Tetrameric triosephosphate isomerase from hyperthermophilic Archaea

Michael Kohlhoff[a,*], Anke Dahm[b], Reinhard Hensel[a]

[a]*FB 9 Mikrobiologie, Universität Essen, Universitätsstr.5, D-45117 Essen, Germany*
[b]*Abt. Biophysikalische Chemie, Biozentrum der Universität Basel, Klingelbergstr. 70, CH-4056 Basel, Switzerland*

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Abstract Triosephosphate isomerase (TIM) of the hyperthermophilic Archaea *Pyrococcus woesei* and *Methanothermus fervidus* have been purified to homogeneity. The enzymes from the two hyperthermophiles represent homo-tetramers of 100 kDa, contrary to all known bacterial and eukaryotic TIMs, which are dimers of 48–60 kDa. Molecular size determination of the TIM from the mesophilic methanogen *Methanobacterium bryantii* yielded the usual molecular mass of only 57 kDa, indicating that the tetrameric aggregation state does not represent an archaeal feature but rather correlates with thermoadaptation. A similar preference for higher protein aggregates in hyperthermophilic Archaea has previously been demonstrated for 3-phosphoglycerate kinases. The gene of the *P. woesei* TIM was cloned and sequenced. The archaeal TIM proved to be homologous to its bacterial and eukaryotic pendants. Most strikingly, the deduced protein sequence comprises only 224 residues and thus represents the shortest TIM sequence known as yet. Taking the three-dimensional structure of the eucaryal TIM as a basis, from the shortenings of the chain considerable rearrangements at the bottom of the α/β barrel and at its functionally inactive flank are expected, which are interpreted in terms of the formation of new subunit contacts.

Key words: Quarternary structure; Thermoadaptation; Thermostability; Nucleotide sequence; *Pyrococcus woesei*; *Methanothermus fervidus*; *Methanobacterium bryantii*

1. Introduction

Recent structure analyses of proteins from extreme thermophilic and hyperthermophilic organisms over the last two years provide a closer insight into nature's strategies for stabilizing the native protein conformation under extreme temperature conditions. From the various studies different features have been discussed as responsible for the increased conformational stability at all three levels of protein structure (core region and secondary structure elements, interactions between domains and subunits): e.g. reduction of solvent accessible area and increase in the fraction of buried atoms [1], stabilization of α-helices, strengthening of polypeptide chain termini and loops [2], shortening of loops [3], increase of ion bridges and hydrophobic interactions [4–6]. The observation that especially the subunit contacts are intensified by a striking increase in ion pairs (as found for the malate dehydrogenase from *Thermus flavus* [6] and the glutamate dehydrogenase from *Pyrococcus furiosus* [5]) and hydrophobic interactions (as reported for the glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima* [4]) suggests that the subunit contacts are highly critical sites for protein stability. Site-directed mutagenesis experiments with aldolase [7] point further to the importance of the subunit contacts for the thermostability of the whole molecule and explain the preferred strengthening of the subunit interactions in proteins from thermophiles.

To enlarge the data set for deduction of common traits in protein thermoadaptation we focus on triosephosphate isomerases (TIM; EC 5.3.1.1) of the hyperthermophilic Archaea *Methanothermus fervidus* and *Pyrococcus woesei* with growth optima at 83°C or 100°C, respectively [8,9]. No data are available yet about structure and function of archaeal TIMs, although their presence in glycolytic and gluconeogenic pathways of archaeal organisms has been proved [10–12].

TIM catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate and represents one of the most thoroughly investigated enzymes. More than 30 sequences from bacterial and eukaryotic TIMs have been published so far, for three eukaryotic enzymes the three-dimensional structure [13–16] could be solved and the enzyme mechanism has been extensively studied with wild-type and mutant enzymes [17]. All naturally occurring TIMs studied so far are homomeric dimers. A certain exception is represented by the bifunctional 3-phophoglycerate kinase/triosephosphate isomerase fusion protein from *T. maritima*, which forms a homo-tetramer [18]. The tertiary structure of TIM is characterized by a compact eight-stranded α/β barrel, a motif strikingly often used in proteins with different functions (cf. [19]). Virtually all active site residues are located in the loops connecting β-strands and the following α-helices at the top of the barrel. The subunit contacts are very extensive, characterized by a hydrophobic patch and a large protruding loop ('interface loop') fitting into a cervice of the neighbouring subunit near the active site.

2. Material and methods

2.1. Organisms

Cells of *P. woesei* (DSM 3773) and *M. fervidus* (DSM 2088) were grown as described [20]. For cloning the *tim* gene of *P. woesei*, the *E. coli* strain XL1-Blue was used [21]. Cells of *Methanobacterium bryantii* were a gift from Prof. H. König (University of Ulm).

