Identification of triosephosphate isomerase (TIM) genes from *Microcystis* (Cyanobacteria: Chroococcales) and theoretical analyses of the potential of cyanobacterial TIM as a target for designing specific inhibitors

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The genes encoding triosephosphate isomerase (TIM) in three species of Microcystis (*M. aeruginosa, M. viridis* and *M. wesenbergii*) were investigated. Reverse transcriptase–polymerase chain reaction indicated that they were transcribed in the cells. Analyses showed that their DNA and deduced amino acid sequences were highly conserved between all the three species, only a single nonsynonymous substitution was seen at position 31, from an Asp in *M. aeruginosa* and *M. viridis* to Glu in *M. wesenbergii*. Sequence alignment of these with 12 other known cyanobacterial TIM sequences showed that all the cyanobacterial TIMs had a very high level of amino acid identity (over 50% between each two). Comparison of the cyanobacterial TIMs with other reported TIMs (from diverse lineages of the three Domains) showed that they possessed common active-site residues and sequence motifs. All cyanobacterial TIMs have two common cysteine residues (Cys127 and Cys176), and the Cys176 is almost cyanobacteria-specific with only one exception in *Streptomyces coelicolor*. Both secondary structure alignment and comparative modelling of Synechocystis sp. TIM showed that Cys176 was located at the hinge region of the flexible loop-6 and might therefore be critical to the movement of TIM’s loop-6, which is important to the function of the enzyme. Thus, the cyanobacterial TIM-specific Cys176 may be a potential site for the discovery of suitable drugs against cyanobacteria, and such drugs may have utility in controlling water blooms due to cyanobacteria.

INTRODUCTION

Triosephosphate isomerase (TIM) is responsible for the reversible isomerization between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. This reaction plays an important role in both glycolysis and gluconeogenesis. TIM has the prototypical α/β barrel structural motif. The kinetic properties (Lambeir et al. 1987, 1991), three-dimensional structure and catalytic mechanism (Knowles 1991; Lodi et al. 1994; Kursula et al. 2001) have been described previously in a series of studies.

From a practical application perspective, TIM has been considered as a target for designing inhibitors used as medicines against pathogenic parasites (Garza-Ramos et al. 1996, 1998; Velanker et al. 1997; Maithal et al. 2002). For this purpose, inhibitors of TIM must be highly selective because TIM is a ubiquitous enzyme existing in both hosts and invading organisms. Gomez-Puyou et al. (1995) suggested that it was feasible to achieve specific inhibition of homologous enzymes with a high level of selectivity by targeting amino acid residues that were not conserved in evolution but were important for the proper functioning of these enzymes. Using this hypothesis, specific inhibition of TIMs have been studied to restrain some parasitic protozoans such as *Trypanosoma, Leishmania* and *Plasmodium* (Garza-Ramos et al. 1996, 1998; Velanker et al. 1997; Maithal et al. 2002).

In recent years, the problem of cyanobacterial blooms, resulting from water pollution and eutrophication, has become more and more serious (Pitois et al. 2001). Some cyanobacteria, such as the toxin-producing strains of *Microcystis*, contribute to cyanobacterial blooms and release toxins, which result in health and environmental hazards in waters (Dawson 1998; Frazier et al. 1998; van Buynder et al. 2001). Today, the prevention and control of cyanobacterial blooms is still an outstanding unsolved issue. So far, eight cyanobacterial genomes have been sequenced, and only one TIM gene has been identified (GenBank accession number: CAE07338.1, NP_442075.1, NP_923986.1, NP_681756.1, NP_488425.1, CAE20992.1, NP_875293.1, CAE19288.1) in each of their genomic databases. In addition, one TIM gene was reported in each of four other cyanobacteria (GenBank accession number: ZP_00325988.1, ZP_00164362.2, ZP_00174972.1, ZP_00111349.1). Thus, unlike photosynthetic eukaryotes, which have two copies of TIM genes, cyanobacteria have only one copy of the TIM gene in their genomes, and the same TIM functions in both their glycolysis and photosynthesis. This means that if TIM is inhibited, both these metabolic pathways in cyanobacteria will be blocked simultaneously. Therefore, cyanobacterial TIM might be an effective target enzyme for designing cyanobacterial inhibitors to control water blooms.

