*. The cyano group of the ligand is hydrogen bonded to the amide group of Val198. d) Binding mode of $preQ_1$-modified RNA with QueTGT from *Z. mobilis*. Asp280, which is responsible for the nucleophilic attack in QueTGT, is firmly kept in position by Gly261 and Tyr258. e) Movement of the RNA ribose 34 upon product formation. In the covalent intermediate state (pale pink), C1′ is covalently bound to Asp280. The 2′-OH group sticks into a hydrophobic subpocket formed by Val45 and Leu68. Upon product formation (cyan) the ribose rotates about some 40° towards $preQ_1$. The 2′-OH group now forms a hydrogen bond with Asp102 and a water molecule is expelled from the binding pocket. f) Binding mode of unmodified tRNA to ArcTGT from *P. horikoshii*. Due to the similar and conserved structure of the binding pocket, as compared to that of QueTGT (Figure 2d), Asp249 is predicted to perform the nucleophilic attack in ArcTGT. (b)–(f) were produced by using the PYMOL program.[50]









Scheme 3. Subsequent steps of the base-exchange mechanism catalyzed by eubacterial TGT as determined from crystal structure analysis. $Asp280^{Q}$ performs a nucleophilic attack on the ribose carbon atom C1′ (a) to form a covalent intermediate state (b). $Asp102^{Q}$ serves as a general acid/base for the deprotonation of $preQ_1$ (c), thereby inducing product formation (d). An alternative mechanism for the reprotonation of guanine after cleavage from tRNA is presented in (b).

The aforementioned crystal structures, in combination with amino acid sequence alignments of TGT enzymes from different species, allow for the first time the assignment of a comprehensive set of crucial residues involved in catalysis and responsible for substrate specificity in the three kingdoms of life.

2. Global Recognition of tRNA by TGTs

At first glance, a direct comparison of substrate recognition by ArcTGT and QueTGT seems rather difficult, as the guanine residues recognized and replaced by the two TGTs are located at completely different positions on the tRNA strand (Figure 1e). While QueTGTs perform the base exchange with G^{34} in the wobble position of the anticodon loop, ArcTGTs replace G^{15}, which is present in the dihydrouridine loop (D-loop). Thus, the overall binding geometry of tRNA with respect to the catalytic domain is remarkably different (Figure 1f). Furthermore, to make these bases addressable by TGTs, tRNAs have to undergo specific conformational changes in both cases (Figure 1e).

In canonical L-shaped tRNAs, G^{15} is involved in the formation of tertiary interactions with the bases C^{48} and A^{59} and is therefore buried within the tRNA core. To make G^{15} accessible to ArcTGTs, tRNA has to undergo a pronounced conformational rearrangement. The crystal structure of TGT from *P. horikoshii* in complex with $tRNA^{Val}$ (Figure 1d) gave an insight into this marked process of structural reorganization, which results in a formerly unknown λ-shaped tRNA (Figure 1e).[26] In this conformation, the usually rigid D-arm protrudes and the D-loop becomes accessible. This tRNA conformation is stabilized by a helical element specific for λ-shaped tRNA and not found in canonical L-shaped tRNA. This so-called "DV" helix is formed by bases from the variable loop and bases normally involved in formation of the D-stem. This led to the postulation of an additional function of the variable loop with respect to tRNA maturation. As a consequence of this spatial rearrangement, the former position of G^{15} is now occupied by G^{23} at the end of the "DV" helix. A crucial factor for the stabilization of the λ conformation by ArcTGT is supposedly the β18–β19 hairpin within the ArcTGT-specific C2 domain, which interacts with tRNA core bases after "DV"-helix formation (Figure 1c and d).

By contrast, the conformational changes of substrate tRNA due to its binding to QueTGT seem to be less pronounced. Structural superimposition of the RNA-stemloop substrate, used for crystal structure analysis with *Z. mobilis* TGT, onto the structure of the entire $tRNA^{Phe}$ from yeast (PDB code: 1EHZ)[38] showed that the anticodon loop is twisted into another direction to enable specific recognition of the $U^{33}G^{34}U^{35}$ sequence (Figure 1e).[32]

