Fig. S1: Multiple sequence aligment of TrmIs ordered with decreased *ClustalW* score compared to $_{Pab}$TrmI. This figure was drawn with *ESPript* (1). Putative TrmIs most similar to $_{Pab}$TrmI and belonging to different taxonomic groups are indicated together with TrmIs with known structure. The consensus sequence motifs are indicated underneath. The secondary structural elements of $_{Pab}$TrmI are shown above. The cysteine residues forming the intermolecular disulfide bonds between two different monomers and Histidine at position 78, which are present only in the *Pyrococcus* and *Thermoccus* species, are labeled with a star.

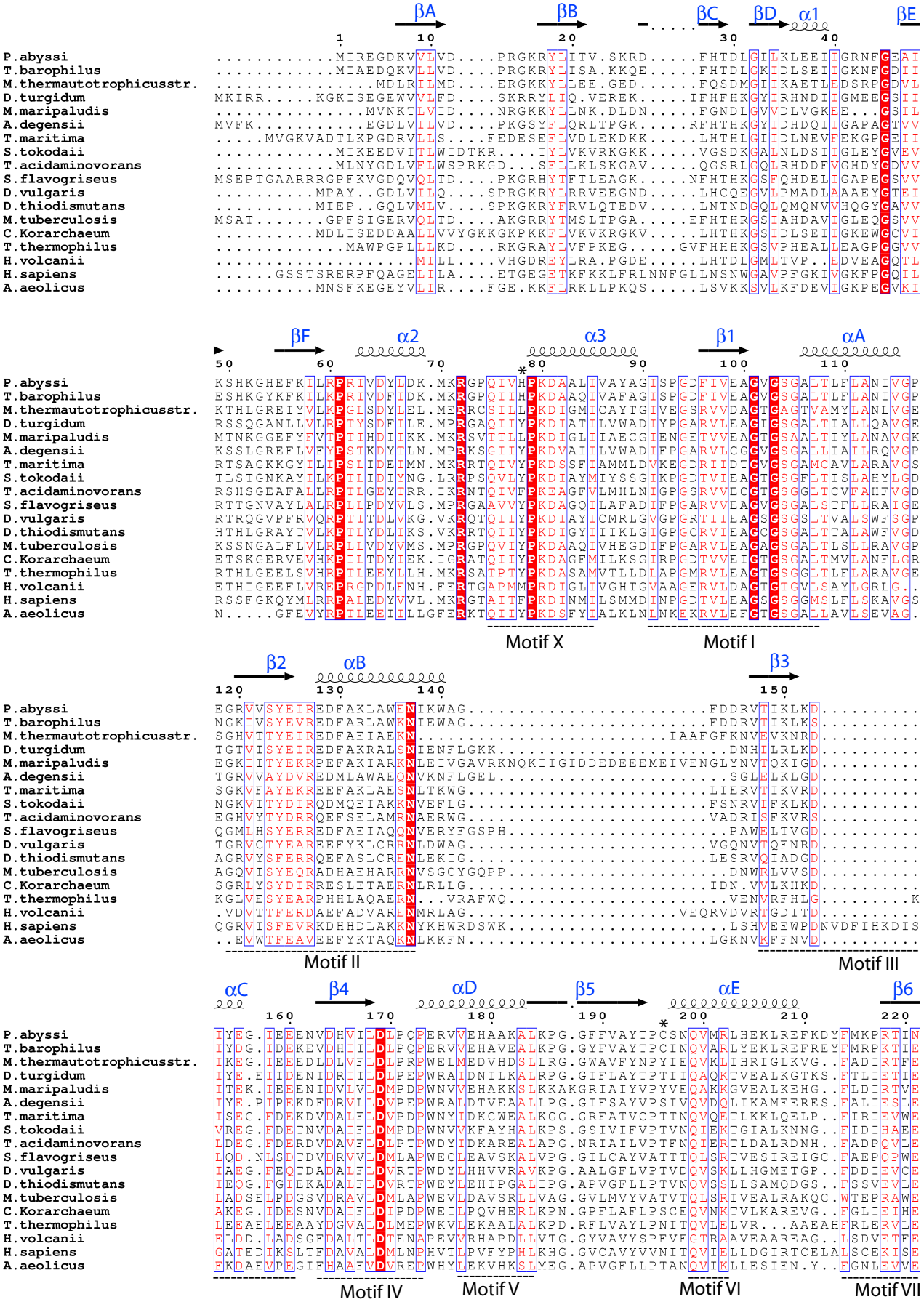
Fig. S2: Stereo view of the SAH binding sites in Crystal Forms I and II. A Fobs-Fcalc electron density map omitting SAH calculated at the level of 1.5 standard deviation is superimposed on the structure. **A** In Crystal Form II, the dihedral angle (C4', C5', S, Cγ) of SAH is -74°. **B** The folded conformation observed in Crystal form I, molecules C and D. The dihedral angle (C4', C5', S, Cγ) of SAH is -152°. **C** The folded conformation observed in Crystal form I, molecules A and B. The dihedral angle (C4', C5', S, Cγ) of SAH is -170°.

