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Molecular interaction of artemisinin with translationally controlled tumor protein (TCTP) of *Plasmodium falciparum*

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ABSTRACT

Malaria causes millions of death cases per year. Since *Plasmodium falciparum* rapidly develops drug resistance, it is of high importance to investigate potential drug targets which may lead to novel rational therapy approaches. Here we report on the interaction of translationally controlled tumor protein of *P. falciparum* (*PfTCTP*) with the anti-malarial drug artemisinin. Furthermore, we investigated the crystal structure of *PfTCTP*. Using mass spectrometry, bioinformatic approaches and surface plasmon resonance spectroscopy, we identified novel binding sites of artemisinin which are in direct neighborhood to amino acids 19–46, 108–134 and 140–163. The regions covered by these residues are known to be functionally important for TCTP function. We conclude that interaction of artemisinin with TCTP may be at least in part explain the antimalarial activity of artemisinin.

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1. Introduction

The translationally controlled tumor protein (TCTP) was first described in the 1980s [1–3]. After being named fortilin, Q23, p23 or p21, it was finally termed translationally controlled tumor protein, because of its regulation at the translational level in cancer cells [4]. As histamine release was identified as a function of this protein, histamine releasing factor (HRF) was used as a synonym to TCTP [5]. In the meantime, there is mounting evidence that TCTP is not a cancer-specific protein, but is ubiquitously expressed in all eukaryotic organisms from protozoa such as *Plasmodium spec.* to plants and mammals [6]. Recently, TCTP has caught the attention of scientists in biomedicine, because its protein levels vary in response to a wide range of external stimuli, including stress. TCTP is involved in cell cycle regulation, malignant transformation, apoptosis as well as histamine release and other immunological functions [7]. Recently, a role of TCTP as target molecule for tumor reversion and malaria treatment has been discussed [8–10].

Artemisinin is a highly valuable anti-malarial drug because of its efficacy in otherwise drug-resistant *Plasmodium* strains [11–13]. However, the mechanisms of its anti-malarial activity are still not completely understood. Generation of free radicals following breakage of the endoperoxide bridge of artemisinin resulting in intracellular oxidative stress is one proposed mechanism [14,15]. The endoperoxide bridge is cleaved by the reaction with ferrous iron in erythrocytic heme and carbon-centered free radicals are generated [16]. TCTP was determined as a target molecule of artemisinin in *Plasmodium falciparum* [10], but the site of interaction remains to be elucidated. Iodoacetamide reduced the interaction of TCTP and artemisinin [9]. This led to the hypothesis that the single cysteine residue of the protein is responsible for drug binding. Subsequently, the interaction of artemisinin with TCTP was modeled using a homology model for *PfTCTP*, since a three dimensional structure of the protein was not available [17].

Abbreviations: TCTP, translationally controlled tumor protein; MS, mass spectrometry; MS/MS, tandem mass spectrometry; *m/z*, *mass* to charge ratio; LC, liquid chromatography; ESI, electron spray ionization; (Q)TOF, (quadrupole) time-of-flight; DTT, dithiothreitol.

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Here we present the analysis the interaction of human and plasmodial TCTP with artemisinin, the *PfTCTP* crystal structure and that the single cysteine of *PfTCTP* may not be neccessary for the interaction of artemisinin.

2. Material and methods

2.1. Mass spectrometry

All chemicals were obtained from Sigma (Deisenhofen, Germany); proteases were from Roche (Mannheim, Germany) and solvents from Biosolve (Valkenswaard, The Netherlands). Protein bands of interest were excised from the SDS gel, cut into pieces, transferred to micro tubes and destained. Cysteine residues were alkylated in the gel using iodoacetamide in the presence of DTT. Proteins were digested in the gel by chymotrypsin or endoproteinase AspN and the peptides were extracted according to Winter and Steen [18]. The volume of the samples was reduced in a vacuum centrifuge. Peptide samples were desalted using ZipTips (Millipore) and eluted by 50% acetonitrile 2% formic acid. Static nano electrospray analyses were performed using in house produced gold coated spray tips. The mass spectrometer was a QTOF2 system (Waters Micromass, Manchester, UK) Sample solutions were filled in the spray tips and electrospray was established by applying a voltage of 1000 V. Peptide precursor ions were fragmented applying five different collision energies depending on the precursor ion mass. Raw files were processed to *.pkl files using MassLynx 4.1 and searched against SwissProt using MASCOT 2.2. Search settings included the proteolytic cleavage site of the respective protease with one allowed missed cleavage as well as carboxamidomethyl (C) and oxidation (M) as variable modifications. Peptide tolerance was set to ±1.2 Da for 2+ and 3+ charged peptides and MS/MS tolerance was ±0.6 Da.

