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Galactonolactone oxidoreductase from *Trypanosoma cruzi* employs a FAD cofactor for the synthesis of vitamin C

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ABSTRACT

Trypanosoma cruzi, the aetiological agent of Chagas' disease, is unable to salvage vitamin C (L-ascorbate) from its environment and relies on *de novo* synthesis for its survival. Because humans lack the capacity to synthesize ascorbate, the trypanosomal enzymes involved in ascorbate biosynthesis are interesting targets for drug therapy. The terminal step in ascorbate biosynthesis is catalyzed by flavin-dependent aldonolactone oxidoreductases belonging to the vanillyl-alcohol oxidase (VAO) protein family. Here we studied the properties of recombinant *T. cruzi* galactonolactone oxidoreductase (TcGAL), refolded from inclusion bodies using a reverse micelles system. The refolded enzyme shows native-like secondary structure and is active with both L-galactono-1,4-lactone and D-arabinono-1,4-lactone. At odd with an earlier claim, TcGAL employs a non-covalently bound FAD as redox-active cofactor. Moreover, it is shown for the first time that TcGAL can use molecular oxygen as electron acceptor. This is in line with the absence of a recently identified gatekeeper residue that prevents aldonolactone oxidoreductases from plants to act as oxidases.

1. Introduction

Trypanosomatids are protozoan parasites belonging to the order of *Kinetoplastida*. They include the medically relevant human parasites *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp., the causative agents of African sleeping sickness, Chagas' disease and leishmaniasis, respectively. Together these diseases threat over 30 million people worldwide. Current treatments are unsatisfactory, since the available drugs have a limited efficacy and exhibit toxic side effects (http://www.who.int).

During their life cycles, trypanosomatids are exposed to reactive oxygen species generated by their own aerobic metabolism and by the host's immune response. The antioxidant response in the parasites is distinct from their mammalian hosts and includes targets that may be exploited therapeutically. Trypanosomes lack catalases and glutathione peroxidases [1,2] and detoxify hydrogen peroxide using a plant-like ascorbate peroxidase [3,4]. Furthermore, they possess the unique dithiol trypanothione which is a conjugate of two glutathione molecules with one molecule of spermidine [1]. The flavoenzyme trypanothione reductase, which keeps trypanothione in the reduced state, is an essential enzyme for the parasite as it is the only enzyme that connects hydrogen peroxide detoxification to NAD(P)H redox biology in these parasites [2].

Trypanosomes contain significant levels of ascorbate (vitamin C), which is synthesized in the glycosome, a unique single membrane organelle. Genome analysis has indicated that ascorbate biosynthesis in trypanosomes is similar to that in plants [5,6]. Most eukaryotic organisms can synthesize ascorbate to their own requirements. Humans and other primates, however, have lost this ability during evolution [7] because the gene encoding the enzyme responsible for the final step is highly mutated and non-functional [8]. The trypanosomal enzymes involved in ascorbate biosynthesis are interesting targets for drug therapy, since the parasites lack the ability to scavenge ascorbate from the environment, and rely on *de novo* synthesis for their survival [1,5].

The terminal step of ascorbate biosynthesis is catalyzed by so-called aldonolactone oxidoreductases. These enzymes belong to the vanillyl-alcohol oxidase (VAO) flavoprotein family [9,10]. Aldonolactone oxidoreductases have been isolated from various sources including animals, yeasts, fungi and plants. Most aldonolactone oxidoreductases are oxidases containing covalently bound FAD, while plants utilize a strictly cytochrome *c*-dependent galactonolactone dehydrogenase containing non-covalently bound FAD [11,12]. The arabinonolactone oxidoreductase (TbALO) from *T. brucei* and galactonolactone oxidoreductase (TcGAL) from *T. cruzi* have been isolated and characterized to

Abbreviations: AOT, bis(2-ethylhexyl)sulfosuccinate; AtGALDH, *Arabidopsis thaliana* L-galactono-1,4-lactone dehydrogenase; CD, circular dichroism; DCPIP, 2,6-dichlorophenolindophenol; DTT, dithiothreitol. GSH, reduced glutathione; GSSG, oxidized glutathione; RM, Reverse micelles; TcGAL, *Trypanosoma cruzi* galactonolactone oxidoreductase; VAO, vanillyl-alcohol oxidase

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some extent [5,6]. Both enzymes were annotated as oxidases, but the activity with molecular oxygen has never been established. Instead, cytochrome *c* was used as electron acceptor in the activity assays [5,6]. TcGAL and TbALO were reported to contain FMN rather than FAD as redox-active cofactor [5]. Nevertheless, the PP loop for FAD binding [9] is in both enzymes conserved (Fig. 1).

