STRUCTURAL INSIGHT INTO THE ACTIVATION MECHANISM OF TOXOPLASMA GONDII NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASES BY DISULFIDE REDUCTION

Ulrike Krug1, Matthias Zebisch1,2, Michel Krauss1, Norbert Sträter1

From the 1Institute of Bioanalytical Chemistry, Center for Biotechnology and Biomedicine, University of Leipzig, D-04103 Leipzig, Germany, and the 2Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, UK.

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To whom correspondence should be addressed: Norbert Sträter, Institute of Bioanalytical Chemistry, University of Leipzig, Deutscher Platz 5, 04103 Leipzig, Germany. Fax: +49-(0)341-9731319. E-mail: strater@bbz.uni-leipzig.de

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Background: Disulfide-regulated NTPDases from T. gondii are related to the virulence of the parasite.

Results: Crystal structures of the active and inactive state were determined.

Conclusion: The 258-268 disulfide bridge acts like a clamp, which upon reduction allows concerted motions of the subunits and domains of the tetrameric enzyme.

Significance: First molecular model of the disulfide regulated activation mode.

SUMMARY

The intracellular parasite Toxoplasma gondii produces two nucleoside triphosphate diphosphohydrolases (NTPDase1 and -3). These tetrameric, cysteine-rich enzymes require activation by reductive cleavage of a hitherto unknown disulfide bond. Despite a 97% sequence identity both isozymes differ largely in their ability to hydrolyze ATP and ADP.

Here, we present crystal structures of inactive NTPDase3 as apo form and in complex with the product AMP to resolutions of 2.0 Å and 2.2 Å, respectively. We find that the enzyme is present in an open conformation that precludes productive substrate binding and catalysis. The cysteine bridge 258-268 is identified to be responsible for locking of activity. Crystal structures of constitutively active variants of NTPDase1 and -3 generated by mutation of C258-C268 show that opening of the regulatory cysteine bridge induces a pronounced contraction of the whole tetramer. This is accompanied by a 12° domain closure motion resulting in the correct arrangement of all active site residues. A complex structure of activated NTPDase3 with a non-hydrolyzable ATP analog and the cofactor Mg2+ to a resolution of 2.85 Å indicates that catalytic differences between the NTPDases are primarily dictated by differences in positioning of the adenine base caused by substitution of R492 and E493 in NTPDase1 by glycines in NTPDase3.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasitic protozoan which can infect a wide variety of warm-blooded animals and humans. About 1/3 of the human world population is infected with the pathogen but chronic infections are normally asymptotic (1). However, toxoplasmosis can cause dangerous complications for patients with AIDS, developing fetuses or organ-transplant recipients undergoing immune-suppressive treatment (2).

Several microbial pathogens including T. gondii express nucleoside triphosphate diphosphohydrolases (NTPDases, E.C. 3.6.1.5). In vertebrates cell surface located homologs (< 15% sequence identity) are well established to control purinergic signaling events via degradation of extracellular nucleotides (3-6). In contrast, the exact function of NTPDases from microbial pathogens is less clear (7). In recent years, evidence is accumulating that the expression of NTPDase genes is required for virulence of many pathogens (7-11). As host ATP exerts a proinflammatory action via P2 receptors and acts as a danger signal it appears
likely that microbial NTPDases interfere with this host response to suppress inflammatory responses and evade immune reactions (7). However, the intracellular location of *T. gondii* and the millimolar *K_m* values of *TgNTPDases* argue against a function to interfere with the canonical extracellular purinergic signaling. Alternatively, a role in purine salvage has been proposed, as many pathogens including *T. gondii* are purine-auxotroph and need to scavenge adenine or uracil for growth (12).

Two NTPDase genes, *TgNTPDase1* (NTPase II) and *TgNTPDase3* (NTPase I) can be expressed in *T. gondii* (7,8,13). Both enzymes consist of 628 amino acids including 15 cysteines (8,13) and form non-covalent homo-tetramers in solution. Despite their high sequence identity of 97%, they exhibit significant differences in substrate specificity: NTPDase1 cleaves ATP and ADP about equally well, whereas NTPDase3 is much more specific towards ATP. Also, *TgNTPDase3* cleaves ATP at 4.5 times the rate of *TgNTPDase1* (8,14). Caused by a short patch of dissimilar amino acids, these differences in substrate specificity are correlated to the virulence of the parasite: NTPDase1 is found in virulent and avirulent *T. gondii* strains whereas NTPDase3 is only found in virulent strains (8,14). Via antisense RNA inhibition it was demonstrated that NTPDase3 is required for replication of the parasite inside the host cells but appeared not to influence parasite invasion (15). In contrast, pretreatment of parasites with a monoclonal antibody inhibiting both NTPDases significantly decreased the number of infected cells, suggesting that one or both isoforms are involved in invasion of the parasites into host cells (16).

Strikingly, *TgNTPDases* are produced as inactive proforms that accumulate in the dense granules and are secreted in large quantities (up to 8% of the total protein) into the parasitophorous vacuole that surrounds the parasite after infection (13,17).

In *vitro*, activation of the dormant enzymes can be achieved by incubation with dithiol compounds. Hence, activation appears to be induced by reduction of a yet unidentified disulfide linkage. How and when exactly the activation occurs *in vivo* is currently unclear. In cellular assays activation of NTPDases by exogenously added dithiols leads to rapid depletion of host cell ATP and an increase in host calcium levels and results in an abrupt exit of parasites from cells (18). It has been speculated that *T. gondii* itself is responsible for the activation of the *TgNTPDases* (7,18,19). Glutaredoxin can activate *TgNTPDase3 in vitro*, and it may be secreted by the parasite for stimulating exit from host cells (19,20). In summary, the presence of NTPDases and their activation has been implicated in *T. gondii* invasion, replication, and egress.

Based on crystal structures we explain here the activation mechanism of *TgNTPDases*. The cysteine bridge responsible for suppression of enzyme activity was identified and appears to lock the enzyme in an open, inactive conformation. Reduction of this cysteine bridge induces a pronounced conformational change that involves a contraction of the whole tetramer and closure of the active site clefts. Complex structures with the substrate analog adenosine 5'- (β,γ-iminotriphosphate) (AMPPNP) and the product AMP help to explain the different substrate specificities of *TgNTPDase1* and -3.

**EXPERIMENTAL PROCEDURES**

**Protein preparation**

The cDNA coding for the mature *T. gondii* NTPDase1 and -3 were kindly provided by Michael Johnson (University of Technology, Sydney/Australia) and cloned via *NdeI-* and *XhoI*-sites into the pET20b(+) vector (Novagen, Madison, WI). The expressed proteins consisted of residues T26 to L628 which corresponds to the mature protein without the signal peptide (8). In addition to these residues, the constructs contained an N-terminal methionine as well as an additional glutamate and a hexahistidine tag at the C-terminus.