3. Functional Aspects of the Base Exchange

3.1. tRNA recognition by the active site

Once the pronounced difference in overall tRNA substrate recognition is realized, a closer look at the surroundings of the binding pocket and a more detailed analysis of its structural features reveal some surprising conservations between ArcTGTs and QueTGTs (Figure 1 f and g). The substrate-binding pockets of both QueTGTs and ArcTGTs accommodate a trinucleotide sequence consisting of the guanine nucleotide addressed by the respective enzyme as well as of the directly preceeding and following nucleotides. Thereby, the QueTGT-bound trinucleotide is present in a very similar conformation to the one bound to ArcTGT. In the case of QueTGTs, this trinucleotide is $U^{33}G^{34}U^{35}$, which is strictly conserved in all Q-specific tRNAs. U^{33} and U^{35} are specifically recognized by QueTGTs through the formation of polar interactions with functional groups of the uracil bases. As U^{33} is present in all tRNAs, the amino acids interacting with U^{35} are particularly highly conserved to guarantee specific recognition.[32] By contrast, the G^{15} residue addressed by ArcTGTs is not embedded within a conserved sequence motif. In the crystal structure of the *P. horikoshii* TGT·tRNA complex, binding of the G^{15} residue flanking A^{14} and U^{16} is mainly achieved through hydrophobic interactions between the bases and amino acid residues lining the substrate-binding pocket.[26] This feature obviously permits ArcTGTs a pronounced promiscuity in base recognition. In this respect, it should be noted that this guanine is not modified in all archaebacterial tRNAs containing a guanine at position 15. The structural prerequisite for G^{15} modification in archaebacterial tRNAs is still unknown,[1] but it is thought to be associated with "DV"-helix formation ability.[26]

By contrast, the composition of the active site responsible for recognition of the central guanine residue (G^{15} or G^{34}, respectively) is highly conserved among all TGTs. Most of the residues involved in QueTGT and ArcTGT are retained or at least conservatively replaced. The following numbering refers to the *Z. mobilis* TGT representative for QueTGTs (residues^{Q}) and the *P. horikoshii* TGT representative for ArcTGTs (residues^{A}). The residues contributing to the recognition of the guanine-like skeleton of QueTGT substrates are $Asp102^{Q}$, $Ser103^{Q}$, $Asp156^{Q}$, $Gln203^{Q}$, and $Gly230^{Q}$. With $Asp95^{A}$, $Ser96^{A}$, $Asp130^{A}$, $Gln169^{A}$, and $Gly196^{A}$, they have identical counterparts in ArcTGTs (Figure 2 b and c).[27,37] While $Tyr106^{Q}$ and $Met260^{Q}$ perform a sandwich-like hydrophobic stacking with the base of the substrate in *Z. mobilis* TGT, only the $Phe229^{A}$ residue that corresponds to $Met260^{Q}$ hydrophobically stacks with the base of the substrate in *P. horikoshii* TGT. $Phe99^{A}$, corresponding to $Tyr106^{Q}$, cannot perform a similar stacking in *P. horikoshii* TGT as the peptide backbone of $Phe99^{A}$ is shifted slightly off from the recognition base (Figure 2 b and c). This difference in recognition results from the most remarkable difference between both pockets, a $Gly105^{Q}$/$Ser98^{A}$ exchange. This causes a deviating stabilization pattern of specific binding-pocket residues. While in *Z. mobilis* TGT $Ser103^{Q}$ is hydrogen bonded to $Asp156^{Q}$, in *P. horikoshii* TGT the $Ser96^{A}$ side chain is hydrogen bonded to $Ser98^{A}$ within the same loop, thereby resulting in a deviating loop geometry and subsequently in an altered substrate-recognition pattern. Remarkably, in RNA-complexed structures of *Z. mobilis* TGT the $Tyr106^{Q}$ side chain is found in a similar position to the $Phe99^{A}$ side chain in *P. horikoshii* TGT. In these structures both residues stabilize the ribose ring of the respective nucleotide substrate (Figure 2 d and f). Obviously, $Tyr106^{Q}$ does not sandwich the substrate base as observed in binary *Z. mobilis* TGT·base complexes (Figure 2 b). The functions of the nonconserved residues $Val197^{A}$/$Leu231^{Q}$ and $Val198^{A}$/$Ala232^{Q}$, which dominate substrate specificity, will be discussed later. The sequence alignment shown in Figure 2 a provides an overview of the conservation of residues involved in substrate binding, substrate recognition, and the base-exchange mechanism.