Fig. S3: A Nucleotide sequence of $_{Pab}$tRNA^{Asp}. **B** $_{Pab}$TrmI and $_{Tt}$TrmI modify positions 57 and/or 58 in $_{Pab}$tRNA^{Asp} but not position 59. Purified enzymes were incubated in the presence of SAM with [α-^{32}P]ATP or [α-^{32}P]UTP labeled T7 transcript of $_{Pab}$tRNA^{Asp} for one hour at 70°C. The resulting [^{32}P]-labeled tRNA was hydrolyzed by nuclease P1 or T2 and the generated nucleosides were analyzed by 2D-TLC on cellulose plates and autoradiography. The nucleosides were quantified by scintillation counting.

Fig. S4: Time course for the dimethylation of *P. abyssi* tRNA^{Asp} by wild-type $_{Pab}$TrmI (triangles) and the H78Y mutant (circles). An equimolar amount of tRNA and enzyme was incubated in the presence of SAM at 70°C for different incubation times and was then digested with RNase A. The digest was analyzed by MALDI mass spectrometry (Fig. 6). The area of the peak corresponding to the dimethylated fragment m^{1}Am^{1}AAUp at m/z 1340.22 was compared to that of the peaks corresponding to the non methylated (m/z 1312.19) and monomethylated (m/z 1326.20) fragments to obtain the percentage of dimethylated tRNA.

1. Gouet, P., Courcelle, E., Stuart, D.I. and Metoz, F. (1999) ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics*, **15,** 305-308.

Figure S1


βA
βB
βC
βD
α1
βE
βF
α2
α3
β1
αA
β2
αB
β3
αC
β4
αD
β5
αE
β6
P.abyssi
T.barophilus
M.thermautotrophicusstr.
D.turgidum
M.maripaludis
A.degensii
T.maritima
S.tokodaii
T.acidaminovorans
S.flavogriseus
D.vulgaris
D.thiodismutans
M.tuberculosis
C.Korarchaeum
T.thermophilus
H.volcanii
H.sapiens
A.aeolicus
Motif X
Motif I
Motif II
Motif III
Motif IV
Motif V
Motif VI
Motif VII