2.2. Cloning

Purified genomic DNA from *P. falciparum* 3D7 strain was provided by Dr. Michael Lanzer, Hygiene Institute of the University of Heidelberg, Germany. The *pf-tctp* (sequence PFE0545c from the PlasmoDB database; www.plasmodb.org) was amplified with Pfu (Fermentas, St. Leon-Rot, Germany) and the primers *PfTCTP*-Fw (GCGGGAATTCCATATGGAATTCCGCATGAAAGTATTTAAAGACGTTTTTAC containing a NdeI restriction site), and PfTCTP-Rev (CGCCGCTCGAGATATTTTTCTTCAAAAAGTCCATCAGATATATAAAC with an XhoI site). PCR conditions were as follows: one cycle at 94 °C for 2 min followed by 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and one cycle at 72 °C for 10 min. The PCR products were digested with NdeI and XhoI and ligated in the pET21a vector (Invitrogen, Darmstadt, Germany). *Escherichia coli* NovaBlue cells (Novagen, Nottingham, UK) were transformed with the pET21a/*pf-tctp* plasmid and grown in 2 × YT medium. The amplified plasmid was purified and sequenced in both directions by MWG Biotech (Ebersberg, Germany). Only *PfTCTP* genes with a point mutation (TGT to TAT) causing a cysteine to tyrosine mutation at position 14 were obtained. Through site-directed mutagenesis using QuickChange site-directed mutagenesis kit (Agilent, Stratagene products, Böblingen, Germany) with primers Mut_*PfTCTP*-Fw (5′-GAAAGTATTTAAAGACGTTTTTACAAATGATGAAGTATGTTCCG-3′) and Mut_*PfTCTP*-Fw (5′-CGGAACATACTTCATCATTTGTAAAAACGTCTTTAAATACTTTC-3′) following the manufacturers protocol, Tyr14 was re-mutated into Cys14. The re-mutated plasmid was named pET21a/*pf-tctpM*.

2.3. Expression and purification of recombinant TCTP molecules

Competent *E. coli* (DE3) RIL cells (Stratagene, La Jolla, CA, USA) were transformed with the two plasmids. Ten milliliters of an overnight culture were diluted 1:100 in 2 × YT medium supplemented with 100 μg/mL carbenicillin (Serva, Heidelberg, Germany) and grown at 37 °C to a ΔOD_{600} of 0.6. Expression of wild-type or Tyr14Cys TCTP was induced with 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). After overnight cultivation at 16 °C, the cells were harvested by centrifugation, suspended in 15 mL buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.0) containing 20 μM PMSF (phenylmethyl sulfonyl fluoride), 150 nM pepstatin and 4 nM cystatin and incubated for 30 min with 200 μg/mL lysozyme (Sigma, Deisenhofen, Germany) and 20 μg/mL DNase (Sigma, Deisenhofen, Germany) at 20 °C. After sonication (3 × 1 min, 60 W), the suspension was centrifuged (30 min at 4 °C, 35,000 × *g*) and the supernatant was kept on ice. The pellet was re-suspended in 15 mL buffer A, sonicated and centrifuged again. Both supernatants were combined. The purified proteins were named *PfTCTP* (*P. falciparum* TCTP) and *PfTCTP*-Tyr14 (*P. falciparum* TCTP with Cys- > Tyr mutation at position 14).

2.4. Affinity chromatography on Ni-NTA superflow

The combined supernatants were applied onto a Ni-NTA superflow C10/10 column (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A. The column was washed at a flow rate of 1.0 mL/min with buffer A followed by buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 7.0) until the eluent had a $\Delta A_{280} \leq 0.01$ in each washing step. *PfTCTP* was eluted with buffer C (50 mM NaH_2PO_4, 300 mM NaCl, 100 mM imidazol, pH 7.0).

2.5. Surface plasmon resonance (SPR) spectroscopy

SPR measurements were performed at 20 ± 0.1 °C on an IBIS dual channel cuvette measurement system (Intersense Instruments, Amersfort, Netherlands). The SPR sensor chip carboxymethyldextran CMD 20 m was used for measuring interactions with TCTP. Artemisinin was dissolved in DMSO and diluted in the assay buffer (10 mM MOPS, 150 mM NaCl, 0.001% Nonidet P40, pH 7.2) whereas the final concentration of DMSO in each measurement was 0.5% (v/v). K_d values were calculated according to the monophasic association model depending on the non-linear regression of the primary data. Using Evilfit software [*] the data were additionally evaluated.

2.6. Crystallization

PfTCTP was crystallized at 4 °C by the hanging drop procedure. The protein solution (6 mg/mL in buffer A) was mixed with the crystallization buffer (21.5% (w/v) PEG 4000, 3% (w/v) 1,6-hexanediol, 50 mM bis-tris methane, 50 mM magnesium actetate, pH 6.2) in an 1:1 ratio to a final volume of 10 μL. All chemicals were purchased from Sigma (Deisenhofen, Germany). Needle-shaped crystals of 1.3 × 0.18 mm size were obtained within 3–7 days.

2.7. Data collection

An X-ray diffraction dataset was collected at 100 K. Prior to cryo-cooling, the crystal was step-soaked in reservoir solution containing up to 20% (v/v) glycerol. Native data were recorded on a Mar300 detector system (Mar0research) using Cu Kα radiation (λ = 1.5418 Å) and φ-scans with a scan width of 1.0° and 20 min exposure per image. Data processing including reflections up to 2.55 Å resolution (guided by the R_{merge} factor) was carried out using MOSFLM [19] and SCALA, which is part of the CCP4 [20] software suite.

2.8. Structure determination

The crystals obtained for *PfTCTP* belonged to space group $P4_32_12$. The structure was determined by molecular replacement

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