2.2. Plasmids, enzymes, chemicals

Vector pBluescript II KS$^+$ [21] was used for cloning and sequencing. Fluorescein-15-dATP was purchased from Pharmacia (Freiburg, Germany), the Digoxigenin kit from Boehringer (Mannheim, Germany). The TSK gel for gel filtration experiments was from Toyosoda (Tokyo, Japan). All other enzymes and chemicals applied for molecular-genetic and biochemical experiments were the same as described elsewhere [20].

2.3. Standard enzyme assay

The assay (conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate) was performed at 70°C and contained 100 mM Tris/HCl (pH 7.4 at 70°C), 5 mM arsenate, 10 mM NAD$^+$, 4 mM

*Corresponding author. Fax: (49) (201) 183 2529.
E-mail: bmb000@aixrs1.hrz.uni-essen.de

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Table 1
Purification of TIM from 20 g *P. woesei* or *M. fervidus*

Purification step	*P. woesei*					*M. fervidus*				
	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	2950	4600	2	–	100	1580	970	1	–	100
Heat precipitation (95/80 °C)	1860	4780	3	2	105	1230	1100	1	2	110
Ammonium sulfate fractionation	n.d.	2600	n.d.	n.d.	n.d.	630	1425	2	4	150
Ion-exchange chromatography	3.11	930	300	90	20	24	1820	76	130	190
Hydrophobic interaction chromatography	0.42	760	1800	1130	17	3	1400	465	775	145
Gel filtration	0.02	90	3900	2450	2	0.03	70	2230	3720	7

dihydroxyacetone phosphate, 20 U glyceraldehyde-3-phosphate dehydrogenase of *P. woesei* [22] at a total volume of 1 ml.

2.4. Purification of the TIM of P. woesei *and* M. fervidus

20 g wet cells were suspended in 30 ml buffer A (50 mM potassium phosphate, pH 7.35 containing 30 mM 2-mercaptoethanol) and disrupted three times in a French pressure cell at 150 MPa. After centrifugation (30 min, 40 000×*g*) the supernatant was heated to 95°C (*P. woesei*) or 80°C (*M. fervidus*), respectively, for 30 min and then centrifuged again (30 min, 40 000×*g*). The supernatant was fractionated by ammonium sulfate (*P. woesei*, 45%–75% saturation; *M. fervidus*, 50%–80% saturation). After dialysis against buffer A, the protein solution was loaded on an ion-exchange column (Q-Sepharose FF (Pharmacia), diameter 26 mm, volume 100 ml, flow 4 ml/min) equilibrated with buffer A containing 10 mM KCl. After rinsing the column with buffer A containing 50 mM KCl (*P. woesei*) or 330 mM KCl (*M. fervidus*) the enzyme was eluted by increasing the KCl concentration either to 150 mM in one step (*P. woesei*) or by a linear gradient from 300 mM to 450 mM (*M. fervidus*). The pooled fractions containing activity were dialyzed against buffer A and centrifuged (30 min, 40 000×*g*) to remove precipitates. After addition of ammonium sulfate (final concentration 1.3 M), the solution was loaded on a fast-flow phenyl-sepharose column (Pharmacia; diameter 16 mm, volume 20 ml, flow 2 ml/min). The enzyme was eluted with a linear gradient (500 ml) from 1.3 M to 0.0 M ammonium sulfate in buffer A without 2-mercaptoethanol, but containing 30% ethylene glycol. Pooled fractions were then loaded onto a gel filtration column (HiLoad 26/60 Superdex 200 prep grade, Pharmacia) equilibrated with 10 mM phosphate buffer, pH 7.35, containing 300 mM KCl. The purity of the fractions was checked by SDS/PAGE.

2.5. Cloning and sequencing the tim gene of P. woesei

Preparation and blotting of the genomic DNA of *P. woesei* was done as previously described [20,22]. Sequencing was carried out according to the protocol of Wiemann et al. [23] using an Automated Laser Fluorescent DNS Sequencer (Pharmacia, Germany). Based on the N-terminal amino acid sequence of the *P. woesei* TIM (AKLKEPIIAINFKTYIEATGKRALEIAK) two degenerated oligonucleotides were designed corresponding to the heptapeptide NFKTYIE (oligo1: aay tty aar acn tay ath ga; oligo2: aat ttt aaa acw tat aaa ga) to screen for the coding gene in Southern blot experiments using genomic DNA digested with different restriction enzymes. Hybridization and detection of the signals was carried out with the Digoxigenin kit (Boehringer) following the recommendations of the manufacturer. A 3.3-kb *Xba*I fragment, which was found to specifically hybridize with the probe, was cloned using pBluescript II KS$^+$ as vector and *E. coli* XL1 Blue as recipient. Positive clones were analyzed by restriction analysis. The sequence of the gene was determined on both strands.