3.2. Catalytic mechanism

For *Z. mobilis* TGT the base exchange of guanine 34 for $preQ_1$ was shown to follow a ping-pong mechanism through a covalent intermediate state (Scheme 3 b).[31] Crystal structures of the RNA-complexed *Z. mobilis* TGT trapped in this intermediate state, as well as of *Z. mobilis* TGT complexed to the final $preQ_1$-modified RNA product, gave novel insights into the catalytic mechanism.[32] Thus, a more detailed reaction pathway can be postulated (Scheme 3). Due to unambiguous crystallographic evidence, the initial assignment of residues involved in catalysis had to be revised. Surprisingly, $Asp280^{Q}$ was identified as the catalytic nucleophile instead of the formerly discussed $Asp102^{Q}$. Furthermore, it became possible to interpret specific adaptations of the QueTGT active site in terms of catalysis.[32] Along the reaction pathway a covalent bond is formed through $Asp280^{Q}$ to RNA-ribose 34 and G^{34} is released. During this step the respective ribose moiety performs a rotation of about 40°. This rotation is supported and stabilized by the neighboring phosphate groups (Figure 2 e).[32] The side chain of $Asp280^{Q}$, strictly conserved in all TGTs, is firmly fixed in position through two hydrogen bonds formed by $Gly261^{Q}$ and $Tyr258^{Q}$ (Figure 2 d). Accordingly, $Asp280^{Q}$ is most likely present in its deprotonated state and performs the nucleophilic attack towards the RNA-ribose 34 sugar carbon atom through its β-carboxy group. $Tyr258^{Q}$ itself is arrested and kept in position through several hydrophobic interactions ($Met43^{Q}$, $Leu100^{Q}$, $Met153^{Q}$, $Phe199^{Q}$, $Met260^{Q}$, $Met278^{Q}$). $Asp280^{Q}$, $Gly261^{Q}$,and $Tyr258^{Q}$ as well as the neighboring hydrophobic residues are conserved in eubacterial and eukaryotic TGTs, thereby emphasizing their particular role to guarantee accurate adjustment of the nucleophile (Figure 2 a).[32] The new conformation of the ribose sugar covalently attached to $Asp280^{Q}$ shows an unexpected geometry. Its 2′-OH group orients towards a small hydrophobic pocket formed by $Leu68^{Q}$ and $Val45^{Q}$ (Figure 2 e). In this orientation the polar group cannot form any hydrogen bonds to the enzyme. The 2′-OH group is obviously arrested in this position solely through steric constraints. $Val45^{Q}$ is strictly conserved in eukaryotic and eubacterial TGTs; similarly, $Leu68^{Q}$ is also highly retained with only a few, structurally conservative exceptions. This hydrophobic subpocket seems to play a crucial role for the stabilization of the RNA-ribose 34 geometry

during nucleophilic attack and formation of the subsequent covalent intermediate. While the covalent bond to Asp280^{Q} is formed, the 2′-OH group has to penetrate the described local hydrophobic environment, where it experiences only weak and rather unfavorable interactions. Upon product formation, the 2′-OH group is released again from this "unpleasant" pocket while the covalent bond to the RNA-ribose 34 is disintegrated and both groups gain in mutual distance. Supposedly, the unfavorable intermediate occupancy of the hydrophobic pocket through the 2′-OH group serves as a kind of tense-spring state and stores energy for the conformational movements required during the ping-pong reaction pathway. After release of guanine and accommodation of preQ$_1$, the RNA-ribose 34 moiety rotates back again by about 40°, simultaneously placing the 2′-OH group into a position suited for hydrogen-bond formation to Asp102^{Q}.[32] Asp102, also strictly conserved, is involved in the specific recognition of 9-deazaguanine/preQ$_1$. Apart from this function, it is assumed that Asp102^{Q} serves as a general base (Scheme 3c). Prior to product formation, bound preQ$_1$ most likely gets deprotonated, with assistance from the carboxy group of Asp102^{Q}.[32] Furthermore, Asp102^{Q} is hydrogen bonded to a water molecule located at the upper rim of the binding pocket and mediating a contact between Asp102^{Q} and Gln107^{Q}, another strictly conserved residue (Figure 2e). This water could possibly assist the proton relay into and out of the binding pocket.[32] However, during product formation the 2′-OH group of RNA-ribose 34 moves towards Asp102^{Q} to form a hydrogen bond. This recognition can only be achieved once Asp102^{Q} also rotates its carboxy function in such a way as to optimize the geometry of the hydrogen bond to be formed. As a consequence of this rotation, the water molecule is released from the binding pocket. Upon liberation from the unfavorable hydrophobic pocket and in the due course of hydrogen-bond formation to Asp102^{Q}, the 2′-OH group subsequently drags the ribose moiety into the position required for the formation of the covalent bond to N9 of preQ$_1$. Accordingly, Asp102^{Q} in addition to its function as general acid/base imposes a strong directional driving force, thus controlling the structural changes upon product formation.

Due to the fact that no crystal structure of a corresponding covalent intermediate with an ArcTGT is available, residues likely to be important for catalysis can only be suggested by taking reference to QueTGT. Archaebacterial Asp249^{A} is in an equivalent position to eubacterial Asp280^{Q} and supposedly performs the nucleophilic attack onto the ribose carbon atom.[32] An aspartic acid in this position is conserved in the TGTs of all kingdoms. Similarly to QueTGTs, in the available ArcTGT structures the side-chain position of Asp249^{A} is stabilized through hydrogen bonds with two adjacent residues (Figure 2f): on one side to His227^{A}, a residue only conserved in archaebacteria, and on the other side through the backbone NH of Gly230^{A}. In QueTGT the conserved Tyr258^{Q} occupies the equivalent position to His227^{A}. Gly230^{A} is structurally conserved in all three kingdoms and corresponds to Gly261^{Q} in QueTGT. While in *Z. mobilis* TGT, Asp280^{Q} is firmly clamped by Tyr258^{Q} and Gly261^{Q} (Figure 2d), in *P. horikoshii* TGT the Asp249^{A} side-chain distances of the carboxy group towards the two residues His227^{A} and Gly230^{A} fall into a range of 2.5–3.5 Å depending on the presence of the bound substrate. Whether there is mechanistic reason for this structural difference with respect to the nucleophilic attack in ArcTGT still remains to be elucidated.