The proteins were expressed in *E. coli* Rosetta pLysS cells after induction with 1 mM IPTG in form of inclusion bodies which were isolated and purified as described (21). Denatured and reduced NTPDases at a concentration of 3 mg/ml were refolded by rapid dilution into 1.5 l 100 mM Tris pH 8.5, 10% (v/v) glycerol, 1 mM EDTA, 1 mM reduced glutathione and 0.2 mM oxidized glutathione to a final concentration of 100 µg/ml. After incubation for seven days at 12°C, the protein was concentrated 20-fold to approximately 70 ml, dialyzed two times against 50 volumes of 20 mM Tris pH 8.5 and subjected to anion exchange chromatography (5 ml HiTrap Q HP column, GE Healthcare) using 20 mM Tris/Cl, 1 mM EDTA, pH 8.5 as running buffer. A linear salt gradient from 0 to 2 M potassium acetate over 30 column volumes was applied to elute the protein. The most active fractions were pooled,
concentrated and further purified using size exclusion chromatography (Superdex 200 16/60, GE Healthcare). Purification was carried out on FPLC instruments (Äkta Explorer/Äkta Purifier, GE Healthcare). Protein was concentrated to 10 mg/ml and stored in size exclusion buffer (10 mM Tris pH 8, 1 mM EDTA, 1 mM NaN₃) at -80°C.

Protein concentrations of purified protein were analyzed using extinctions at 280 nm and specific extinction coefficients calculated by the ProtParam tool (see SI, Table S1)(22).

**Activity assay**

The reaction buffer in all activity assays contained 50 mM Hepes/NaOH (pH 7.5) and 20 mM magnesium acetate and was chosen according to previous studies (8,13,14,23). Protein samples were diluted in this buffer supplemented with 100 µg/ml BSA to prevent adhesion of NTPDase to the reaction tube. The reaction was started in microtiter plates by addition of 280 µl reaction buffer containing 2 mM ATP or 5 mM ADP and optionally 3 mM DTT to 20 µl of protein sample. Released phosphate was measured colorimetrically using a modified malachite green assay (24).

**Preparation of cysteine to serine mutants**

Cysteine to serine mutants were prepared using the QuikChange-method from Stratagene and positive clones were validated by sequencing. The sense mutagenesis primer for NTPDase3 had the sequence 5'-GAC CCA GCT AGG AGC ATG ATT GAT GAA TAC GG -3' for mutagenesis of cysteine 258 to serine (C258S), 5'-GG GTG AAG CAA TC CCG AAT GAC CTT GCT GG-3' for C268S, 5’-CTC AAG GAG CTT GCT ATT AAC GAT GAG TTT TGT C-3' for C341S, 5’-G CAA GGC GGA ATT TCC TCC AAC CCG TG-3' for C352S and 5'-G AAG ATC GAG AAC TC CAG TAT ATA ATC AAA GGA ACC GG-3' for C433S, containing the respective point mutation (underlined). The proteins were subjected to rapid dilution refolding in 1.5 ml test scale and without further purification subjected to activity tests.

To account for amino acid differences the C268S-mutagenesis primer with the sequence 5' -GAA TAC GGG GTG AAG CAT TC CCG AAT GAC CTT GCT GG-3' was used to generate TgNTPDase1 C258S-C268S. In the following, the mutation C258S-C268S is indicated as ∆CC.

**Isothermal titration calorimetry (ITC)**

ATPase and ADPase activities were determined at 25°C in a VP-ITC isothermal titration microcalorimeter (MicroCal part of GE Healthcare, Freiburg/Germany) as described (25). In this work two buffers were employed that were optimized for low dilution endotherms of the nucleotides. Buffer 1 is identical to the one used in the characterization of rat NTPDase1 (25) and contained 100 mM Tris, 50 mM NaCl, 10 mM CaCl₂, 100 µg/ml BSA, adjusted to pH 7.4 with malonic acid. Buffer 2 contained 10 mM MgAc₂ instead of CaCl₂ to resemble more closely those buffers which have previously been used for characterization of TgNTPDases (8,13,14,23). The molar enthalpy of hydrolysis was determined by subtracting dilution endotherms from heat recordings of experiments with complete hydrolysis of the nucleotide. For the magnesium buffer, the molar enthalpy of hydrolysis was found to be -8.25 ± 1.35 kcal/mol for ATP and -11.17 ± 0.51 kcal/mol for ADP. Values for the calcium buffer were determined previously (25). The catalytic parameters Kₘ and kₗ were then determined for the permanently activated variants (see results section) by using the multiple injection method (26-28). Substrate was added in a step-wise manner from a 30 mM stock solution in reaction buffer to 0.4 nM (TgNTPD1∆CC, ATP), 0.4 nM (TgNTPD1∆CC, ADP), 53.4 pM (TgNTPD3∆CC, ATP) or 4 nM (TgNTPD3∆CC, ADP) enzyme equilibrated in the ITC cell. Thermal power data recorded after compensation of dilution endotherms (determined in pilot experiments) were transformed to apparent rate constants using the molar reaction enthalpy and the molar enzyme concentration (25). Experimental data were fitted based on Michaelis-Menten kinetics to derive the kinetic constants kₗ and Kₘ using OriginPro 8G.

**Crystallization and derivatization**

TgNTPDase3 (T26-L628) wild-type crystallized within two days at 19°C and 8 mg/ml protein concentration by hanging-drop vapor diffusion after mixing 1 µl of protein with 0.7 µl reservoir solution (containing 200 mM ammonium acetate, 25 mM CaCl₂, 50 mM Na-cacodylate pH 6.2, 11% (w/v) PEG4000) and 0.3 µl microseeding solution. Microseeds were prepared using Seed Beads (Hampton Research, Aliso Viejo, CA/USA), 500 µl of 200 mM KCl, 10 mM MgCl₂, 50 mM Na-cacodylate pH 6.5, 10% (w/v) PEG4000 and crystals grown from this condition.
Heavy atom derivatives were produced by soaking the crystals 45 minutes in a new drop with the composition of the original reservoir solution supplemented with 2 mM thiomerosal or cis-dichlorodiammine platinum. The iodide derivative was prepared by soaking of the crystal in the final cryosolution supplemented with 100 mM KI for 30 seconds and immediate cryocooling in liquid nitrogen.

The AMP complex was produced by soaking the crystal during cryoprotection with 20 mM AMP for 2 min.

Crystals of the disulfide mutant $\text{TgNTPDase3}_{\Delta \text{CC}}$ in complex with Mg$^{2+}$×AMPPNP were obtained at 19°C within two days by mixing 1 µl of a 10 mg/ml protein solution (containing 2 mM AMPPNP and 10 mM magnesium acetate) and 1 µl reservoir solution (100 mM KCl, 10 mM MgCl$_2$, 50 mM Tris pH 8.5, 30% (w/v) PEG400). $\text{TgNTPDase1}_{\Delta \text{CC}}$ was crystallized accordingly in 100 mM Tris pH 8.2, 50 mM MgCl$_2$, 25% (v/v) pentaerythritol ethoxylate (17/8 PO/OH).