2.6. Molecular mass determinations

Molecular mass determinations for the native proteins were performed by gel filtration and density gradient ultracentrifugation experiments. For the gel filtration experiments with the TIMs of *P. woesei*, *M. fervidus* and *M. bryantii* a HiLoad 26/60 Superdex 200 prep grade column (Pharmacia) equilibrated with 50 mM potassium phosphate buffer, pH 7.35, containing 300 mM KCl was applied. For calibration the following marker enzymes were used: ferritin (horse), alcohol dehydrogenase (yeast), D-lactate dehydrogenase (*Lactobacillus leichmannii*), TIM (mouse), 3-phosphoglycerate kinase (yeast), cytochrome c (beef). In parallel experiments, the molecular mass of the TIM from *M. fervidus* was determined with other gel matrices (TSK gel, Toyosoda; Sephadex G-100, Pharmacia) under the same conditions. In a second approach, the molecular mass of the *M. fervidus* enzyme was determined by density gradient ultracentrifugation (10–30% sucrose in 100 mM potassium phosphate buffer pH 7.5, total volume 5 ml, 15 h 180 000×*g*, fraction size 100 µl) with alcohol dehydrogenase (yeast), D-lactate dehydrogenase (*L. leichmannii*) and cytochrome c (beef) as marker proteins. Heat stability tests, protein determination and SDS/PAGE were done as previously described [22].

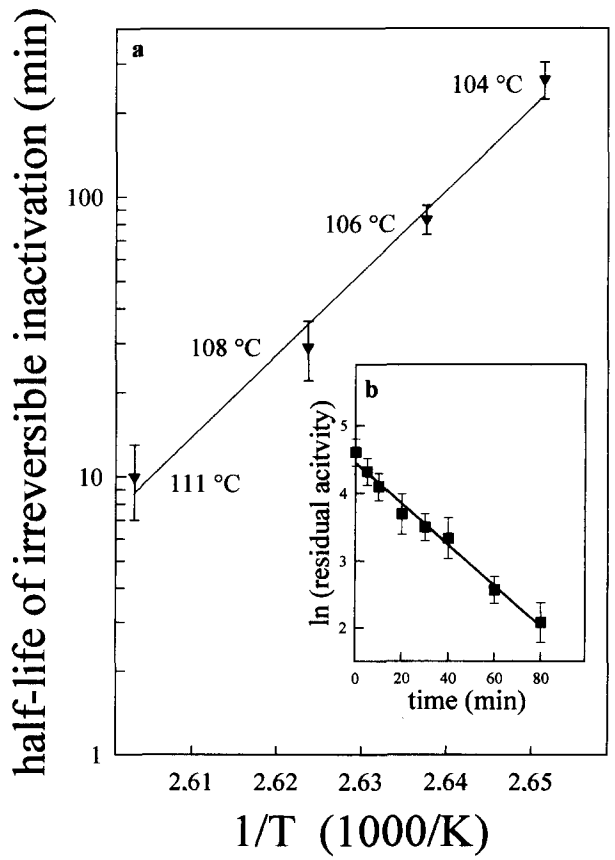

Fig. 1. Thermal inactivation of TIM of *P. woesei*. (a) Arrhenius plot; (b) semilog plot of thermal inactivation at 108°C (protein concentration: 10 µg/ml).

2.7 Protein sequencing

Protein sequencing was performed with the aid of a gas phase sequenator (model 473 A, Applied Biosystems) at the Max-Plack-Institute of Molecular Physiology (Dortmund, Germany).

3. Results and discussion

3.1. Purification of the triosephosphate isomerases from P. woesei *and* M. fervidus

The purification of the TIMs from both organisms (20 g wet cells) is documented in Table 1. After the final purification step, the proteins migrate as single bands on SDS gels with an apparent molecular mass of 24.0 kDa (*P. woesei*) or 27.8 kDa (*M. fervidus*).

3.2. Thermostability of the purified TIMs from P. woesei *and* M. fervidus

The thermostability of the enzyme from *P. woesei* was determined by inactivation kinetics at temperatures between 104 and 111°C (Fig. 1a). The inactivation followed first-order kinetics (Fig. 1b) and the velocity of inactivation proved to be independent of the protein concentration (range tested: 4–20 μg/ml). With a half-life of inactivation of 280 min at 104°C (corresponding to the upper growth limit of the organism) the thermal stability of the *P. woesei* TIM is considerably higher than that of the *M. fervidus* enzyme exhibiting only a half-life of inactivation of 60 min at 88°C (5°C above the optimal growth temperature). Like other enzymes from this organism [20,24], also the *M. fervidus* TIM obviously depends on extrinsic stabilizers for its in vivo function. Detailed analyses of solute–protein interactions to investigate the stabilizing influence of the cellular milieu are under way.