The tRNA-ribose 15 moiety is able to place its 2′-OH group into a hydrophobic pocket of archaebacterial TGTs in a similar way to that observed for tRNA-ribose 34 when bound to eubacterial TGT. In archaebacterial TGTs, this pocket is composed of the conserved residues Val39^{A} and Ile61^{A}, corresponding to Val45^{Q} and Leu68^{Q} in *Z. mobilis* TGT (Figure 2f). Asp95^{A} in archaebacterial TGT, a further residue conserved across all kingdoms, is equivalent to Asp102^{Q} in eubacterial TGT, but it adopts a slightly different binding-mode geometry. It does not form a hydrogen bond to the 2′-OH group in the complex with bound tRNA. However, one has to note that at the stage that corresponds to the determined crystal structure, the unmodified tRNA with guanine at position 15 is still bound. With consideration of the spatial restraints imposed by preQ$_0$ once it is accommodated in the binding pocket, the presence of preQ$_0$-modified tRNA could force the adjacent ribose ring to penetrate deeper into the binding pocket and enable hydrogen-bond formation through its 2′-OH group to Asp95^{A}. Thus, a binding mode similar to the eubacterial TGT·preQ$_1$-tRNA complex seems likely.

As a summary of the observations for both structures, all features necessary to perform the base-exchange reaction are similarly exhibited in either QueTGTs or ArcTGTs. Hence, an evolutionarily highly conserved mechanism must be assumed to originate from an ancient ancestor that existed even before the separation of the three kingdoms.

3.3. Substrate-specificity regulation

Another tempting puzzle to be solved across the TGT superfamily is the question of how the pronounced differences in substrate specificity and promiscuity are regulated. Compared to ArcTGTs, QueTGTs show an extended reservoir of substrates (Table 1).[15,22,30,39,40] In QueTGTs both preQ$_1$ (PDB code: 1P0E) and the archaebacterial substrate preQ$_0$ (PDB code: 1P0B) are accepted.[37] For preQ$_1$ recognition, the carbonyl group of the Leu231^{Q}/Ala232^{Q} peptide bond plays a crucial role in identifying the probably protonated aminomethyl moiety of preQ$_1$ (Figures 2b and 3a). Interestingly enough, crystal structure analyses revealed that this peptide bond does not occur in

Table 1. Substrate specificity of TGTs in the three kingdoms of life.[a]

Enzyme	Guanine[b]	preQ$_0$[b]	preQ$_1$[b]	Queuine[b]
archaebacterial TGT[30,40]	+	+	−	−
eubacterial TGT[15,39]	+	+	+	−
eukaryotic TGT[22]	+	+	+	+

[a] + = accepted as a substrate, − = not accepted as a substrate. [b] For chemical formulae, see Scheme 1.

one single orientation. In the presence of other substrates such as $preQ_0$ or in the case of a bound pyridazindion-type inhibitor (PDB code: 1N2V)[36] the peptide bond surprisingly adopts a flipped orientation, thereby presenting an NH-donor functionality towards the binding pocket (Figure 3a and c). In the case of a bound imidazole skeleton, the protein presents its peptidic NH group towards the ligand and a contact is mediated through a well-defined interstitial water molecule (Figure 3a). Due to the fact that the imidazole moiety of the inhibitor superimposes well with the pyrrolo moiety of 9-deazaguanine in the structure of the RNA complex, we assume that the NH-exposing conformation is also adopted in the case of the bound natural substrate G^{34}-tRNA and a water molecule intercedes a hydrogen bond towards the NH group of $Ala232^Q$. Unfortunately, the moderate resolution of the *Z. mobilis* TGT·RNA complex structure (2.9 Å) does not allow reliable information to be extracted from the electron density in order to confirm this assumption (Table 2). Similarly to the anticipated situation in QueTGTs, in the binary ArcTGT·guanine complex structure (PDB code: 1IT7) a water molecule is found connecting guanine with the $Val197^A$/$Val198^A$ peptidic NH group, which corresponds to the $Leu231^Q$/$Ala232^Q$ peptide bond (Figure 3b).[27] This suggests an identical guanine-binding mode in ArcTGTs and QueTGTs. From a mechanistic point of view, the water molecule at this position also provides an alternative scenario to formulate the reprotonation step of guanine after tRNA cleavage. With the two most important tautomers of guanine taken into consideration, reprotonation could either occur at position N9 or N7 of the imidazole moiety (Scheme 1). Reprotonation at N9 could be performed as described above by the general acid/base $Asp102^Q$. Alternatively, reprotonation at N7, as suggested by Iwata-Reuyl,[1] in this case through the interstitial water molecule could be possible as well (Scheme 3b).[1] The fact that the methyl group of $Ala232^Q$ in the NH-exposing conformation reduces the available space of the binding pocket and perfectly shields this water molecule speaks in favor of this possibility (Figure 3a).