Data collection and structure determination

X-ray data were collected at 100 K. Cryoprotection was achieved by stepwise transfer to a cryoprotection buffer which was the reservoir or soaking solution plus 20% (w/v) PEG200 for $\text{TgNTPDase3}$ and $\text{TgNTPDase1}_{\Delta \text{CC}}$ or 50% (w/v) PEG400 for $\text{TgNTPDase3}_{\Delta \text{CC}}$. X-ray data sets were collected at BL14.1 and BL14.2 at Protein Structure Factory/Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung (Berlin, Germany) and beamline ID23-2 at the European Synchrotron Radiation Facility (Grenoble, France). Data sets were processed and scaled using XDS (29) or MOSFLM and SCALA (30) (Table 1). For the data sets of active $\text{TgNTPDase1}_{\Delta \text{CC}}$ and $\text{TgNTPDase3}_{\Delta \text{CC}}$ many crystals had to be tested to find a suitable specimen for structure analysis because the diffraction spots were diffuse along the short reciprocal unit cell axis c* and were overlapping for most of the crystals.

The structure of $\text{TgNTPDase3}$ was determined by multiple isomorphous replacement with anomalous scattering (MIRAS) using data of the iodide, mercury and platinum derivatives in the program autoSharp (31). Experimental phases were significantly improved with the program DM (32) applying NCS-operators which were manually determined from the heavy atom positions. An initial model was built by Buccaneer (33) and refined using REFMAC5 (34) and Buster-TNT (35). The structure of the active disulfide mutant $\text{TgNTPDase3}_{\Delta \text{CC}}$ was determined by molecular replacement using Phaser (36). Molecular replacement trials with the tetrameric structure or the monomer as search models failed. A first monomer could be placed by searching for truncated N- and C-terminal domains separately. Additional monomers were then found using this monomer as search model. Two half-tetramers are present in the asymmetric unit. Full tetramers can be generated by the crystallographic symmetry operators. Given the limited resolution of the active $\text{TgNTPDase3}_{\Delta \text{CC}}$ variant, high NCS restraints were used during refinement. The structure of $\text{TgNTPDase1}_{\Delta \text{CC}}$ was determined with the program Molrep (37) using a $\text{TgNTPDase3}_{\Delta \text{CC}}$ monomer as search model. Two monomers are present in the asymmetric unit of the crystals of $\text{TgNTPDase1}_{\Delta \text{CC}}$ and the full tetramer is generated by the crystallographic symmetry operators. The crystal structure was refined with Buster-TNT (35). TLS (Translation/Libration/Screw) refinement was employed in refinement for all structures. TLS groups were assigned based on the output of the TLS Motion Determination Home Server (38,39) and were defined for each chain as I: 35-58, II: 59-256 and 586-629, III: 257-268, IV: 269-394 and 425-585, V: 395-424.

Structure analysis

The buried surface area was determined using the program AREAIMOL (30) by subtracting the accessible surface area of the tetramer from the sum of the accessible surface areas of the dimers. The tetramer contact areas were calculated as half of the buried surface area. All molecular figures were generated with the program PyMOL (www.pymol.org). For an automatic determination of dynamic domains, the program DynDom (40) was used. Residues that move together as a rigid body during the conformational change are assigned to a dynamic domain based on a clustering algorithm. A window size of 5 residues was used and the ratio of interdomain to intradomain displacements was set to a relatively low value of 0.7 for the domain assignment. The large intradomain displacements are due to large changes in the conformation in particular of residues 257 to 269 and 459 to 471.
RESULTS AND DISCUSSION

Crystal structure of the dormant state of T. gondii NTPDase3

TgNTPDase3 was refolded from purified bacterial inclusion bodies, separated from misfolded protein and crystallized. The crystal structure was determined at 2.0 Å by the MIRAS method using crystals derivatized by thiomerosal, potassium iodide and cis-Pt(NH3)2Cl2 (Table 1). An almost complete thiomerosal, potassium iodide and cis-method using crystals derivatized by structure was determined at 2.0 Å by the MIRAS

interface via interactions with symmetry mates of these helices generated by the r-dyad (Figure 2C). In general, hydrophobic interactions are interspersed with hydrogen bonds and salt bridges. Closely adjoining interface I, subunit interactions are formed via the β-hairpin of strands 6 and 7. This interaction appears to be central for the activation mechanism as discussed below.

Interface II (Figure 2D) is formed along the p-axis between the C-terminal domains of chains A and B as well as C and D with a contact area of 3000 Å² per tetramer. The interface includes residues of loop 289 to 301 (between β-strands 9 and 10), the region 405 to 412 (partially involved in α-helix L) and α-helix P. Based on visual inspection, interface II is estimated to be relatively loose, lacking specific polar interactions and strong hydrophobic contacts.

The structure of the dormant state of TgNTPDase3 represents an open, inactive conformation.

Superposition of TgNTPDase3 and RnNTPDase2 (41) shows that the cleft between the two domains is more open in the microbial enzyme as a result of a domain rotation of about 12° (Figure 3). This leads to a relative orientation of the catalytic residues that would not allow productive binding of nucleotides, as shown by superposition with the complex structure of RnNTPDase2 with Ca²⁺×AMPPNP (41) (Figure 3B). G280 of TgNTPDase3 is expected to bind to the β/γ-bridging oxygen of

Tetramer structure

The TgNTPDases form homotetramers with 222 symmetry (a dimer of dimers). In the following discussion, we denote the three perpendicular 2-fold axes as p, q and r as defined in Figure 1C and Figure 2A. The TgNTPDase3 tetramer has dimensions of ≈147 Å × 101 Å × 85 Å along the p, r, q axes, respectively. Mostly hydrophobic interactions account for the formation of the tetramer (Figure 2). Two major interfaces are involved in oligomerization.

Interface I (Figure 2B,C) is formed between the N-terminal domains of chains A and D as well as B and C and symmetrical around the r-axis. A large surface contact area of 5334 Å² per tetramer is formed by this interaction. About half of this area (2433 Å²) can be attributed to the N-terminal helix alone which protrudes about 30 Å into the neighboring monomer and forms interactions with helices C, D and G as well as residues P225 and F226 (Figure 2B). The latter residues are positioned for this interaction as a result of the formation of the β-bulge of strand 5. Helix A is indispensable for the formation of active protein as it was not possible to refold denatured protein into an activatable form from an N-terminally shortened variant (amino acids L55 to L628, data not shown). In proximity to the N-terminal helix, the C-terminal helices U and V form the remaining contact area of this interface via interactions with symmetry mates of these helices generated by the r-dyad (Figure 2C). In general, hydrophobic interactions are interspersed with hydrogen bonds and salt bridges. Closely adjoining interface I, subunit interactions are formed via the β-hairpin of strands 6 and 7. This interaction appears to be central for the activation mechanism as discussed below.

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ATP, but the observed distance of 5.5 Å of this residue to the substrate is 2.5 Å longer than the corresponding hydrogen bond in RnNTPDase2 (41). Furthermore, S282 cannot bind to the γ-phosphate group of ATP, as this distance of 4.7 Å is 1.5 Å longer compared to the bond in RnNTPDase2 (41). In addition, E277 cannot position the metal-coordinating water molecules and the salt bridge between R192 and the catalytic base E236 can not be formed as a result of the open active site cleft in TgNTPDase3. The latter interaction was found to be important for correct positioning of the catalytic base (41,43,44). These findings indicate that in the dormant form of TgNTPDases the open state may be stabilized and activation might be achieved by induction of a domain closure motion.