Prior to this work, no TIM genes were identified in *Microcystis*, which is generally the dominant genus contributing to cyanobacterial blooms (Romanowska-Duda & Tarczynska 2002). It was not known yet whether all cyanobacterial TIMs shares some common features. However, in cyanobacterial
TIMs, to employ the strategy suggested by Gomez-Puyou et al. (1995), it is important to identify an amino acid residue that is not conserved in evolution but plays an important role in the function of the enzyme. In the present work, firstly, TIM genes in three species of Microcystis (M. aeruginosa Kzing Lemmermann, M. viridis Lemmermann and M. wessenbergii Komrek) were amplified, cloned and sequenced. Then, the three Microcystis TIMs, together with TIMs of other cyanobacteria and noncyanobacteria, were analyzed to find potential sites for specific inhibitors of cyanobacterial TIMs.

**MATERIAL AND METHODS**

**Materials**

Microcystis aeruginosa (FACHB-315), M. viridis (FACHB-930) and M. wessenbergii (FACHB-574) were purchased from Algal Culture Collection of the Institute of Hydrobiology, CAS, and were cultured with Endo medium (Lu et al. 2001) in sunlight at 20–25°C.

**Amplification, cloning and sequencing of TIM genes**

Genomic DNA was extracted from Microcystis spp. by a modified phenol–chloroform method (Lu et al. 2001). Two pairs of primers were used in polymerase chain reaction (PCR). The first pair was designed by matching two highly conserved regions of all the reported TIM amino acid sequences through alignment, and the nucleotide sequences of the primers were according to TIM gene of Synechocystis sp.: P1, 16–36: 5'-ATTGCTGGTAATTGGAAAATG-3'; Pd1, 507–488: 5'-AGCCAAATCGGTTCGTAGG-3' (the nucleotide numbers was according to the TIM gene of Synechocystis sp.) and was used to amplify an expected segment of ~ 500 bp of TIM genes from Microcystis genomic DNA. The PCR was performed as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 1 min, 48°C for 1 min and 72°C for 1 min, followed by 72°C for 10 min. The PCR products of the expected size were purified from 2% agarose gel by using gel extraction kits (Watson Biotechnological Inc., Shanghai, China). The purified products were cloned into pMD 18-T Vectors (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China), and at least five clones of each sample were sequenced by ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) with BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, USA). To get much longer sequences, a second pair of primers was designed: Pu2, 236–255: 5'-AAATCTCGGGCGGATATGTA-3' (according to a conserved region of the three Microcystis TIM gene segments already obtained through alignment), Pd2, 718–700: 5'-AG(AG)AT(TC)G(C(TG)(AC)G(C)AA-3' (corresponding to the C-terminal conserved region of the reported cyanobacteria Synechocystis sp. and Nostoc sp. TIM genes). The PCR, cloning and sequencing were carried out as described above.

**Reverse transcriptase–polymerase chain reaction and PCR with the same primer pair**

Total RNA was isolated from axenic M. viridis using the UNIQ-10 total RNA minipreps classic kit (Shanghai Sangon Biologcal Engineering and Technology and Service Co., Ltd., Shanghai, China) according to the manufacturer’s instructions. The RNA was subsequently incubated with RQ RNase-free DNase (Promega Co., Madison, WI, USA) for 15 min at 37°C to remove contamination of genomic DNA, and terminated by adding Terminator mix. After treatment with chloroform and ethanol, the total RNA was purified with a column and used as templates for reverse transcriptase–polymerase chain reaction (RT-PCR). The reverse transcription reaction (with Pd1 as the RT primer) was carried out at 50°C for 30 min using the TaKaRa RNA PCR Kit (Avian Myeloblastosis Virus (AMV)) V.2.1 (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China) according to the manufacturer’s instructions. A control without adding AMV Reverse Transcriptase XL was set simultaneously. Subsequent PCR with the primer pair of Pu1/Pd1 was carried out using the same Kit for 35 cycles at 94°C for 30 s, 48°C for 30 s and 72°C for 45 s. In contrast with the RT-PCR, a PCR with the same primer pair was performed using M. viridis genomic DNA as templates. Both the RT-PCR and PCR products were analyzed in the same 1.5% agarose gels.

**Analyses of sequences**

DNA sequence analyses were performed by DNASTar v.5.0 (DNASTAR, Inc., USA). Multiple alignments of nucleotide sequences and amino acid sequences were carried out using CLUSTALW (Thompson et al. 1994).