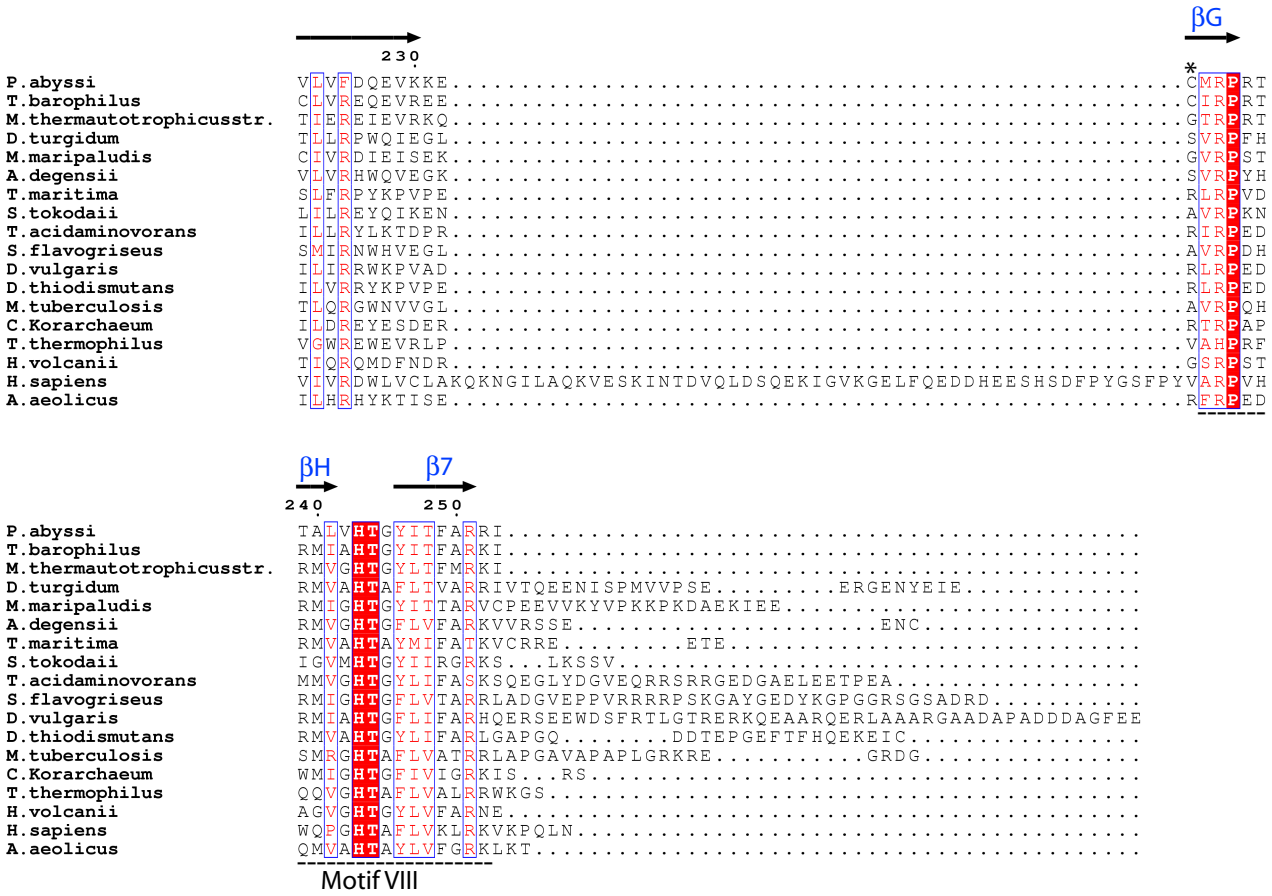
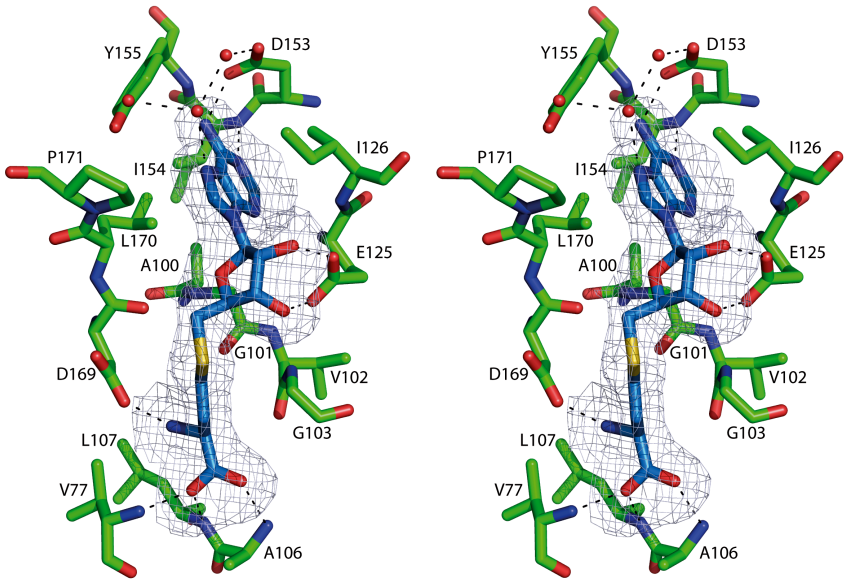
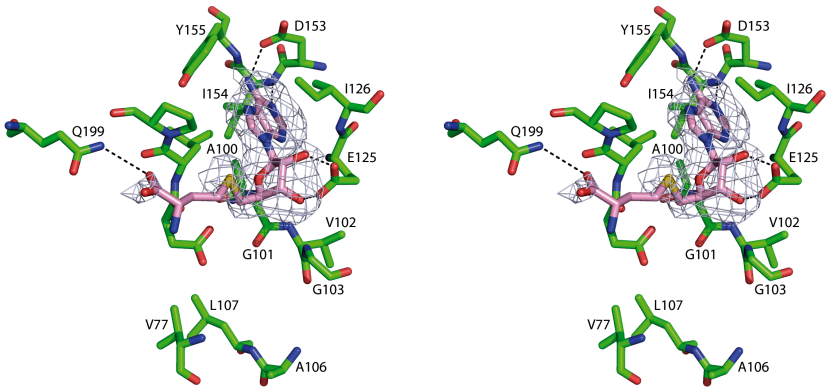