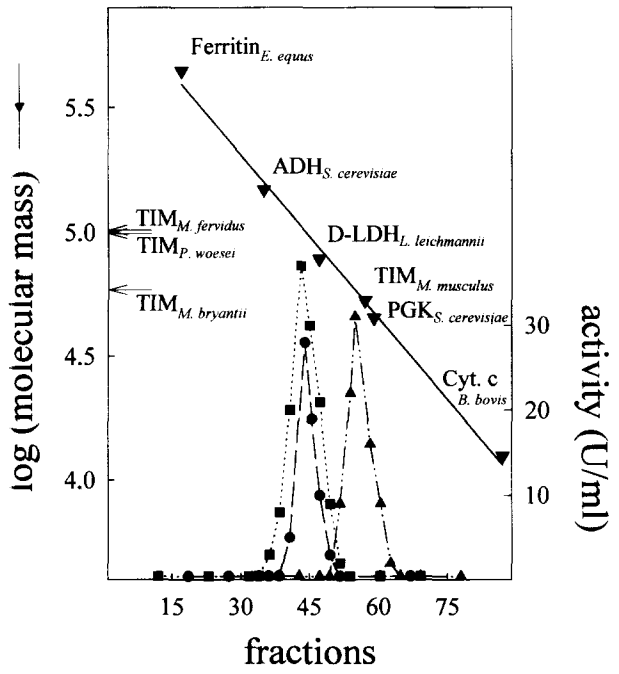

Fig. 2. Molecular mass determination of the TIMs from *P. woesei*, *M fervidus* and *M. bryantii* by gel filtration on HiLoad 26/60 Superdex 200. The elution diagram of the archaeal TIMs is shown (■ *M. fervidus*; ● *P. woesei*; ▲ *M. bryantii*) together with the elution maxima (▼) of different marker enzymes used for calibration of the column. The arrows indicate the deduced molecular masses.

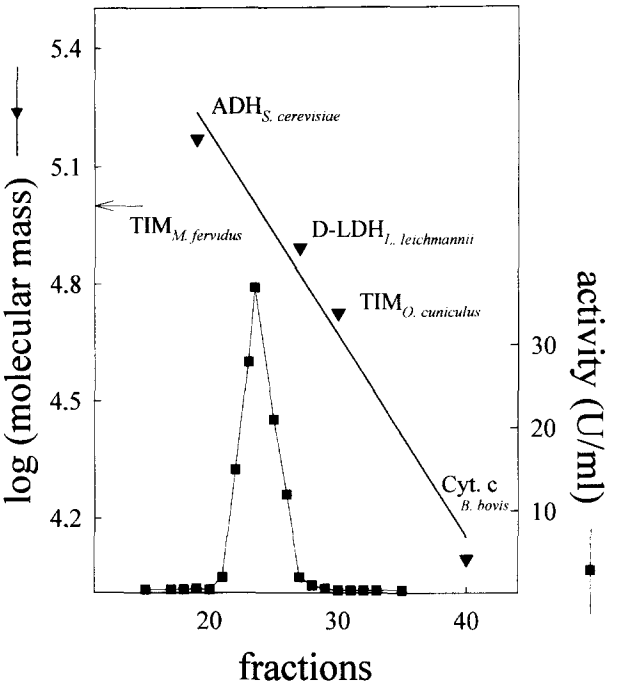

Fig. 3. Molecular mass determination of the TIM from *M. fervidus* by sucrose density centrifugation. The activity profile of the *M. fervidus* TIM is respresented together with the activity maxima of the marker enzymes (▼). The arrow indicates the deduced molecular mass.

3.3. Molecular mass determinations

Molecular mass determination under non-denaturing conditions yielded values of 99.6 ± 6.0 kDa (TIM of *P. woesei*; by gel filtration on Superdex 16/200; Fig. 2) or 100 ± 6.0 kDa (TIM of *M. fervidus*; by gel filtration on three different matrices (see section 2) and sucrose density ultracentrifugation; Figs. 2 and 3), respectively. Considering the subunit masses determined by SDS/PAGE, the data account for homo-tetramers under native conditions. These findings are in contrast to the known TIMs from Bacteria and Eucarya, which form dimers with molecular masses of 48–60 kDa. Molecular mass determinations with protein extracts of the mesophilic Archaeum, *Methanobacterium bryantii* (optimal growth temperature: 37°C) enriched by ammonium sulfate fractionation yielded values of 57 ± 6.0 kDa for the TIM (Fig. 2) indicating the usual dimeric association. From that, one should conclude that the tetramer formation of the TIM of *P. woesei* and *M. fervidus* does not represent a domain-specific feature, but rather correlates with the adaptation to higher temperatures. Interestingly, a similar "thermophilic" trend to a higher aggregation state is also observed by comparing 3-phosphoglycerate kinases of *M. bryantii* (monomeric enzyme) and the hyperthermophiles *P.woesei* and *M. fervidus* (dimeric enzyme) [20]. Also the tetramer formation of the PGK/TIM fusion protein isolated from the hyperthermophilic bacterium *T. maritima* [18] seemingly follows this trend. Since the unfused PGK protein, which is also produced in this organism, forms "normal" monomers, one may suggest that — in analogy to the TIMs from thermophilic Archaea — the higher association state of the bifunctional fusion protein is caused by the TIM moiety. However, it cannot be ruled out that the higher association is due to fusion-specific rearrangements of the polypeptide chains.