**Modelling of Synechocystis sp. TIM**

The tertiary structure of mature Synechocystis sp. TIM was predicted by comparative modelling using a combination of the Swiss-Pdb Viewer and the SWISS-MODEL server (Peitsch 1995, 1996; Guex & Peitsch 1997). The Bacillus stearothermophilus TIM [PDB entry: 1BTM; ExPDB entry: IBTMA and IBTMB (Delboni et al. 1995)] was chosen as the template to model the Synechocystis sp. TIM because it was the best hit and shared 50.2% identity with the Synechocystis sp. TIM. When using Synechocystis sp. TIM as the target sequence to find suitable modelling templates in the ExPDB template database. Optimise Mode method was used to carry out the modelling. First, the sequences alignment between Synechocystis sp. TIM and B. stearothermophilus TIM was modified manually with Swiss-Pdb Viewer. Then the modelling request was submitted to Swiss-Model server to get the initial model. Fixing a carbon atoms of the initial model, energy minimization was performed by 500 steps steepest descent and 500 steps conjugate gradient. Root-mean-squared (RMS) values for the topologically equivalent α carbon atoms between the model and the template were calculated. Finally, protein structure superposition of the modelled Synechocystis sp. TIM over Homo sapiens TIM (PDB entry: 1HTI), Saccharomyces cerevisiae TIM (1YPI, 3YPI) and Escherichia coli TIM (1TRE) was performed with the DiCE structural alignment program (Sali & Blundell 1990).

**RESULTS AND DISCUSSION**

**Amplification, sequencing and identification of TIM genes in Microcystis spp.**

With the two pairs of primers (Pu1 and Pd1 for the 5’ end of the gene, and Pu2 and Pd2 for the 3’ end), two expected over-
lapping PCR products (both about 500 bp), were obtained from all the three species (*M. aeruginoisa*, *M. wesenbergii* and *M. viridis*). After sequencing and assembling the two overlapping sequences, pairwise sequence comparisons among the nucleotide sequences of the three species showed that they shared over 98% identity. The deduced amino acid sequences were more highly conserved: only one residue difference was observed, the Asp31 in *M. aeruginoisa* and *M. viridis* TIMs was substituted by Glu31 in *M. wesenbergii*. When the three deduced polypeptides were aligned with 153 reported TIM sequences (from diverse lineages of the three Domains, including 47 eukaryotes, 74 eubacteria and 22 archaebacteria) obtained from the NCBI Protein database, a high degree of homology was observed. The three deduced amino acid sequences of *Microcystis* genes had an identity of greater than 50% to each of those sequences from 12 previously reported cyanobacterial sequences. These alignments clearly demonstrated that we had obtained the TIM gene sequences for the three species of *Microcystis* (GenBank accession number AY238889, AY238890 and AY238891). A sequence file with the accession numbers and alignments reported here is available from the authors upon request.

In this work, the first pair of primers was designed to correspond to two highly conserved regions of all known TIM gene sequences, and the PCR products, which showed a single ~500 bp band in agarose gels, were very pure; the sequences obtained from five clones were completely identical. All these suggested that, as for the eight cyanobacteria whose genomes had already been sequenced, TIM genes probably occur as a single copy in the three *Microcystis*.

To verify the transcription of the three genes, RT-PCR and PCR with the same primer pair were carried out simultaneously on *M. viridis*. Both of the products showed the same expected size amplicons of about 500 bp (the calculated length is 500 bp) (Fig. 1, Lanes a and c). The negative result of the control of RT-PCR verified that the products of RT-PCR were derived from RNA and not from DNA contamination (Fig. 1, Lane b). This implied that the gene was transcribed in the cells and might not contain introns.

**Analyses of potential sites of cyanobacterial TIM as targets for designing specific inhibitors**

Including the present work, there are now 15 cyanobacterial TIM sequences known. When they were aligned with the other 153 known TIMs mentioned above, all the 15 cyanobacterial TIM sequences were shown to share common features with the other TIMs. For example, they also possessed the conserved active-site residues (Lys14, His96 and Glu165) and some common sequence motifs (part of the alignment results are shown in Fig. 2). In contrast to TIMs of other eubacteria and eukaryotes, the cyanobacterial TIMs had one deletion before the seventh β-strand (see Fig. 2). All the cyanobacterial TIMs had two common cysteine residues (Cys127 and Cys176). Cys127 was also found in other noncyanobacterial TIMs and was a strictly conserved residue of TIMs, but Cys176 was found almost exclusively in cyanobacterial TIMs (with the only exception of TIM of a noncyanobacterium *Streptomyces coelicolor*), and was almost a cyanobacterial TIM-specific cysteine.