In this research we have investigated the cofactor, substrate and electron acceptor specificity of recombinant TcGAL, refolded from inclusion bodies using an AOT-isooctane reverse micelles system. In contrast to an earlier report [5], we found that TcGAL employs a non-covalently bound FAD as redox active cofactor. Furthermore, we established that TcGAL can use molecular oxygen as efficient electron acceptor.

2. Materials and methods

2.1. Chemicals

FAD, FMN, L-galactono-1,4-lactone, D-arabinono-1,4-lactone, bovine heart cytochrome *c*, 1,4-benzoquinone and bis(2-ethylhexyl) sulfosuccinate (AOT) were from Sigma-Aldrich (St Louis, MO, USA). 2,6-Dichlorophenolindophenol (DCPIP) was from Merck (Darmstadt, Germany). Mini-Complete protease inhibitor was from Roche Molecular Biochemicals (Mannheim, Germany). Restriction endonucleases, T4-DNA ligase and dNTPs were purchased from Invitrogen (Carlsbad, CA, USA). Pfu DNA polymerase was obtained from Fermentas GmbH (St Leon-Rot, Germany). Oligonucleotides were synthesized by Eurogentec (Liege, Belgium). All other chemicals were from commercial sources and of the purest grade available.

2.2. Recombinant expression of TcGAL

The pTrcHisC vector containing the TcGAL gene (from *T. cruzi* X10/6 clone) N-terminally fused to a His6-tag, XpressTM epitope and enterokinase cleavage site [5], was kindly provided by Prof. M.W. Fraaije (University of Groningen). To remove the large non-native N-terminal tag, the TcGAL gene was re-cloned into a modified pBAD-vector. An NdeI restriction site in the TcGAL gene was first removed using the QuikChange II site-directed mutagenesis method (Stratagene, La Jolla, CA, USA) using pTrcHisCTcGAL as a template. The primers used are TcGAL-qcf (5′- GCT TCG ATG TGT TCC GTC GTA CGT ACT CAC GAC GAT TGC TG- 3′) and TcGAL-qcr (5′-CAG CAA TCG TCG TGA GTA CGT ACG ACG GAA CAC ATC GAA GC-3′); changed nucleotides are underlined. Successful mutagenesis was confirmed by digestion with NdeI. The native TcGAL gene was amplified from the mutated pTrcHisCTcGAL vector by PCR using the oligonucleotides TcGAL-f (5′-GGA ATT CCA TAT GCG TTG TGA CGT TTC CAT GCG GC-3′) introducing a new NdeI restriction site (underlined), and TcGAL-r (5′-CCC AAG CTT ACA AAT GAC TAT TGG TGC TG-3′), introducing a HindIII restriction site (underlined). The amplified fragment was cloned between the NdeI and HindIII restriction sites of a modified pBAD/Myc-HisA expression vector, kindly provided by Prof. M.W. Fraaije (University of Groningen). A stop-codon was introduced in the reverse primer to prevent fusion to the myc epitope and His6-tag present in the pBAD vector. The resulting pBAD-TcGAL construct was verified by automated sequencing of both strands and electroporated into *Escherichia coli* TOP10 electrocompetent cells for recombinant expression.

2.3. Purification of TcGAL

For enzyme production, *E. coli* BL21(DE3) cells harboring the pBAD-TcGAL plasmid, were grown in Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin until the OD_{600} reached ~0.6. Expression of TcGAL was induced by addition of 0.02% L-arabinose and the incubation was continued for 16 h at 28 °C. The cells were harvested and resuspended in 50 mM Tris–HCl, 5 mM DTT, pH 7.4, supplemented with 1 tablet of mini-Complete protease inhibitor, 10 mM $MgCl_2$ and DNase I and subsequently passed twice through a pre-cooled French Pressure cell (SLM Aminco, SLM Instruments, Urbana, IL, USA) at 10000 psi. The resulting homogenate was centrifuged at 25 000*g* for 30 min at 4 °C and the insoluble inclusion bodies were collected. The soluble extract was loaded onto a HiLoad 26/10 Q-Sepharose FF column