From the suggested importance of the intersubunit contacts for thermostabilization, as deduced from experimental and comparative studies on various oligomeric enzymes [4–7], one should expect that the benefit of the higher association state resides in an increase of protein thermostability. The observation that the preference of a higher association state does not generally apply (or even countercurrent trends occur, as shown in the case of the enolases [25]) indicates that the advantage of association is limited by functional and/or structural restraints. Possibly, proteins whose low volume/surface ratio needs additional intermolecular interactions for stabilizing the core structure like proteins with short peptide chains (TIM) or proteins consisting of rather independent domains with lose contacts between them (like PGK) especially profit from the higher aggregation state. Additionally, we must assume that the stabilization potential of the additional contacts can only be used by proteins whose topology allows subunit association without restriction of the conformational flexibility of the active site region.

3.4. *The nucleotide sequence of the tim gene of* P. woesei

The *tim* gene encompasses 678 bp (Fig. 4). The G+C content of the gene has been calculated to be 44.8%, exceeding significantly the average G+C content of the genomic DNA (37.5% [9]). The codon usage is characterized by a preference for codons with A or T at the third position with the only exceptions of the triplets coding for Lys and Tyr.

In front of the coding region, sequences could be identified which showed similarities to the complementary consensus sequence of the 3′ end of the archaeal 16S rRNA (pos. −6 to −12) as well as to consensus sequences established for the

```
          -50                                                    -1
TGAAGAGTATTGTTAGAAACATTTAAGAATATTGAAGTAAATTTTCACGATTGGTGATAAGCT
1
ATG GCT AAA CTC AAG GAG CCA ATT ATA GCA ATC AAT TTT AAG ACG TCA
 M   A   K   L   K   E   P   I   I   A   I   N   F   K   T   Y
 50
ATA GAG GCA ACT GGA AAA AGG GCT TTA GAA ATA GCA AAG GCC GCT GAA
 I   E   A   T   G   K   R   A   L   E   I   A   K   A   A   E
    100
AAA GTG TAC AAA GAA ACA GGT GTA ACA ATT GTG GTC GCT CCC CAG CTT
 K   V   Y   K   E   T   G   V   T   I   V   V   A   P   Q   L
      150
GTA GAT CTA AGA ATG ATT GCC GAA AGC GTT GAA ATA CCT GTA TTC GCT
 V   D   L   R   M   I   A   E   S   V   E   I   P   V   F   A
          200
CAA CAT ATA GAC CCG ATA AAA CCT GGG AGT CAT ACT GGA CAC GTT CTT
 Q   H   I   D   P   I   K   P   G   S   H   T   G   H   V   L
              250
CCT GAG GCT GTG AAG GAA GCT GGA GCG GTA GGG ACT CTC CTC AAT CAC
 P   E   A   V   K   E   A   G   A   V   G   T   L   L   N   H
                  300
TCT GAG AAT AGA ATG ATC CTT GCA GAT CTC GAG GCT GCA ATA AGG AGG
 S   E   N   R   M   I   L   A   D   L   E   A   A   I   R   R
                      350
GCC GAA GAA GTA GGT CTA ATG ACA ATG GTC TGC TCT AAT AAT CCT GCT
 A   E   E   V   G   L   M   T   M   V   C   S   N   N   P   A
                          400
GTA TCG GCG GCT GTA GCC GCT TTG AAC CCA GAT TAT GTT GCT GTA GAG
 V   S   A   A   V   A   A   L   N   P   D   Y   V   A   V   E