Table 2. PDB accession codes, structural details, and references of presented crystal structures.

PDB code	Proteins and substrates	Maximum resolution [Å]	Ref.
1PUD	apo QueTGT crystallized at pH 8.5	1.85	[2]
1P0D	apo QueTGT crystallized at pH 5.5	1.9	[37]
1P0E	QueTGT·$preQ_1$	2.4	[37]
1P0B	QueTGT·$preQ_0$	1.7	[37]
1N2V	QueTGT·2-butyl-imidazo-pyridazindione inhibitor	2.1	[36]
1Q2R	QueTGT·RNA stemloop (covalently bound)	2.9	[32]
1Q2S	QueTGT·preQ1-modified RNA stemloop	3.2	[32]
1IT7	ArcTGT·guanine	2.3	[27]
1IT8	ArcTGT·$preQ_0$	2.5	[27]
1J2B	ArcTGT·$tRNA^{Val}$	3.3	[26]
1TPH	triose phosphate isomerase·phosphoglycolohydroxamate	1.8	[47]
1EHZ	$tRNA^{Phe}$	1.93	[38]

If the presence of this water molecule with its mediating function in QueTGT is assumed, the properties of the binding pocket have to be altered during the course of G^{34} cleavage. Proper recognition of the aminomethyl group of $preQ_1$ requires the NH-donor functionality to be replaced by the CO-acceptor functionality through a switch of the $Leu231^Q$/$Ala232^Q$ peptide bond. Also, a slightly different geometry of the binding pocket is required to accommodate the kinked aminomethyl group of $preQ_1$ (Figure 3a). Interestingly enough, upon nonphysiological binding of the archaebacterial substrate $preQ_0$ to QueTGT, this peptide switch is not provoked and the peptide bond remains in its NH-exposing conformation (Figure 3c). The peptide switch is stabilized from the side opposite to the binding pocket by the side-chain carboxyl group of $Glu235^Q$, which is strictly conserved in the QueTGTs. Depending on the protonation state of its carboxyl group, $Glu235^Q$ either donates a hydrogen bond towards the backwards-exposed carbonyl group or accepts a hydrogen bond from the amide group of the reoriented peptide bond, thus serving as a general acid/base mediating the peptide switch (Figure 3a). As expected for such a general acid/base, the peptide switch gated by $Glu235^Q$ is already functional in uncomplexed QueTGT depending on the applied pH conditions (Figure 3c). In *Z. mobilis* TGT, crystallized at pH 5.5, $Glu235^Q$ is probably protonated and forms a hydrogen bond with the CO group of the peptide bond. In consequence, the NH group is exposed towards the binding pocket (PDB code: 1P0D).[37] At pH 8.5, $Glu235^Q$ experiences deprotonation which triggers the switch of the peptide bond now binding the $Glu235^Q$ γ-carboxyl group through its NH group (PDB code: 1PUD). Accordingly, the CO group is exposed towards the binding pocket.

By contrast, ArcTGTs feature an exclusive substrate specificity towards guanine and $preQ_0$.[30,40] This results from a significantly reduced adaptability of the binding pocket. The peptide bond corresponding to the $Leu231^Q$/$Ala232^Q$ bond that performs the peptide flip in QueTGTs is represented by $Val197^A$/$Val198^A$ in ArcTGTs. However, it strictly presents an NH-donor group towards the substrate. This peptide bond lacks the peptide-switching functionality observed in QueTGTs due to an entirely different stabilization mechanism. In *P. horikoshii* TGT, the two invariant backbone NH bonds of $Leu200^A$ and $Leu201^A$, which will not support a peptide switch as mediated by $Glu235^Q$, are present in the second sphere of amino acids around the active site (Figure 3b).[37] With respect to substrate specificity and from a mechanistic point of view, the switching functionality of the peptide bond is not required for ArcTGTs. The interstitial water molecule that bridges guanine with the $Val197^A$/$Val198^A$ peptidic NH group in the binary *P. horikoshii* TGT·guanine complex structure can be directly replaced by the acceptor nitrile group of $preQ_0$ (PDB code: 1IT8) without any adaption of the recognition properties or geometry of the binding pocket.[27] Apart from this lacking adaptability, the adopted binding mode is almost identical to that of $preQ_0$ in QueTGT (Figure 3c). This provides a conclusive explanation for why QueTGTs exhibit an enhanced substrate promiscuity that