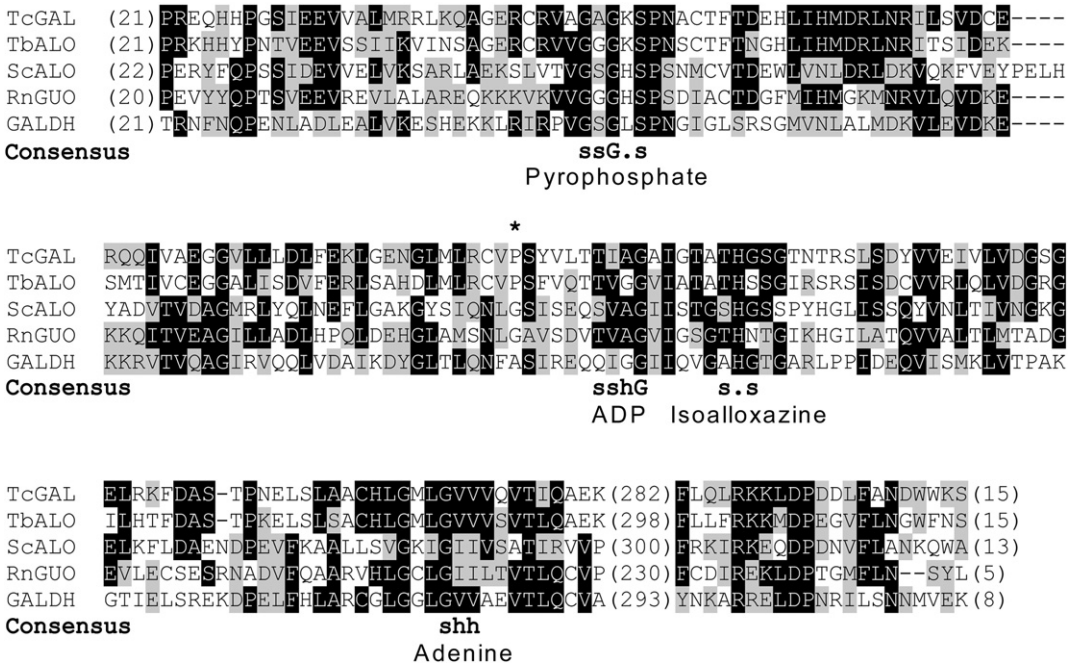

Fig. 1. ClustalW multiple sequence alignment [39] of the FAD-binding domains of TcGAL and related aldonolactone oxidoreductases. Identical residues are shaded in black, similar residues are shaded in gray. The consensus of conserved residues specifically interacting with parts of the FAD cofactor is shown under the alignment (G, glycine; s, small: G,A,S,T; h, hydrophobic: I,L,V, ·, any residue) [9]. The asterisk (*) marks the residue that determines the oxygen reactivity [23]. The number of residues present in gaps in the sequence and at the termini are indicated in parentheses. The accession numbers used are as follows: TcGAL, Q4DPZ5; TbALO, Q57ZU1; ScALO, P54783; RnGUO, P10867; GALDH, Q8GY16.

(Pharmacia Biotech, Uppsala Sweden) equilibrated with 50 mM Tris–HCl, 5 mM dithiothreitol (DTT), pH 7.4. Proteins were eluted with a linear gradient of NaCl (0–0.5 M) in the same buffer. The TcGAL activity was collected in the flow-through, pooled and applied onto a Ceramic hydroxyapatite XK 26/11 (Pharmacia Biotech) column equilibrated with 25 mM sodium phosphate, 5 mM DTT, pH 6.5. Proteins were eluted with a gradient of sodium phosphate (25–500 mM). Again all the TcGAL activity was collected in the flow-through. The active fractions were pooled and brought to 20% ammonium sulfate saturation and applied onto a Phenyl-Sepharose CL-4B (Pharmacia Biotech) column equilibrated with 50 mM sodium phosphate, 5 mM DTT, 20% ammonium sulfate saturation, pH 7.4. Proteins were eluted with a linear gradient of ammonium sulfate (2–0%) in the same buffer.