A complex structure with the substrate fragment AMP shows that the position of the nucleoside binding site is conserved between RnNTPDase2 (41) and TgNTPDase3. However, weaker and fewer contacts are made to the nucleoside in TgNTPDase3. This may explain the much larger $K_m$ values of TgNTPDases for nucleotides compared to their mammalian orthologs (8,21). In TgNTPDase3 the adenine base is sandwiched between L553 and the peptide bond between G492 and G493. In contrast, in RnNTPDase2 the nucleobase is bound between the large planar side chain groups of R394 and Y350 (41). L557 in TgNTPDase3 lies in the plane of the nucleobase but in contrast to Y398 in RnNTPDase2 (41), it cannot form direct or indirect hydrogen bonds with it. The hydrophobic binding pocket for the base is further formed by G492 and A496.

Identification of the cystine linkage involved in enzyme activation

The crystal structure of TgNTPDase3 revealed the so far unknown disulfide pattern of TgNTPDases. They contain 15 cysteines (Figure 1 and SI Figure S1) that form seven disulfide bonds (1, C59-C88; 2, C258-C268; 3, C341-C352; 4, C356-C445; 5, C365-C433; 6, C396-C413; 7, C526-C558) and one free cysteine (C186). The free cysteine is located on β-strand 4 and is relatively buried inside the molecule and isolated from other cysteines. Mutation of the free cystine C186 yielded a variant that retained ~75% of the activity of the wild-type after activation with DTT or TCEP (data not shown). Sequence alignments using all available NTPDase sequences (Protein families database, PFAM 01150) shows that disulfides 4 and 7 are strictly conserved in the NTPDase family. These disulfides are required for correct folding of NTPDases as determined by mutations to serine residues for mammalian NTPDases (45,46).

Knowing the disulfide pattern of TgNTPDases cysteine mutations were introduced to identify the disulfide responsible for enzyme activation. Conserved disulfide bonds were not considered for mutagenesis experiments. Variants C258S-C268S, C341S-C352S and C433S of TgNTPDase3 were prepared. In particular, the disulfide bridge C258-C268 was chosen as a preferred target due to its location in an exposed loop between the monomers.

Subsequent activity assays with protein out of small-scale refolding batches identified variant C258S-C268S to be active with and without addition of DTT, whereas no activity at all was measured for the two other variants. This cystine bridge is formed between the strands of a β-hairpin that protrudes between domain I and II (Figure 1A) and reaches across the internal void of the tetramer to interact with the diametrically positioned monomer close to interface I (Figure 1C, Figure 2B). Since the kinetic characterization (see below) of the C258S-C268S variant demonstrated that this disulfide bridge is solely responsible for the activation of the enzyme by thiol compounds, variants of other disulfide bridges were not characterized in this study.

To gain further insight into catalysis and the activation mechanism of TgNTPDases, large scale refolding and purification of C258S-C268S variants were carried out for TgNTPDase1 and -3 (further referred to as TgNTPDase1 and -3 ACC). The permanently active variants were subjected to kinetic analysis and crystallization trials.

Kinetic characterization

The activity of the ACC-variants was found to be ~3 to 6-fold higher for ADP and ATP than that of the DTT-activated wild-type enzymes (Figure 4A,B; see SI Table S2). Addition of DTT did not raise activity of the ACC-mutants any further. Thus, mutagenesis of the C258S-C268S linkage can fully activate TgNTPDases without the requirement of further activation by thiol reagents. These findings strongly indicate that the reduction of this disulfide bridge alone is responsible for the activation of the wild-type enzymes. Hence, the generation of permanently activated variants allowed us for the first time to measure $k_{cat}$ and $K_m$ values unbiased by
incomplete activation of the latency-associated disulfide bridge or partial unspecific reduction of other structurally important disulfide bonds. To derive the kinetic constants we utilized a novel calorimetric activity assay (25). Briefly, the heat release from hydrolysis of the energy-rich anhydride bonds is measured in an isothermal titration calorimeter (Figure 4C). This assay allows to determine $K_m$ and $k_{cat}$ with high accuracy in a single experiment (Figure 4D, Table 2). No labeling of substrates or separation of substrates and products is required.

We measured substrate turnover in two buffers, (1) 100 mM Tris/malonate, 50 mM NaCl, 10 mM CaCl$_2$ (pH 7.4) and (2) 100 mM Tris/malonate, 50 mM NaCl, 10 mM MgAc$_2$ (pH 7.4). Substrate was titrated to the enzyme from a 30 mM solution in reaction buffer (see example in Figure 4C,D). Table 2 shows the magnesium buffer to be preferred for ATP, whereas for TgNTPDase3CC the measurements indicate slightly higher activities in CaCl$_2$-buffer.

$K_m$ values were found to be in the low mM-range. Specificity constants for TgNTPDase3CC show a 202-fold (Mg$^{2+}$) or 177-fold (Ca$^{2+}$) higher specificity constant ($k_{cat}/K_m$) for ATP over ADP (Table 2). The preference of TgNTPDase3CC for ATP is also shown in the malachite green assay (Figure 4B) and is in agreement with the kinetic data of the wild-type enzymes activated by thiol reduction (8).

TgNTPDase1 was described to hydrolyze ATP and ADP equally well (8), which could be confirmed in the malachite green assay for TgNTPDase1 and TgNTPDase1ACC, whereas ITC-based saturation kinetics indicate a slight preference of TgNTPDase1ACC for ADP.

**Activation mechanism**

Insight into the activation mechanism was gained from crystal structures of TgNTPDase1ACC at 2.5 Å resolution and TgNTPDase3ACC in complex with Mg$^{2+}$×AMPPNP at 2.85 Å. Due to mutation of C258 and C268 to serines and the full activation of these protein variants, these structures represent the reduced state of TgNTPDases.

In the following we will refer to the extended β-hairpin loop carrying the disulfide bridge responsible for regulation of enzyme activity as the "activation loop" (Figure 1). Comparison of the structures of TgNTPDase3 and TgNTPDase3ACC reveals large conformational rearrangements of the activation loop, the relative position of the subunits of the tetramer and of the two domains of each subunit (Figure 5 and supplementary Movie S1). These rearrangements were also observed in the absence of bound substrate for TgNTPDase1ACC. This finding demonstrates that the conformational switch results from the opening of the disulfide linkage and not from substrate binding.

**Global changes.** The dimensions of the tetramer shrink along the r-axis by approximately 13 Å. The accessible surface area decreases from 94391 Å$^2$ for the inactive form (TgNTPDase3) to ~85200 Å$^2$ for the activated variant (TgNTPDase3ACC). The largest contribution to this change comes from the increase of the contact area at tetramerization interface II (along the p-axis) by around 800 Å$^2$. In contrast, the total contact area at interface I (around the r-axis) hardly changes.

The contraction of the whole tetramer results mainly from a movement of the two dimers A/D and B/C towards each other (see Figure 5A,B). This approximation is accompanied by a relative rotation of the dimer pairs around the r-axis by ~10°. Due to their strong interactions across interface I, the domain I pairs of A/D and B/C move together as rigid bodies. In parallel to the convergence of the domain I pairs, domain II of all monomers experiences a rotational movement which closes the active site cleft between domain I and II. Therefore, the inactive and active states can also be described as open and closed conformations, respectively.