                              450
CCT CCT GAG TTG ATA GGA ACT GGT ATC CCA GTC AGT AAG GCC AAG CCT
 P   P   E   L   I   G   T   G   I   P   V   S   K   A   K   P
                                  500
GAA GTG ATA ACC AAC ACA GTG GAG TTG GTT AAG AAG GTA AAT CCT GAG
 E   V   I   T   N   T   V   E   L   V   K   K   V   N   P   E
                                      550
GTT AAG GTT CTT TGT GGA GCT GGA ATA TCC ACG GGA GAA GAC GTT AAG
 V   K   V   L   C   G   A   G   I   S   T   G   E   D   V   K
                                          600
AAG GCA ATA GAG CTT GGA ACG GTA GGG GTT CTT TTA GCA AGT GGA GTA
 K   A   I   E   L   G   T   V   G   V   L   L   A   S   G   V
                                              650
ACA AAG CAA AGA TCC AGA GAA GGC GAT ATG GGA TCT TGT TTC GGG AAT
 T   K   Q   R   S   R   E   G   D   M   G   S   C   F   G   N
   678                                  700
AAT TAA GGAGTAGAAGCTAAATCTCTATCTTTACTGTTTTTCCTGTTTTTGAGGATTCATA
 N   *

TGCTGCA
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Fig. 4. Nucleotide sequence of the *tim* gene of *P. woesei* TIM and adjacent regions. The deduced protein sequence is given in the one letter code, the stop codon is marked by (*). The putative expression signals are underlined.

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                1                   20                  40
sec. struct.        B1          H1          B2    H2
T. brucei       MS-KPQPIAAANWKCNGSQQSLSELIDLFNSTSINHD--VQCVVASTFVHLAM
P. woesei       -AKLKEPIIAINFKTYIEATGKRALEIAKAAEKVYKETGVTIVVAPQLVDLRM
B. stearo.      M-RKP--IIAGNWKMHKTLAEAVQFVEDVKGHVPPA-DEVISVVCAPFLFLDR
E. coli         MRHP---LVMGNWKLNGSRHMVHELVSNLRKE-LAGVAGCAVAIAPPEMYIDM
S.cerevisiae    --AR-TPFVGGNFKLNGSKQSIKEIVERLNTASIPEN--VEVVICPPATYLDY
G. gallus       AP-R-KFFVGGNWKMNGDKKSLGELIHTLNGAKLSAD-T--VVCGAPSIYLDF
Z. mais         --GR-KFFVGGNWKCNGTTDQVEKIVKTLNEGQVPPS-VVEVVVSPPYVFLPV
                      ▲ ▲
                60                  80                  100
sec. struct.        B3          H3      B4    H4f
T. brucei       TKERLSHPKFVIAAQNA-IAKSGAFTGEVSLPILKDFGVNWIVLGHSERRAYY
P. woesei       IAESVE---IPVFAQHIDPIKPGSHTGHVLPEAVKEAGAVGTLLNHSENRMIL
B. stearo.      LVQAADGTDLKIGAQTMHFADQGAYTGEVSPVMLKDLGVTYVILGHSERRQMF
E. coli         AKREAEGSHIMLGAQNVNLNLSGAFTGETSAAMLKDIGAQYIIIGHSERRTYH
S.cerevisiae    SVSLVKKPQVTVGAQNAYLKASGAFTGENSVDQIKDVGAKWVILGHSERRSYF
G. gallus       ARQKLD-AKIGVAAQNCYKVPKGAFTGEISPAMIKDIGAAWVILGHSERRHVF
Z. mais         VKSQLR-QEFHVAAQNCWVKKGGAFTGEVSAEMLVNLGVPWVILGHSERRALL
                          ▲   ▲ ▲▲                  ▲ ▲
                120                 140
sec. struct.        H4      B5    H5f     H5
T. brucei       GETNEIVADKVAAAVASGFMVIACIGETLQERESGRTAVVVLTQIAAIAKKLK
P. woesei       ADLEAAIRRAEE----VGLMTMVCSNNPAVSAAVAVLNP--------------
B. stearo.      AETDETVNKKVLAAFTRGLIPIICCGESLEEREAGQTNAVVASQVEKALAGLT
E. coli         KESDELIAKKFAVLKEQGLTPVLCIGETEAENEAGKTEEVCARQIDAVLKTQG
S. cerevisiae   HEDDKFIADKTKFALGQGVGVILCIGETLEEKKAGKTLDVVERQLNAVLEEVK
G. gallus       GESDELIGQKVAHALAEGLGVIACIGEKLDEREAGITEKVVFEQTKAIADNVK
Z mais          GESNEFVGDKVAYALSQGLKVIACVGETLEQREAGSTMDVVAAQTKAIAEKIK