2.4. Isolation and refolding of TcGAL inclusion bodies

The insoluble material collected after cell lysis was washed with 6% Triton X-100 containing 60 mM EDTA and 1.5 M NaCl, pH 7.0 and dissolved in 6 M guanidinium hydrochloride as described [13]. Subsequently, the denaturant was removed by dialysis against 10 mM sodium phosphate, 1 mM DTT, pH 8.0. A final dialysis step was performed against 10 mM sodium phosphate, pH 7.2 to remove excess DTT. After dialysis a turbid suspension of aggregated protein was obtained. This suspension was injected into the water–AOT–isooctane reverse micelles system containing 0.4 M AOT and shaken intensively. The hydration degree, w_0, was varied in the range 20–35 by adding different amounts of 10 mM sodium phosphate, pH 8.5. Oxidized and reduced glutathione solutions (10 mM GSSG and 30 mM GSH) in 10 mM sodium phosphate, pH 8.5 were mixed and an aliquot of 100 μl was added to 1 ml of 0.4 M AOT solution in isooctane. Refolding of the enzyme was initiated by mixing 1 volume of micellar solution containing glutathiones with 3 volumes of TcGAL micellar solution and an aliquot (10 μl per ml) of FAD or (FMN) cofactor solution in water (10-fold molar excess to enzyme concentration). The resultant solution was shaken for 10 min yielding a transparent TcGAL containing system. The final micellar protein concentration was 1–2 mg/ml depending on the surfactant hydration degree.

2.5. Protein analysis

SDS–PAGE was performed using 12.5% acrylamide slab gels essentially as described by Laemmli [14]. Proteins were stained with Coomassie Brilliant Blue R-250. Total protein concentrations were estimated using the Bradford protein assay from BioRad with BSA as standard. Desalting or buffer exchange of small aliquots of enzyme was performed with Bio-Gel P-6DG columns (Bio-Rad).

2.6. Thin-layer chromatography (TLC)

The identity of the flavin cofactor of the soluble TcGAL preparation was determined by TLC. The flavin was extracted with a solvent mixture consisting of butanol/acetic acid/water (5:3:3). Extracted flavin and the reference compounds FAD, FMN and riboflavin were applied onto a Baker-flex silica-gel IB2 TLC plate (JT Baker, Inc., Phillipsburg, NY, USA). Butanol/acetic acid/water (5:3:3) was used as mobile phase. Fluorescent spots were visualized by UV light.

2.7. TcGAL activity in aqueous solution

TcGAL activity was routinely assayed by following the reduction of cytochrome *c* at 550 nm [12]. Initial velocities were calculated using a molar difference absorption coefficient ($\Delta\varepsilon_{550}$ of 21 $mM^{-1}cm^{-1}$) for reduced minus oxidized cytochrome *c*. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate per minute, which is equivalent to the reduction of 2 μmol of cytochrome *c* [15]. Alternatively, TcGAL activity was measured with the artificial electron acceptor, 1,4-benzoquinone ($\varepsilon_{290} = 2.3\ mM^{-1}cm^{-1}$) [12]. For the determination of kinetic parameters, the concentration of lactone substrate was varied in the range from 5 μM to 2 mM with a constant 1,4-benzoquinone concentration of 1 mM.

2.8. TcGAL activity in reversed micelles

A solution of 0.1 M AOT in isooctane was used for the preparation of the reverse micelles system. The particle size distribution of AOT reverse micelles is narrow, i.e. uniformed micelles of equal size are formed at each surfactant hydration degree [16]. The hydration degree of the micelles ($w_0 = [H_2O]/[AOT]$) was varied over the range of 12–40 by addition of corresponding amounts of 25 mM sodium phosphate, pH 7.2. The inner cavity radius of the micelles r_m in Å can be calculated using the empirical equation [17]:

$$r_m = 1.5w_0 + 4 \quad (1)$$

which holds for all enzyme-containing reverse micellar systems studied [16–18].

2.9. Dehydrogenase and oxidase activity

It has been shown previously that due to strong association with the AOT interface [19], the secondary structure of cytochrome *c* is lost in AOT reverse micelles [20]. Thus, dehydrogenase activity of TcGAL in AOT reverse micelles was measured with the artificial electron acceptor, 1,4-benzoquinone. The molar absorption coefficient of 1,4-benzoquinone in AOT reverse micelles is similar to that in aqueous solution ($\varepsilon_{290} = 2.3\ mM^{-1}cm^{-1}$) [12]. The TcGAL activity in reverse micelles was measured at pH 7.2, since at higher pH values the auto-oxidation of 1,4-benzoquinone is significant [21]. L-Galactono-1,4-lactone dehydrogenase from *Arabidopsis thaliana* (AtGALDH) [12] served as a control.