**Changes at the activation loop.** The movements of the subunits and domains are triggered by a conformational change of the activation loop. In the inactive state residues 257-260 and 266-269 form two antiparallel β-strands and the 258-268 disulfide bridge acts as a clamp to hold the two strands together, thus stabilizing this loop conformation. The activation loops of all four monomers show an approximately parallel orientation along the r-axis. By contacting the diametrically positioned subunits at the p-interface, the four loops push apart the A/D- and B/C-dimers in a concerted fashion (Figure 5A,B,C).

Upon reduction the β-hairpin is destabilized at the proximal region and a winding up movement can be observed in the region just prior to the hairpin. The β-strands are shortened and the cysteines form the first and last residues of the two β-strands. In the Ramachandran plot, R257 and R269 undergo...
large shifts from the β-strand to the α-helical region (see SI Figure S2). Both their guanidino groups are translated by more than 16 Å. R269 is oriented towards domain II in the dormant state and becomes fully solvent exposed upon activation. R257 forms a weak salt bridge to D271 before activation. Upon reduction two salt bridges are formed with D254 and D98. D98 is exposed from the bulge in β-strand 3 (Figure 5D), highlighting the importance of this unusual structural element for activation. Notably, analysis with the program SSBOND (47) shows that in spite of the fact that C258 and C268 remain part of the β-hairpin, the distance of their Cα-atoms in the active state is too large for the formation of a disulfide bond. Hence, the wound-up loop conformation cannot be formed in the oxidized enzyme.

The shortening of the activation loop is accompanied by a rotational movement of the loop of approximately 45° relative to domain I. Although the tip of the activation loop (E262, Y263) remains bound to the diametrically opposed monomer, the mode of interaction is altered. In the inactive state, a polar interaction to R53 and a hydrophobic interaction to T57 can be observed. Upon activation the loop slides along helix A to stop close to the exposed cysteine bridge C59-C88. The aromatic ring of Y263 now stacks to the side chain of R58 and its backbone oxygen forms a hydrogen bond with C59. The backbone carboxyl oxygen of E262 forms a hydrogen bond to R86 (Figure 5D).

Changes at interface II. By reduction of the C258-C268 disulfide bridge the tetramer interface along the p-axis changes. The loop between β-strands 9 and 10 (residues 287-301) interacts in the inactive state mainly with helix K, F405 and K406 as well as residues 462-464. After activation it slides along the neighboring monomer to interact with residues F194, E196, W197 and D200, partially involved in helix G (see SI Figure S3). Furthermore, activation of the enzyme induces the formation of two additional helices, F and O. Helix F is located close to the active site (see below). Helix O is formed at the interface across the p-axis by residues 464-468 which are part of a loop in the inactive state.

Electron density of the structures of the inactive form of TgNTPDase3 indicates a partial opening of the C258-C268 disulfide bonds. Based on the obvious differences between the protein crystallized in the inactive state and the structure of the permanently active variant we assume that the partial opening of the bridge in the inactive structure is caused by radiation damage during data collection. This assumption is supported by the relatively strong radiation sensitivity of the crystals (data not shown). The C258-C268 disulfide bridge of the activation loop is probably more easily reduced than the other disulfide bridges, as indicated by the time course of activation and deactivation of the enzymes by thiol reagents (18). This cysteine linkage might thus also open first by radiation damage. An alternative explanation would be that the C258-C268 bridges are partially open in the purified enzyme due to a high redox potential. However, the closed conformation, as observed in the TgNTPDase3ΔC1C structure, forms only upon reduction of all four C258-C268 disulfide bridges due to a cooperative movement of the four subunits upon activation.

Function of the activation loop

From the present data it is not clear whether the function of the activation loop is purely inhibitory in its oxidized state (forcing the monomers and domains apart), activating in its reduced state (drawing the domains together) or a mixture of both. Replacement of the whole loop (residues E253 to R269) by a short flexible linker (sequence: GG) gave rise to a variant that was active without addition of reducing agents but had only strongly diminished activity of 8 U/mg (data not shown). A possible explanation is that the reduced loop indeed stabilizes the enzyme in the active state. However, the activation loop might also be necessary to support a yet uncharacterized domain motion during catalysis, which has been observed for other NTPDases (25). Although it remains unclear how the activation is realized in nature, we note that the exposed position of the four activation loops make them well accessible for an activating reductase (20).

The soluble, monomeric NTPDase from the bacterium L. pneumophila is lacking a β-hairpin loop between the two structural domains (48). However, a putative membrane interaction loop (MIL) is found in the membrane-anchored mammalian NTPDases at a position corresponding to the activation loop of TgNTPDases (Figure 1B)(41). This MIL is considered to insert in or interact with the cell membrane and thereby influence catalytic properties (41). Functional studies analyzing the importance of the MIL in mammalian enzymes are still missing. For these enzymes, a movement of the two protein domains has been suggested to be involved in the effects of the membrane attachment on the kinetic properties (49).
Domain motion involved in enzyme activation

The movement of the subunits within the TgNTPDase tetramer is coupled to a movement of the two protein domains of each subunit (Figure 6). Shortening of the activation loop generates a drawing force along r onto its flanking domains. Domain I forms a sturdy interaction with domain I of a neighboring monomer (interface I) and these domain dimers are free to follow the drawing force generated by the activation loop. Domain II, however, cannot follow this movement along r because it bumps at interface II onto domain II of its neighbor. To concede to the drawing force, domain II evades into a tilting motion relative to domain I leading to closure of the active site clefts (Figure 5A,B).

For an unbiased determination of the rigid domains, the program Dyndom was used (40). Residues that move together as a rigid body during the conformational change are automatically assigned to a dynamic domain. All four subunits of TgNTPDase3 were compared to the four domains in TgNTPDase3ΔCC. Although relatively small ratios of interdomain to intradomain displacements of 0.63 to 1.01 were observed, consistent and plausible results were obtained for all comparisons concerning the presence of two domains that mostly matched the structural domains described in Figure 1. The interdomain rotation angles vary from 5.6° to 12.3° and the translational components of the screw operations are small (between 0.0 to 1.0 Å). The largest difference of the domain orientation was observed for chain C of TgNTPDase3 and chain B of TgNTPDase3ΔCC (Figure 6B).

The line depicted in Figure 6B marks the orientation of the rotation axis. Based on the angle between the rotation axis and the line connecting the domain centers, the movement is characterized as the percentage closure motion between 0% (pure twist motion) and 100% (pure closure motion). For the comparison between chains C and B the axis is characterized by 27% closure motion. For all comparisons the percentage closure motion varies from 12% to 57%, i.e. it resembles more a twist motion. For most of the comparisons, the rotation axis runs approximately parallel to helix T of the domain interface. Helices H and T which are located between the domains are consistently assigned to domain I.

The differences in the magnitude of the interdomain rotation angle obtained for a comparison of the structures of TgNTPDase3 and TgNTPDase3ΔCC are mostly due to different domain orientations of the four subunits of the inactive form of TgNTPDase3, with rotation angles varying between 2.8° and 4.7°. In contrast, the four subunits in TgNTPDase3ΔCC and TgNTPDase1ΔCC are more similar to each other with a maximum interdomain rotation angle of 1.2°.