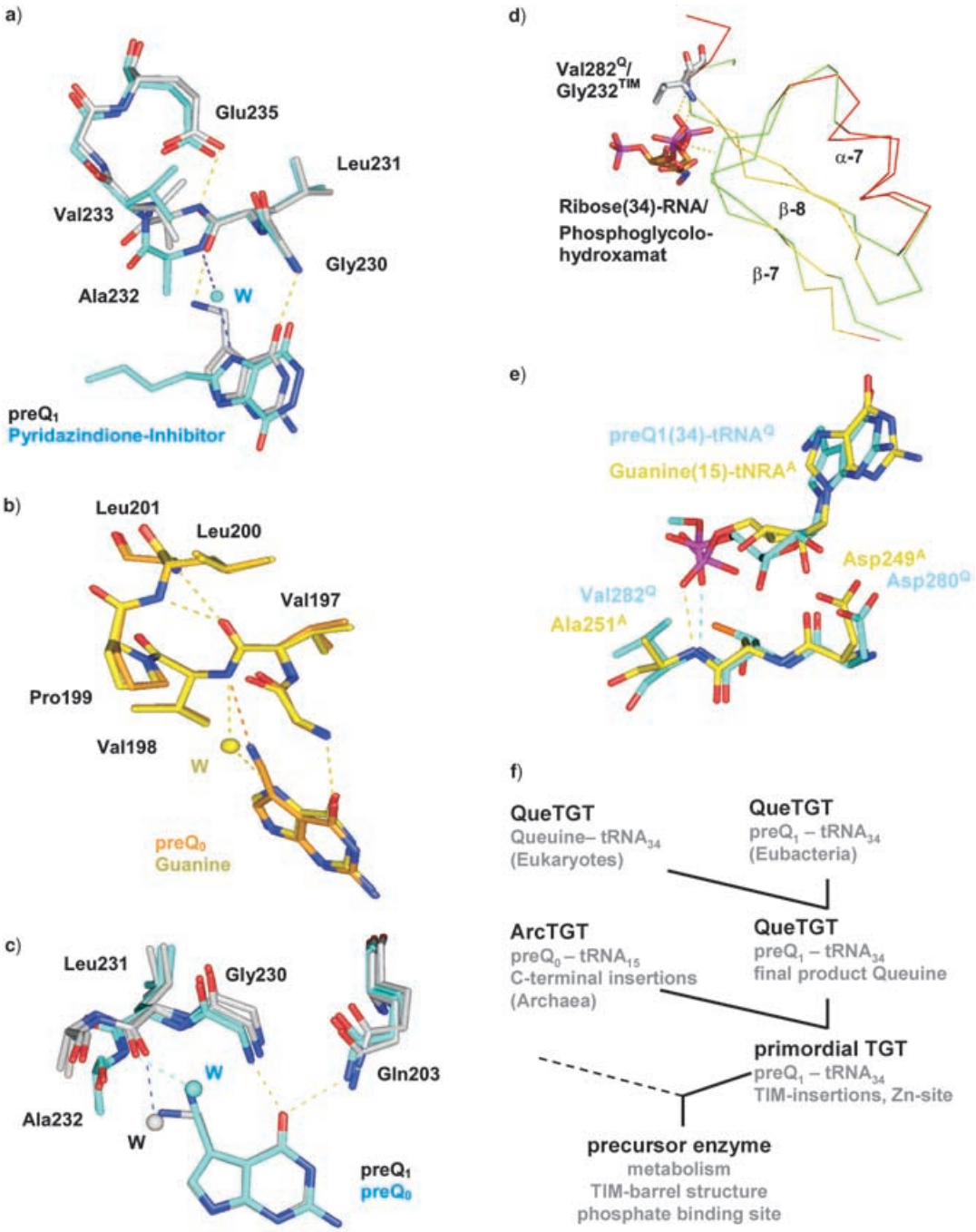


Figure 3. a) Structural alignment of $preQ_1$ (grey) and a pyridazindione-type inhibitor (cyan) in complex with QueTGT. The peptide bond of Leu231/Ala232 can perform a peptide switch controlled by the protonation state of Glu235 and substrate properties. In the case of the inhibitor, a water molecule mediates the contact between the imidazo moiety of the inhibitor and Ala232. b) Structural alignment of $preQ_0$ (orange) and guanine (yellow) in complex with ArcTGT. The peptide bond of Val197/Val198 is invariant due to backbone hydrogen bonding. The donor properties of the water molecule mediating between Val198 and guanine can be directly replaced by the $preQ_0$ cyano group. c) Structural alignment of $preQ_1$·QueTGT (grey), $preQ_0$·QueTGT (cyan), and apo QueTGT crystallized at pH 5.5 (cyan) and at pH 8.5 (grey). Depending on the pH value of crystallization the peptide switch Leu231/Ala232 is provoked in similar fashion to the way it is by the two substrates. Water molecules in the apo QueTGT structures already preadopt the positions of substrate functional groups within a narrow range. d) Structural alignment of the SPB motif ranging from the seventh to the eighth TIM-barrel β strands in *Gallus gallus* triose-phosphate isomerase and *Z. mobilis* QueTGT. At the end of the eighth β-strand, residues $Val282^Q$ and $Gly232^{TIM}$ in conserved positions bind substrate phosphate groups in a similar fashion. Yellow: β strand; red: α helix; green: loop regions. e) Structural alignment of the SPB motif residues in conserved positions of ArcTGT ($Ala251^A$) and QueTGT ($Val282^Q$). Binding to the phosphate group of the respectively modified base of tRNA occurs in a similar fashion. f) Suggested evolutionary tree for TGTs departing from a TIM-barrel-enzyme precursor. (a)–(e) were produced by using the PYMOL program.[50]

enables them to recognize $preQ_0$ and $preQ_1$ in addition to guanine.