The oxidase activity of TcGAL in AOT reverse micelles was determined by measuring the formation of ascorbic acid through its reaction with 2,6-dichlorophenolindophenol (DCPIP). The absorption maximum and corresponding molar absorption coefficient of DCPIP in reverse micelles of AOT in isooctane at pH 7.2 ($\varepsilon_{535} = 11.5\ mM^{-1}cm^{-1}$) are different compared to that in water ($\varepsilon_{600} = 20.6\ mM^{-1}cm^{-1}$) [22]. The A113G variant of AtGALDH, which in contrast to wild-type AtGALDH shows high oxidase activity [23], was used as a control for the measurement of the oxygen reactivity of TcGAL in the reverse micelles system.

In a typical kinetic experiment, solutions of D-arabinono-1,4-lactone (in the case of TcGAL) or L-galactono-1,4-lactone (in the case of wild-type AtGALDH and A113G AtGALDH) in water and 1,4-benzoquinone in dimethylsulfoxide or DCPIP in ethanol were solubilized in a 0.1 M AOT solution in isooctane by intensive shaking. The final concentrations were 1 mM for 1,4-benzoquinone and 100 μM for DCPIP. The hydration degree, w_0, was adjusted by addition of corresponding amounts of 25 mM sodium phosphate, pH 7.2. The reaction was initiated by addition of 20 μl TcGAL-containing micellar system, (0.4 M AOT, $w_0 = 30$). Final assay volume was 1 ml. The background rate for non-enzymatic decomposition of 1,4-benzoquinone was subtracted from the reaction rate measured in the presence of enzyme. In the case of DCPIP, the background rate for the non-enzymatic reaction was negligible. To determine kinetic parameters, the concentration of lactone substrate was varied in the range from 5 μM to 2 mM. The activity of TcGAL in reverse micelles followed Michaelis–Menten kinetics. K_m and V_{max} were calculated using nonlinear regression data analysis.

2.10. Circular dichroism

CD spectra were recorded with a Jasco-715 spectropolarimeter at 20 °C in 1 mm path length quartz cuvettes. The CD spectra of TcGAL were measured in AOT reverse micelles (0.04 M AOT, 10 mM sodium

phosphate, pH 7.2, $w_0 = 22$ and 27). The CD spectra of AtGALDH in aqueous solution (10 mM sodium phosphate, pH 7.2) were measured as a reference. Since AOT has high absorbance in the far-UV region, for CD measurements its concentration has to be as low as possible. At 0.04 M AOT it was possible to obtain good quality spectra in the wavelength range 195–260 nm, which enabled quantitative secondary structure analysis. The protein concentrations were 0.05–0.1 mg/ml. The mean residue ellipticity $[\theta]$ ($\mathrm{deg \times cm^2 \times dmol^{-1}}$) was calculated using the equation:

$$[\theta] = (\theta_{obs} / 10) \times (MRW / lc) \quad (2)$$

where θ_{obs} is the observed ellipticity, MRW the mean residue molecular weight, l the optical path length and c the protein concentration [24].

The secondary structure analysis was performed using CD Spectroscopy Deconvolution program (CDNN, version 2.1, G. Bohm, 1997).

3. Results

3.1. Sequence analysis

The mature TcGAL protein contains 505 amino acid residues and has a theoretical mass of 56740 Da. The TcGAL used in this study, originating from *T. cruzi* clone X10/6 [5], shows 97% sequence identity to the TcGAL sequence in the database (*T. cruzi* genome reference strain CL Brener, GeneDB accession number Tc00.1047053509179.100). The small differences do not involve active site residues or residues essential for flavin binding and are most likely the result of polymorphism between the two sub-species. TcGAL shows 28%, 23% and 21% sequence identity, and 51%, 46% and 46% similarity to gulonolactone oxidase from *Rattus norvegicus*, arabinonolactone oxidase from *Saccharomyces cerevisiae* and GALDH from *Arabidopsis thaliana*, respectively. The TcGAL protein contains a relatively large number of 15 cysteine residues, compared to the 5 cysteines present in GALDH from *A. thaliana* (AtGALDH).