βG
230
*
P.abyssi
T.barophilus
M.thermautotrophicusstr.
D.turgidum
M.maripaludis
A.degensii
T.maritima
S.tokodaii
T.acidaminovorans
S.flavogriseus
D.vulgaris
D.thiodismutans
M.tuberculosis
C.Korarchaeum
T.thermophilus
H.volcanii
H.sapiens
A.aeolicus
βH
β7
240
250
Motif VIII

Figure S2

A



B



C

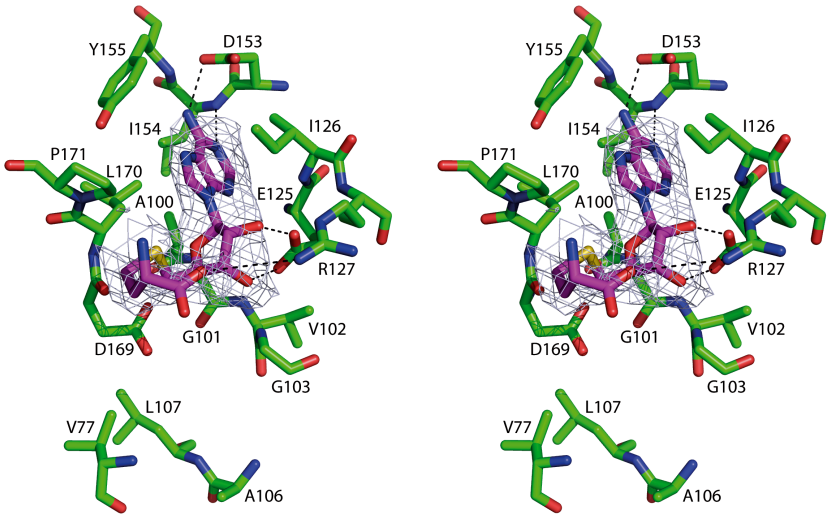
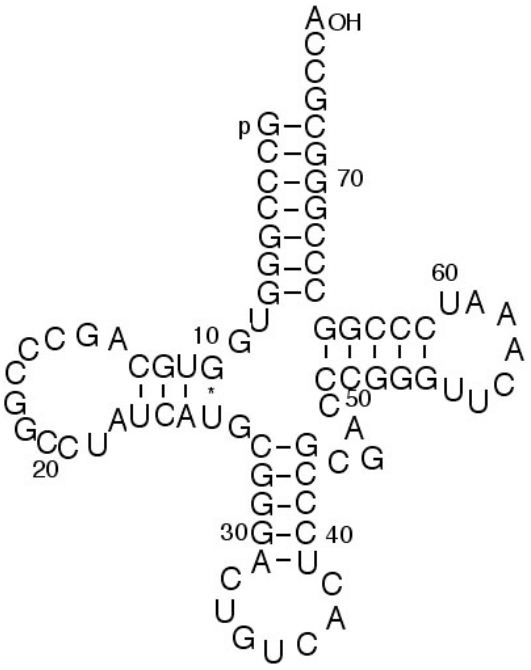