                160                 180                 200
sec. struct.    H6b   B6            H6          H7b   B7
T. brucei       KADWAKVVIAYEPVWAIGTGKVATPQQAQEAHALIRSWVSSKIGADVRGELRI
P. woesei       ------DYVAVEPPELIGTGIPVSKAKPEVITNTVELV----KKVNPEVKVLC
B. stearo.      PEQVKQAVIAYEPIWAIGTGKSSTPEDANSVCGHIRSVVSRLFGPEAAEAIRI
E. coli         AAAFEGAVIAYEPVWAIGTGKSATPAQAQAVHKFIRDHI-AKVDANIAEQVII
S. cerevisiae   --DWTNVVVAYEPVWAIGTGLAATPEDAQDIHASIRKFLASKLGDKAASELRI
G. gallus       --DWSKVVLAYEPVWAIGTGKTATPQQAQEVHEKLRGWLKSHVSDAVAQSTRI
Z. mais         --DWSNVVVAYEPVWAIGTGKVATPAQAQEVHASLRDWLKTNASPEVAESTRI
                          ▲
                220                 240                 260
sec. struct.        H7      B8  H8f   H8
T. brucei       LYGGSVNGKNARTLYQQRDVNGFLVGGASLKPEFVDIIKATQ
P. woesei       GAGISTGEDVKKAIELGTVGVLLASGVTKQRSREGDMGSCFGNN
B. stearo.      QYGGSVKGPDNIRDFLAQQQIDGPLVGGASLEPASFLQLVEAGRHE
E. coli         QYGGSVNASNAAELFAQPDIDGALVGGASLKADAFAVIVKAAEAAKQA
S. cerevisiae   LYGGSANGSNAVTFFKDKADVDGFLVGGASLKPEFVDIINSRN
G. gallus       IYGGSVTGGNCKELASQHDVDGFLVGGASLKPEFVDIINAKH
Z. mais         IYGGSVTAANCKELAAQPDVDGFLVGGASLKPEFIDIINAATVKSA
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Fig. 5. Sequence alignment of TIMs of *P. woesei* and bacteria and eucarya represented by *Bacillus stearothermophilus* (*B. stearo*, [34]), *Escherichia coli* (*E. coli*, [35]), *Trypanosoma brucei* (*T. brucei*, [33]), yeast (*S. cerevisiae*, [36]), chicken (*G. gallus*, [37]), maize (*Z. mais*, [38]). The secondary structural (sec. struct.) elements (H, α-helix; B, β-strand) of the three-dimensional structure of the *T. brucei* TIM [5] are given above the sequences. Introduced gaps for optimal alignment are marked by hyphens (-), conserved functional (K14, H99, E171) and interface residues [16] are indicated by filled triangles.

Box A and Box B elements of the archaeal promoter (pos. −15, −16; pos. −37 to −42; [26,27]) suggesting that these sequences possess functional importance as translational and transcriptional signals. Downstream of the coding region two five-T stretches follow (pos. 709–713; 718–722) corresponding to a putative transcription termination signal [28]. The presence of putative promoter and transcription termination signals suggests that the *tim* gene of *P. woesei* is not cotranscribed with other genes coding for enzymes of the Embden-Meyerhof-Parnas (EMP) pathway as was found for some bacterial species [29–31].

3.5. *Deduced amino acid sequence of the tim gene of* P. woesei

The reading frame codes for 225 residues. Since, however, the N-terminal Met residue is missing in the mature protein (as shown by protein sequencing, cf. section 2), the TIM subunit comprises only 224 residues with a theoretical molecular mass of 23 697 Da. This value corresponds well with the apparent subunit molecular mass of 24 000 Da as determined by SDS PAGE.

The deduced amino acid sequence of the *P. woesei* TIM exhibits sequence motifs, which clearly characterize the ar-