Superposition of the C-terminal domains (Figure 6A) and N-terminal domains (Figure 6B) alone shows that the domains move largely as rigid bodies with the following exceptions. In the N-terminal domain, the loop between helices B and C and the loop between helices F and G undergo an internal displacement to move away from the domain interface, where they are in contact with residues of the C-terminal domain, in particular with the region 395 to 424 including helices L and K. As a result, the latter region of the C-terminal domain also shows significant internal displacements to accommodate the domain movement (Figure 6B). In addition, the activation loop (252-272) and the loop 586-597 between the two domains show large independent motions and are assigned to none of the two domains.

The domain-domain reorientation described above induces the following secondary structure changes in the active site cleft. Helix F is formed and contributes to the correct arrangement of the phosphate binding pocket by positioning of T188 and A189 which coordinate the γ-phosphate group of the substrate and the nucleophilic water. R192 moves closer to the nucleophilic base E236 to position it by formation of a salt bridge.

Domain motion in the actin/hsp70/sugar kinase superfamily

A domain motion has been shown to be involved in the catalytic cycle of many of the enzymes of the actin/hsp70/sugar kinase superfamily, with hexokinase being a prototypical example for a substrate induced domain closure motion (50). A domain movement of rat NTPDase1 is characterized based on a comparison of four independent conformers in the crystal (25). The assignment of dynamic domains is similar to the results obtained for TgNTPDases with the exception that helix H and parts of helix T are assigned to the C-terminal domain in the mammalian enzyme. These helices are located at the domain interface and the interdomain rotation axis passes nearby. Studies on the 28.1° domain motion of a yeast hexokinase showed that the domain motion...
Substrate binding mode

Crystallization and structure determination of TgNTPDase3ΔCC in complex with Mg\(^{2+}\)×AMPPNP allowed us to further substantiate our understanding of the activation mechanism of TgNTPDases and to characterize the substrate binding mode and catalytic mechanism (Figure 7A,B). The ATP analog is located in the cleft between the two domains. Superposition of RnNTPDase2 (41) indicates a similar arrangement of the domains and the active site (Figure 7C). The positions of the terminal phosphate groups and the ACR loops now correspond closely to that of the AMPPNP complex structure of RnNTPDase2 (41) indicating that the structure of TgNTPDase3ΔCC represents a competent substrate binding mode. Residues from both phosphate binding loops (G72-S74, G280-S282) can now bind simultaneously to the nucleotide. Although water molecules are not resolved in the present structure, the side chain of E277 is now in reasonable distance to position a water molecule in the coordination sphere of the metal ion. R192 and E236 were too far apart from each other in the inactive state, but now can form a salt bridge (Figure 7C).

However, in contrast to RnNTPDase2 (41) the conformation of the nucleotide differs in detail and the position of the base is shifted in TgNTPDase3ΔCC. This results in a more extended conformation of the ATP analog in the microbial enzyme. The extended conformation appears to result from a larger distance of the residues involved in binding of the nucleotide base from the binding site of the terminal phosphate group (Figure 7C). In contrast, even in an extended conformation as suggested for RnNTPDase2 (41) ADP cannot optimally bridge the distance between the binding sites for the nucleobase and the terminal phosphate group, explaining the high specificity of TgNTPDase3 for ATP.

As in the complex structure TgNTPDase3×AMP, the adenine base is sandwiched between the planar peptide group of G492 and the side chain of L553. But whereas the terminal phosphate group of AMP is pointing out of the active site cleft in crystals obtained for the inactive state, the triphosphate group of AMPPNP is oriented to the active site residues of the N-terminal domain in the active state (see SI Figure S4) as expected for a catalytically competent binding mode.

For the AMP complex structure differences between the mammalian and the microbial enzyme in binding of the nucleoside moiety were described above. Additionally interactions with the triphosphate group of the nucleotide differ. The side chain of S75, corresponding to H50 in RnNTPDase2 (41), is too far away from the substrate analog to be involved in binding. Instead, H50 (41) is functionally replaced by the side chain of R77, which interacts with the β-phosphate. In addition, R108 interacts electrostatically with the γ-phosphate of the ligand. D201 as part of the conserved DXG-motif of ACR4 in RnNTPDase2 (41) is substituted by E277 in TgNTPDase3, thus forming an EXG-motif. Although water molecules are not visible in the crystal structure presented here, we assume that E236 functions as a general base to deprotonate a water nucleophile for direct attack of the terminal phosphate group as this residue is similarly
positioned as the conserved catalytic base E165 in RnNTPDase2 (41).

Insight into catalytic differences between TgNTPDase1 and TgNTPDase3

Structures of TgNTPDases1 and -3 in the activated state allow us to characterize the molecular basis for the differences in substrate specificity. Analysis on chimeric constructs had previously shown that a block of 12 residues dictates the substrate specificity for ATP vs. ADP ((14), residues 488 to 499 in Figure 1). Remarkably, two glycines in TgNTPDase3 residues are replaced by amino acids with relatively bulky side chains (R492 and E493) in TgNTPDase1 in the immediate vicinity of the base binding site (Figure 7D). The two side chains adopt conformations that would not support binding of the substrate’s nucleobase in the position observed in the TgNTPDase3ΔCC×Mg²⁺×AMPPNP complex structure (Figure 7D). We assume that upon nucleotide binding, R492 adopts a different conformation. This notion is supported by the weak side chain density of this residue in chain B. E493 is located behind the base. The close proximity of R492 and E493 to the nucleobase suggests that these two residues are predominantly responsible for the different substrate specificities of TgNTPDase1 and 3, perhaps by influencing the distance between the base binding site and the binding site for the terminal phosphate group.

CONCLUSIONS

In conclusion, with this study we could derive a detailed molecular model for the activation mechanism of TgNTPDases. Activation is initiated by the reduction of the disulfide bond C258-C268, followed by a conformational change of the activation loop that induces a presumably cooperative movement of the protein subunits of the tetramer and of the two domains of each subunit. The observed conformational change resembles that of a jumping jack, where drawing on the cord (activation loop) induces clapping of the hands (domain I and II). The subunit and domain motions finally result in formation of a competent active site geometry as visualized by the complex structure with an ATP analog. The observed cooperative movement of subunits and domains and the control of these motions by the conformation of the activation loop show that the activation mechanism of TgNTPDases is strongly dependent on the quaternary structure of the enzyme. It is likely to be an important regulatory part for the survival of T. gondii in the host and its controlled egress. The comparative analysis of TgNTPDase1ΔCC and -3ΔCC crystal structures allows us to understand the molecular basis for the significant differences in substrate specificity of the two isoenzymes, which are caused by only 3% sequence deviation but contribute to the formation of virulent vs. avirulent T. gondii strains.
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FOOTNOTES

Abbreviations - ACR, apyrase conserved region; AMPPNP, adenosine 5'-(β,γ-iminotriphosphate); ΔCC, C258S-C268S mutation; DTT, Dithiothreitol; ITC, isothermal titration calorimetry; FPLC, fast protein liquid chromatography; MIL, membrane interaction loop; NTPDase, nucleoside triphosphate diphosphohydrolase; RnNTPDase, Rattus norvegicus NTPDase; TCEP, Tris(2-carboxyethyl)phosphine; TgNTPDase, Toxoplasma gondii NTPDase

Accession numbers - Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 4a57 (TgNTPDase3), 4a59 (TgNTPDase3×AMP), 4a5a (TgNTPDase3ΔCC×Mg2+×AMPPNP) and 4a5b (TgNTPDase1ΔCC).