Figure S3

A


AOH
pG
70
60
10
50
20
30
40

B

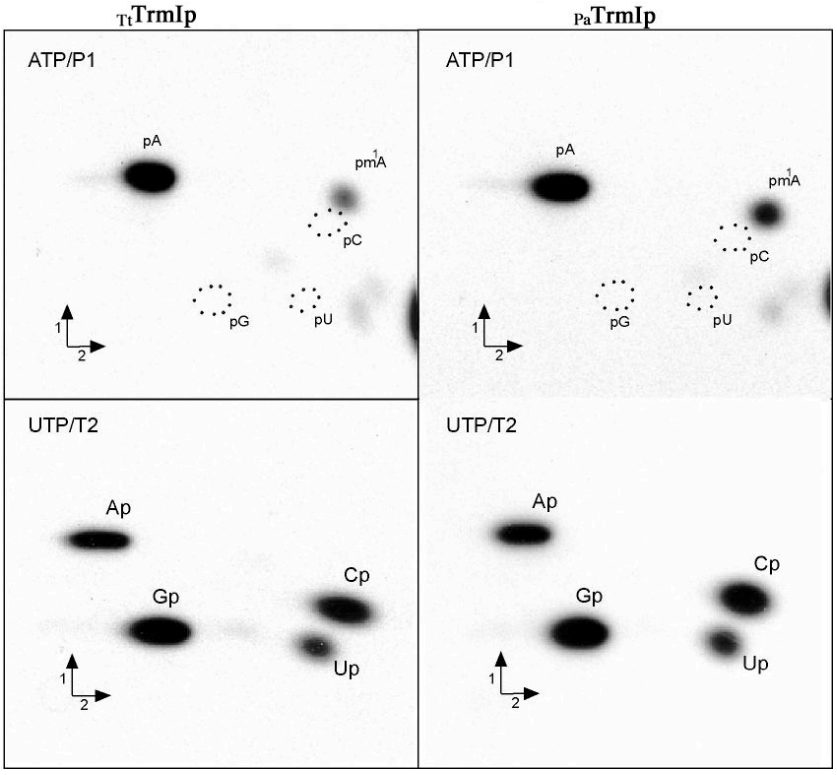
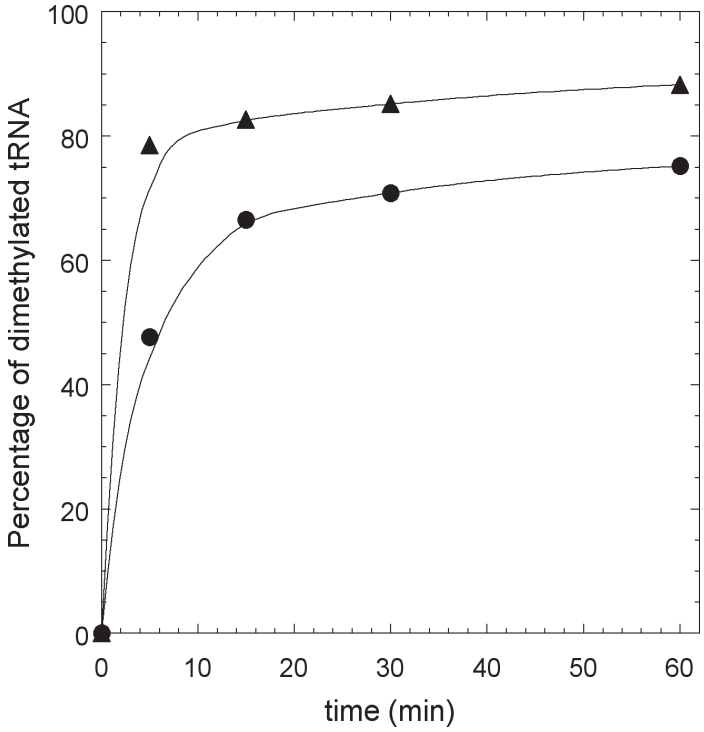

TtTrmIp
PaTrmIp
ATP/P1
ATP/P1
pA
pm1A
pC
pG
pU
pA
pm1A
pC
pG
pU
1
2
1
2
UTP/T2
UTP/T2
Ap
Gp
Cp
Up
Ap
Gp
Cp
Up
1
2
1
2

Figure S4


Percentage of dimethylated tRNA
100
80
60
40
20
0
0
10
20
30
40
50
60
time (min)