The further extended substrate specificity in eukaryotic QueTGTs towards queuine (Table 1)[22] is presumably due to a

spatial extension of the binding pocket. As suggested by a homology model based on the *Caenorhabditis elegans* sequence, a Val233Gly replacement, specific for eukaryotic QueTGTs, significantly enlarges the binding pocket, thereby allowing the binding of extended $preQ_1$-like substrates such as queuine (Figures 2a and 3a).[3]

3.4. Structural relevance of water molecules in binding pockets

It can be assumed that water molecules are found in the active site of the uncomplexed protein in almost all enzyme crystal structures. Most probably, these molecules already indicate favorable positions to be occupied by groups of putative ligands. In the case of *Z. mobilis* TGT this assumption is confirmed by at least one specific substrate functional group. In the uncomplexed pH 5.5/pH 8.5 crystal structures of *Z. mobilis* TGT, water molecules are bound to either the CO- or the NH-exposing peptide switch in distinct orientations (Figure 3c). With respect to the binding mode of $preQ_1$ and $preQ_0$, these water positions already indicate within a narrow range the putative binding sites of the bound 7-deazaguanine substituents. Accordingly, with sufficient resolution and accurate refinement, active-site water molecules could suggest a conclusive picture mapping out likely binding sites of polar functional groups of substrate molecules. In consequence, such water molecules serve as suitable probes to analyze a binding pocket for putative interaction sites of polar groups in structure-based drug design. This fact has already been successfully exploited for the design of inhibitors of this enzyme at two additional sites within the binding pocket.[36,41]

4. Classification of TGT within TIM-Barrel-Fold Enzymes

Crystal structure analysis provides powerful access to information about the evolutionary relationships between proteins and helps to unravel the functional evolution of complex metabolic pathways. In this section we want to show how TGT crystal structures assist the structural classification within the TGT superfamily and help in the discovery of the putative origin among enzymes adopting a TIM/$(\beta\alpha)_8$-barrel fold. The TIM barrel is very abundant and approximately 10% of all enzymes share this geometry.[42] The biological roles of 85% of the known reaction types performed by TIM-barrel enzymes are associated with metabolism.[43] During the last two decades, several evolutionary classification models for TIM-barrel enzymes have been proposed.[44,45] Recent considerations favor the model of divergent evolution starting from a common ancestor, rather than convergent evolution. For about a dozen of the assumed 26–29 homologous superfamilies, sequential, structural, and functional evidence has been presented to support this assumption.[43,46] Among these presumably closer related superfamilies, a standard phosphate-binding (SPB) motif, involved in the recognition of substrate phosphate groups, is widespread.[43,46] It ranges from the seventh β strand to the eighth α helix of the TIM-barrel motif and is characterized by a high structural homology along with conserved phosphate-binding positions at the ends of the adjacent seventh and eighth TIM-barrel strands. This common structural element was also used as one criterion to achieve higher order classification.

A straightforward assignment of TGTs to the TIM-barrel-fold enzymes is difficult. As TGTs catalyze reactions attributed to the information pathway, they constitute one of the few examples for TIM-barrel enzymes not involved in metabolism.[43] Furthermore, TGTs form a separated superfamily within the TIM-barrel fold enzymes (SCOP Version 1.65,[5,6] CATH Version 2.5.1[7,8]). This is due to unusual insertions into the TIM barrel responsible for RNA binding (Figure 1a and c). Therefore, almost no global structural relationship to other superfamilies could be detected.[43] Surprisingly, alignments performed by Nagano et al.[43] revealed that TGTs share the standard phosphate-binding motif with some other mutually related superfamilies (Figure 1a and c). For *Z. mobilis* TGT, the overall C_α root mean square deviation (RMSD) with respect to the SPB motif is only 1.7–2.0 Å. $Gly261^Q$ and $Val262^Q$ at the end of strand β13 (representing the seventh TIM-barrel strand), as well as $Val282^Q$ at the end of strand β14 (representing the eighth TIM-barrel strand) were predicted to be in conserved positions for substrate phosphate binding through backbone interactions. Inspection of the *Z. mobilis* TGT·RNA complex revealed that the predicted $Gly261^Q$ is involved in binding of a substrate ribose hydroxy group, while $Val282^Q$ is indeed involved in binding of the substrate phosphate group of G^{34}. A structural alignment of the SPB motif of QueTGT and triose-phosphate isomerase (TIM) from *Gallus gallus* (PDB code: 1TPH)[47] with their respective ligands shows equivalently positioned phosphate groups, binding residues, and orientation towards $Val282^Q$ and $Gly232^{TIM}$ (Figure 3d). Furthermore, $Val282^Q$ is one of the well-conserved residues next to the active site in QueTGTs (Figure 2a).[41,48] In the available ArcTGT structure from *P. horikoshii*, $Ala251^A$, which corresponds to $Val282^Q$, displays a similar phosphate-binding mode to the phosphate group of G^{15} (Figure 3e). This finding supports the allocation of the TGT superfamily to other presumably homologous SPB-containing superfamilies.