FIGURE LEGENDS

Figure 1: Protein fold of TgNTPDase3. A, stereo view of a TgNTPDase3 monomer in complex with AMP (red). Domains I and II are colored in orange and blue, respectively. Strands are numbered 1 to 24 and helices labeled A to V. Cysteines are depicted in yellow. B, topology diagram: triangles represent β-strands and circles α-helices. The flanking strands 3 and 5 of the first β-sheet are interrupted by β-bulges. Red labeled helices and red marked β-sheet limitations correspond to the topology of the activated variant TgNTPDase3ΔCC. The topology of rat NTPDase2 (41) is underlaid in gray, highlighting the conservation, deletion and addition of structural elements. C, orientation of the subunit domains in the tetramer. D, structure-based sequence alignment between TgNTPDase1, -3 and RnNTPDase2 using RAPIDO (42,52), with 328 aligned residues (rmsd 3.25 Å) and three defined rigid bodies of 159 residues (rmsd rigid bodies 1.59 Å). Aligned residues are shown in capital letters and underlaid in gray. Residues conserved between TgNTPDases and RnNTPDase2 are underlaid in black and positions that are different between TgNTPDase1 and -3 are highlighted in green. The red star in A, B, C and D highlights the activation loop with the disulfide bridge responsible for inactivity of the oxidized form.

Figure 2: Tetramer interactions of TgNTPDase3. Chains A, B, C and D of the protein structure are colored in yellow, red, blue and green, respectively. A, surface representation with the 2-fold symmetry axes p, q, r (see text). Interfaces I and II are indicated. B, C and D, zoom of different areas of the tetramer interfaces. Interacting residues are shown as sticks and labeled with the residue number. Polar interactions are indicated by dashed lines. B, C interactions at interface I. For a better view, the molecules in B were rotated 90° around the r-axis. D, interface II between chains A and B.

Figure 3: Comparison of TgNTPDase3 to the mammalian cell surface NTPDase2. A, Superposition of RnNTPDase2 (gray, bound Ca2+×AMPPNP gray/violet) and TgNTPDase3 (orange/blue, bound AMP green). Residues 45-58,115-127 for domain I of RnNTPDase2 were aligned with corresponding residues 70-83, 181-193 of TgNTPDase3. B, close-up view of the active site. An omit-electron density map (Fo-Fc×φc, contoured at 3 σ) of AMP is shown in gray. Active site ligands from the superimposed RnNTPDase2 structure are shown with half transparency. Amino acids involved in binding of the base and residues expected to interact with the phosphate groups of ATP or ADP are indicated. A selection of polar interactions that occur in the productive substrate binding mode of RnNTPDase2 are shown as dashed lines and drawn red when distances are too long for the formation of hydrogen bonding or salt bridge interaction. The figure illustrates that domain II that binds the nucleoside moiety has to rotate towards domain I for the formation of a productive substrate binding mode.

Figure 4: Kinetic analysis of enzyme activation and specificity towards ATP and ADP. A and B, specific activity ± SD (indicated as error bars) for TgNTPDase variants with 2 mM ATP or 5 mM ADP
ADP and optionally with addition of 3 mM DTT according to a malachite green assay. Reactions were carried out for 10 min at room temperature. Please note the different scale in A and B. C, ITC-based enzyme assay. Titration of TgNTPDase1ΔCC with ADP as substrate. 3× 5µl, 2× 10µl and 12× 20µl of a 30 mM ADP were added to 0.4 nM TgNTPDase1ΔCC in a magnesium reaction buffer with reequilibration times of 300 to 350s. D, calculation of turnover rates from the data in C.

**Figure 5:** Structural changes upon enzyme activation (see also supplementary Movie S1). A, tetrameric structures of TgNTPDase3 (left) and TgNTPDase3ΔCC (right). The chains are colored as in Figure 2. B, schematic representation of the conformational switch shown in A after reduction of C258-C268, which is located in the β-sheet between the monomers. Reduction of the activation loop (β-hairpin) results in tetramer contraction, domain closure and establishment of the correct active site (red star) geometry. C and D, conformational change and interactions of the activation loop. Shown is the loop of monomer A in the same orientation as in panel A. Domains I and II are colored in orange and blue, respectively. Cartoon and sticks of the neighboring subunit are colored in green with a light gray protein surface. Shown are all residues involved in interactions either in the open/inactive form (C) or in the closed/activated conformation (D). The hydroxyl groups of S258 and S268 in the crystal structure of TgNTPDase3ΔCC (D) are colored yellow to indicate the positions of the free cysteines in the reduced activated enzyme.

**Figure 6:** Domain motion upon enzyme activation: comparison between TgNTPDase3 (chain C, light gray) and TgNTPDase3ΔCC (chain B, domain I: orange, domain II: blue). A, superposition of the structures based on domain II. AMPPNP (red) marks the position of the active site. B, stereo view of dyndom assignment of the dynamic domains. The interdomain screw axis (12° rotation, 0.03 Å translation) and AMPPNP are shown in red. The structures are superimposed based on domain I. Residues assigned to none of the two domains are colored in green. Regions undergoing significant internal displacements are colored in black.

**Figure 7:** Substrate binding to TgNTPDases. A, AMPPNP bound to the active site of TgNTPDase3ΔCC. Residues involved in the hydrolysis of ATP are shown as sticks and are colored as described in Figure 1. Water molecules are not present in the crystal structure and were modeled according to the active site of RnNTPDase2 (41). The omit-electron density map (Fobs-Fcalc, contoured at 2 σ) of AMPPNP is shown in gray. B, scheme of the interactions of Mg2⁺×AMPPNP in the active site of TgNTPDase3ΔCC. The size of the blue clouds indicates the solvent accessibility of the ligand atoms. The strength of the interactions to the ligands is indicated by a blue shadow behind the residue. C, superposition of RnNTPDase2 (gray) and TgNTPDase3ΔCC (orange/blue, bound AMPPNP green, Mg2⁺ purple) on all residues. D, superposition of AMPPNP (gray) from the TgNTPDase3ΔCC×Mg2⁺×AMPPNP complex on TgNTPDase1ΔCC with putative base binding residues colored in green.
### TABLES