TIM is a dimeric glycolytic enzyme, consisting of two identical monomers. The monomer has a typical TIM-barrel fold with an inner core cylinder of eight parallel β-strands surrounded by a concentric outer cylinder formed by eight α-helices. The eight central β-strands of the scaffold are labelled from the N-terminus to the C-terminus as β1–8, the corresponding α-helices are labelled α1–8. The loops connecting the β-strands to the subsequent helices are called loop 1–8. In loop-6 the conserved residues form a rigid lid and the non-conserved residues on both sides of the lid form two hinges (Joseph et al. 1990). According to the secondary structural definitions for TIMs of several species (including *Homo sapiens*, *S. cerevisiae*, *E. coli* and *B. stearothermophilus*; PDB entries 1HTI, 1YPI, 1TRE, 1BTM, respectively), the cyanobacterial Cys176 was located in the nonconserved region between the conserved lid region (WAIGTG) and the α6 helix, that is, in the hinge region of loop-6.

In our modelling of *Synechocystis* sp. TIM, the final total energy of the *Synechocystis* sp. TIM structure model was −13,184.065 kJ/mol after energy minimization, and the RMS of 240 topologically equivalent α carbon atoms between the model and the template was 0.96 Å when using the DiCE structural alignment program to superpose the modelled *Synechocystis* sp. TIM structure over the A-chain structures of 1HTI, 1YPI, 3YPI, 1TRE mentioned above, the backbone atoms aligned very closely (Fig. 3). All these strongly suggest that the tertiary structural model of *Synechocystis* sp. TIM had the same structural features of all reported crystallographic TIM molecules.

In addition, the model also showed that the cyanobacterial TIM-specific Cys176 was clearly located at the hinge of loop-6 (Fig. 3). The importance of the flexible loop-6 had been
Fig. 2. Partial results of TIM sequence alignment. Five cyanobacteria: *Synechocystis* sp. PCC 6803 (S.yn), *Nostoc* sp. PCC 7120 (N.os), *Trichodesmium erythraeum* IMS101 (T.er), *Microcystis aeruginosa* (M.ae) and *Prochlorococcus marinus* str. MIT 9313 (P.ma); two nonphoto- synthesic bacteria: *Escherichia coli* (E.co) and *Bacillus stearothermophilus* (B.st); two eukaryotes: *Homo sapiens* (H.sa) and *Saccharomyces cerevisiae* (S.ce). Cys176 of *Synechocystis* sp. TIM is indicated with an asterisk (*) above the sequence. The secondary structural elements of the three-dimensional structure of TIMs of *H. sapiens* (1HTI), *S. cerevisiae* (1YPI), *E. coli* (1TRE), *B. stearothermophilus* (1BTM) and *Synechocystis* sp. (modelled) are labelled with underlines (thin line, α-helix; bold line β-strand) and shadows (loop-6).

...demonstrated very clearly in previous work. The proposed role of this loop is to ensure efficient throughput of substrate to product and to stabilize the reaction intermediate (cis-enediolate) (Knowles 1991). During the catalysis, the binding of substrates or substrate analogues was accompanied by a change in the conformations of loops-5, 6 and 7. The most remarkable change occurred in loop-6—the tip of the loop undergoes a Cα movement of about 7 Å in response to ligand binding to provide new hydrogen bonds to the substrate phosphate group, and the motion of the loop-6 lid was caused by the change of dihedral angles of hinge residues (Joseph et al. 1990). These imply that the hinge residues of loop-6 in TIM play a pivotal role in catalysis.

In our work, both the secondary structural comparisons and the modelled *Synechocystis* sp. TIM structure indicated that Cys176 meets the criteria suggested by Gomez-Puyou et al. (1995), that is,
it is not a conserved amino acid residue, rather it is a cyanobacteria TIM-specific one, and it plays an important role in the function of TIM. Therefore, we suggest that Cys176 is suitable choice as a target residue for designing cyanobacteria TIM-specific inhibitors. Although Cys176 also exists in TIM of S. coelicolor, this will not affect the use of such inhibitors in the control of water blooms. Certainly, further work is needed to validate this potential target site, which includes designing, synthesis and testing of inhibitors that specifically interact with cyanobacterial TIM Cys176.

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