The SPB motif could also be a key structural element for understanding why TGT is not involved in metabolism as most of the other TIM-barrel enzymes are. Comparison of evolutionarily related TIM-barrel enzymes involved in metabolism shows no strict correlation with the metabolic pathway that they are involved in. Often, related enzymes perform tasks in different metabolic pathways.[46] This prompted Copley and Bork to suggest that recruitment between different pathways is one possible driving force to develop new protein functions.[46] This model requires an ancient enzyme possessing a broader substrate specificity as a starting point. New functions can evolve in the case of different compounds sharing a common structure and being accepted as substrates by this enzyme. Specific groups (for example, phosphate groups) represent such common substructures. Duplication and diversification of the respective gene could result in two distinct protein functions involved in different metabolic pathways.[46]

By applying such arguments to TGT, the following consideration with respect to the evolutionary origin of TGTs can be drawn (Figure 3 f). The ancient predecessor of the presently existing TGTs was possibly involved in central metabolism in accepting and binding phosphorylated substrates through its SPB motif. Due to the fact that tRNA also contains phosphate moieties and occurs in cells with a rather high concentration, it could have served as some sort of substrate for this ancestor. After gene duplication and evolutionary modification, one such copy could have evolved with specific tRNA recognition and modification properties as primordial TGT. This could explain why the TGT ancestor possibly departed from pure metabolism and developed as a catalyst with a function rather unusual for an enzyme with a TIM-barrel fold. With respect to evolution across the TGT superfamily, we assume the following scenario. As TGTs from all three kingdoms share a core structural domain and a common reaction mechanism, an ancient TGT had obviously evolved before the three kingdoms diverged. TGTs modifying G^{34} appear to represent the ancient version of the enzyme. As QueTGTs are absent in some organisms, such as *Saccharomyces cervisiae*, *Mycoplasma*, *Treponema pallidum* and Mycobacterial species,[19] the Q modification in tRNAs seems not to be absolutely crucial for the persistence of a species. As a QueTGT is also absent in most of the archaebacterial species sequenced up to now,[1] we suggest that in archaea TGT has undergone an evolutionary transition followed by the loss of G^{34}-modification ability to take over a new and more important task. As a result, a protein with highly sophisticated domain architecture was developed, thereby equipping TGT with three supplementary domains. This adapted TGT, now altered towards ArcTGT, became part of the archaeosine modification pathway. The ultimate purpose of the archaeosine modification is most presumably the improvement of tRNA stability to maintain the translational process even under the rough living conditions of archaea.

5. Final Summary

The present review summarizes and discusses distinct properties of the TGT enzyme superfamily. This superfamily displays the unique function of catalyzing specific base-exchange reactions in tRNAs. TGTs are present across all three kingdoms of life and show a conserved structure. In all cases they operate on guanine residues. Throughout the different family members, the base exchange follows the same reaction mechanism, even though guanine residues at different positions of the tRNA are replaced. Comparison of the TGT structures, however, demonstrates that they exhibit very similarly shaped recognition sites that result in a similar RNA conformation in the surroundings of the active site after binding to the enzyme. The conserved reaction mechanism involves nucleophilic attack of an aspartate oxygen atom onto C1′ of the ribose sugar and proceeds through a covalent intermediate. Pronounced induced-fit adaptations parallel the substrate-binding process and the various steps along the reaction pathway. The manifold TGTs from the three kingdoms of life exhibit pronounced differences in their substrate promiscuity. A structural comparison shows that the level of substrate promiscuity is governed by the extent of binding-site adaptation along with the involvement of active-site water molecules. These molecules actively support substrate promiscuity by taking a role as either versatile space fillers once ligands with smaller side chains are bound or as mediators of polar interactions by taking advantage of their ambivalent donor and acceptor functionality. The TGT enzymes adopt the very abundant TIM-barrel fold even though they do not belong to the group of metabolizing enzymes. However, some important and unusual insertions make them form a unique superfamily within the TIM/$(\beta\alpha)_8$-barrel enzymes that shares hardly any global structural relationships with other superfamilies. Interestingly enough, they exhibit a standard phosphate-binding (SPB) motif in common with typical TIM-barrel metabolizing enzymes. This suggests that the superfamily of TGT enzymes has evolved from an ancient ancestor involved in central metabolism and recognizing phosphorylated substrates.

Keywords: enzymes · protein structures · structure–activity relationships · substrate recognition · tRNA

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