**Table 1:** Data collection and refinement statistics (values in parentheses refer to the highest-resolution shell).

|                | TgNTPDase3 apo | TgNTPDase3 thiomerosal | TgNTPDase3 KI | TgNTPDase3 cis-Pt(NH3)2Cl2 | TgNTPDase3 ×AMP | TgNTPDase3ΔCC×Mg2+×AMPPNP | TgNTPDase1ΔCC |
|----------------|----------------|------------------------|---------------|-----------------------------|-----------------|-----------------------------|----------------|
| **Data collection** |                |                        |               |                             |                 |                             |                |
| X-ray source    | BL14.1 / HZB, BESSY | BL14.1 / HZB, BESSY | BL14.1 / HZB, BESSY | BL14.1 / HZB, BESSY | BL14.1 / HZB, BESSY | BL14.2 / HZB, BESSY | ID23-2/ESRF |
| Wavelength (Å)  | 0.91841        | 0.83                   | 1.9           | 0.89                        | 0.91841         | 0.91841                     | 0.8726         |
| Space group     | P2₁            | P2₁                    | P2₁           | P2₁                         | P2₁             | C222₁                       | C222₁          |
| **Cell dimensions** |                |                        |               |                             |                 |                             |                |
| a (Å)           | 89.0           | 88.9                   | 89.4          | 88.9                        | 89.2            | 72.0                         | 73.7           |
| b (Å)           | 165.9          | 164.4                  | 165.8         | 164.7                       | 166.0           | 150.8                        | 150.4          |
| c (Å)           | 97.5           | 97.6                   | 97.3          | 97.3                        | 97.9            | 487.1                        | 242.4          |
| β (°)           | 97.0           | 97.1                   | 97.4          | 97.1                        | 97.2            | 90                            | 90             |
| Wilson B factor (Å²) | 21.5         | 55.5                   | 72.8          | 51.3                        | 33.0            | 49.6                         | 42.1           |
| Resolution range (Å) | 40.39-2.00 (2.11-2.00) | 54.00-2.90 (3.06-2.90) | 55.22-3.20 (3.37-3.20) | 60.19-2.90 (3.06-2.90) | 43.40-2.20 (2.30-2.20) | 39.00-2.85 (3.00-2.85) | 39.30-2.60 (2.60-2.50) |
| Unique reflections | 183360       | 61578                  | 46364         | 61544                       | 142618          | 61587                        | 47598          |
| Average multiplicity | 5.1 (5.0)   | 5.1 (5.1)              | 5.3 (5.2)     | 4.7 (4.6)                   | 3.0 (2.9)       | 3.7 (3.4)                    | 4.4 (3.9)      |
| Completeness (%) | 97.3 (97.9)   | 99.9 (99.9)            | 100 (100)     | 100 (100)                   | 96.9 (93.3)     | 98.3 (94.3)                  | 99.9 (99.6)    |
| Rmerge (%)¹ | 10.1 (2.9)    | 8.2 (2.9)              | 9.3 (3.3)     | 8.3 (2.8)                   | 7.6 (2.0)       | 5.0 (2.0)                    | 11.9 (3.0)     |
| Rfree (%)¹ | 11.7 (56.6)   | 16.4 (54.7)            | 16.9 (59.0)   | 16.7 (57.0)                 | 11.5 (68.9)     | 22.7 (60.4)                  | 10.9 (55.5)    |
| Rpim (%)¹ | 5.0 (24.5)    | 7.2 (23.9)             | 7.3 (25.8)    | 7.7 (26.4)                  | 6.4 (40.0)      | 15.1 (39.7)                  | 5.1 (27.7)     |
| **Refinement** |                |                        |               |                             |                 |                             |                |
| Resolution range (Å) | 40.39-2.00 | 43.40-2.20             | 39.00-2.85    | 39.30-2.60                  | 39.00-2.85      | 39.30-2.60                   | 39.00-2.85     |
| Rwork/Rfree (%) | 17.4/20.9     | 18.0/22.8              | 23.4/28.4     | 17.6/22.6                   | 17.6/22.6       | 17.6/22.6                    | 17.6/22.6      |
| No. of amino acids | 2369         | 2369                   | 2360          | 1185                        | 2371            | 2360                         | 1185           |
| Water           | 1274          | 884                    | 0             | 247                         | 884             | 0                            | 247            |
| Ligands (Mg²⁺, AMPPNP, Cl⁻) | 4           | 4                      | 8             | 0                           | 4               | 8                            | 0              |
| Average B factor (Å²) | 31.9         | 41.6                   | 20.4          | 36.5                        | 37.2            | -                            | 30.8           |
| Protein         | 31.9          | 41.6                   | 20.4          | 36.5                        | 37.2            | -                            | 30.8           |
| Water           | 33.0          | 46.8                   | 59.4          | -                           | -               | -                            | -              |
| rmse bond/angles (Å)/(°) | 0.009/1.03 | 0.010/1.14             | 0.010/1.45    | 0.010/1.19                  | 0.010/1.19      | 0.010/1.19                   | 0.010/1.19     |
| Ramachandran Plots |                  |                        |               |                             |                 |                             |                |
| favored (%)     | 98.7          | 98.8                   | 97.1          | 98.5                        | 99.9            | 100                          | 100            |
| allowed (%)     | 100           | 100                    | 100           | 100                         | 100             | 100                          | 100            |
| number of outliers | 1            | 2                      | 0             | 0                           | 2               | 0                            | 0              |
| PDB accession code | 4a57        | 4a59                   | 4a5a          | 4a5b                        | 4a57            | 4a59                         | 4a5b           |

¹as defined in (53)
Table 2: Kinetic constants determined for ADP and ATP hydrolysis of TgNTPDase1ΔCC and TgNTPDase3ΔCC in magnesium or calcium buffer using the ITC-based enzyme assay.

| TgNTPDase1ΔCC | $k_{\text{cat}}$ (s$^{-1}$) | $K_m$ (mM) | $k_{\text{cat}}/K_m$ (s$^{-1}$/mM) | ratio | (ratio $(k_{\text{cat}}/K_m)^{ATP}/(k_{\text{cat}}/K_m)^{ADP}$) |
|---------------|-----------------|--------|-----------------|------|-------------------------------|
| ADP/Mg$^{2+}$ | 238 ± 14        | 1.17 ± 0.05 | 204 ± 15       |      | 0.40                          |
| ATP/Mg$^{2+}$ | 276 ± 27        | 3.4 ± 0.5  | 81 ± 15        |      |                               |
| ADP/Ca$^{2+}$ | 333 ± 15        | 1.30 ± 0.16 | 255 ± 34       |      | 0.57                          |
| ATP/Ca$^{2+}$ | 349 ± 22        | 2.4 ± 0.2  | 150 ± 20       |      |                               |

| TgNTPDase3ΔCC | $k_{\text{cat}}$ (s$^{-1}$) | $K_m$ (mM) | $k_{\text{cat}}/K_m$ (s$^{-1}$/mM) | ratio | (ratio $(k_{\text{cat}}/K_m)^{ATP}/(k_{\text{cat}}/K_m)^{ADP}$) |
|---------------|-----------------|--------|-----------------|------|-------------------------------|
| ADP/Mg$^{2+}$ | 18 ± 1          | 1.25 ± 0.15 | 15 ± 2        |      | 202                           |
| ATP/Mg$^{2+}$ | 2290 ± 74       | 0.8 ± 0.2 | 3000 ± 800     |      |                               |
| ADP/Ca$^{2+}$ | 13.2 ± 0.3      | 2.3 ± 0.2  | 5.8 ± 0.6      |      | 177                           |
| ATP/Ca$^{2+}$ | 588 ± 52        | 0.572 ± 0.009 | 1030 ± 90   |      |                               |
Figure 2:
Figure 3:
Figure 4:

A

B

C

D

figure4.png
Figure 6:
Figure 7:

A

B

C

D

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Structural insight into the activation mechanism of Toxoplasma gondii nucleoside triphosphate diphosphohydrolases by disulfide reduction
Ulrike Krug, Matthias Zebisch, Michel Krauss and Norbert Sträter

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