chaeal TIM as homologous to the bacterial and eukaryotic enzymes (Fig. 5). As expected, the functionally important residues (Lys 14, His 99, Glu 171 (Fig. 5); numbering of the residues according to the *T. brucei* sequence: Lys 13, His 95, Glu 167) are conserved. Out of the 14 highly conserved residues involved in the dimer interface interactions of the eucaryal TIMs [16], 8 are present in the *P. woesei* sequence indicating similar subunit contacts also in the archaeal enzyme.

Despite unequivocal homology, the average similarity between the *Pyrococcus* TIM sequence and the bacterial or eukaryotic sequences is rather low, ranging from 17.4% to 23.8% identity as compared to the identities between bacterial and eucaryal sequences, which exceed 38%. Thus, the relationship between archaeal, bacterial and eucaryal TIMs corresponds to that already found for the enzymes of the EMP pathway, glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase exhibiting a lower similarity between the archaeal and bacterial or eukaryotic homologues than between the latter ones [20,32].

Most strikingly, the TIM of *P. woesei* represents, with 224 amino acids, the shortest TIM sequence known up to now. For optimal alignment 4 gaps have to be inserted, with the greatest between pos. 146 and 165 (Fig. 5). Assuming a similar secondary structure arrangement as determined for the TIM of *T. brucei*, one gap is located within the loop between helix H2 and β-strand B3 (pos. 60–62), one gap eliminates α-helix H5 completely (pos. 146–165) and two further gaps shorten the helices α-H4 (pos. 119–122) and H6 (pos. 198–201). On the basis of the known spacial TIM structure [19], these gaps cluster at the bottom of the barrel and/or on that flank of the barrel without obvious functional importance (Fig. 6). From these changes one would expect a considerable structural rearrangement at the barrel bottom and at the barrel flank by eliminating α-helix H5. These rather localized changes may be correlated with the formation of new subunit binding sites in the tetrameric TIM. We propose a model, in which the dimerization of the TIM dimers occurs via the bottom and the functionally inert flank of the barrel (Fig. 7). Assuming that the TIM dimers are connected by the usual interface [16], a rather compact tetramer with four freely accessible active sites would result, whose globular structure is also claimed by gel filtration experiments. Ongoing crystallographic studies will reveal the validity of this model and give insights into the contribution of the additional subunit contacts to protein stability.

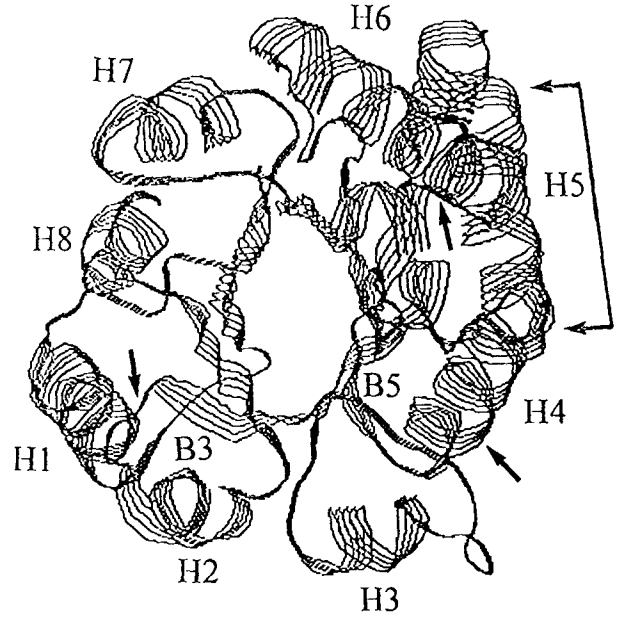

Fig. 6. Ribbon structure of *T. brucei* TIM monomer indicating the approximate positions (marked by arrows) of deletions found in the *P. woesei* structure. View: on the top of the α/β-barrel (drawn by RasMol V2.5, designed by R. Sayle, Glaxo Research and Development, Greenford, UK).

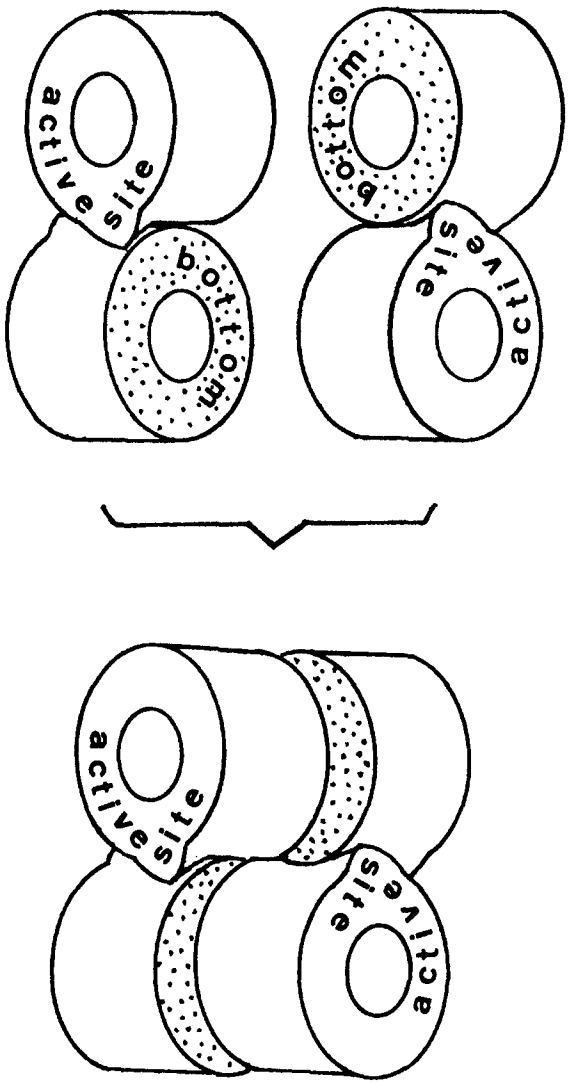

Fig. 7. Model of the monomer arrangement in the *P. woesei* TIM tetramer assuming new subunit contacts across the bottom and the functionally inert flank of the α/β-barrel. The interactive loop involved in the dimer contacts is represented by the protruding nose.

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