Multiple sequence alignment of the N- and C-terminal parts of the FAD-binding domains of various aldonolactone oxidoreductases (Fig. 1) shows that residues specifically interacting with the pyrophosphate and adenine parts of the FAD-cofactor are conserved in TcGAL. Furthermore, a gatekeeper residue preventing oxidase activity similar to Ala113 in AtGALDH [23] is absent in TcGAL, suggesting that the enzyme can use molecular oxygen as electron acceptor.

3.2. Expression and purification of recombinant TcGAL

The recombinant expression of native TcGAL from the pBAD-TcGAL vector in *E. coli* yielded, at best, a mixture of soluble protein and insoluble inclusion bodies (Fig. 2). Using cytochrome *c* as electron acceptor, TcGAL activity was confirmed in the crude *E. coli* extract for L-galactono-1,4-lactone (0.10 U $\mathrm{mg^{-1}}$) and D-arabinono-1,4-lactone (0.23 U $\mathrm{mg^{-1}}$). Thin-layer chromatography of the flavin released from the partially purified TcGAL preparation yielded a retention behavior similar to that of commercial FAD ($R_f = 0.28 \pm 0.04$), and quite different from FMN ($R_f = 0.52 \pm 0.02$). The insoluble inclusion bodies did not contain flavin and were inactive.

Purification of soluble TcGAL (Fig. 2) resulted in considerable loss of enzyme activity (Table 1). Changing the order of purification steps did not improve the outcome, as the protein had a tendency to 'stick' to any column once removed from the crude extract. Altogether we were unable to obtain pure active TcGAL from the soluble fraction, which prompted us to recover the enzyme from the insoluble inclusion bodies fraction [25–27]. Indeed, the production of recombinant protein in inclusion bodies can be in some cases advantageous, since (i) a large amount of highly enriched protein can be easily separated from other proteins, (ii) protein trapped in inclusion bodies is protected from proteolytic degradation and aggregation, and (iii) the inclusion bodies do not have toxic or lethal effects on the host cell.

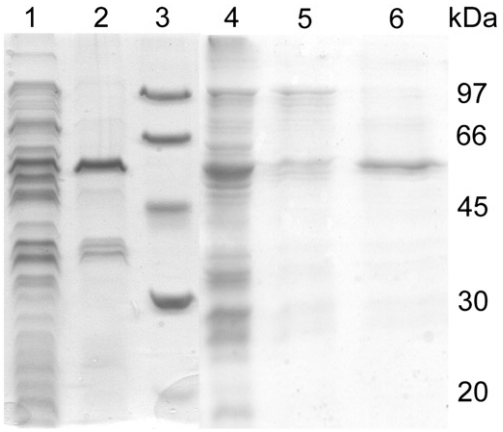

Fig. 2. SDS–PAGE analysis of the expression and purification of soluble TcGAL in *E. coli*. TOP10 cells harboring the pBAD-TcGAL plasmid were induced with 0.2% L-arabinose for 16 h at 28 °C. Lane 1, soluble crude extract; lane 2, insoluble fraction inclusion bodies; lane 3, LMW marker; lane 4, Q-sepharose pool; lane 5, hydroxyapatite pool; lane 6, phenyl-sepharose pool. The predicted molecular mass of native TcGAL is 56.7 kDa.

3.3. Refolding of TcGAL inclusion bodies using reverse micelles

To obtain soluble and active TcGAL from inclusion bodies we applied a recently developed approach based on reverse micelles (RM) [28]. RM spontaneously form in a tertiary system containing surfactant, water and non-polar organic solvent. The size of the inner cavity of micelles where protein molecules and other hydrophilic molecules can be entrapped can be strictly controlled by varying the surfactant hydration degree (w_0), which represents the molar ratio of water to surfactant. RM can be considered as a "nanoreactor" of molecular size where one can obtain the desired supramolecular form of the protein or its complexes by controlling of the micellar inner cavity size. The method therefore provides the modulation of the enzyme oligomeric composition, as it has been demonstrated for a number of enzymes of different classes [28–30]. This approach is a perspective for refolding of large, hydrophobic, multidomain, multisubunit, or highly disulfide bridged proteins, such as TcGAL. Indeed, in reverse micelles each protein molecule is localized in an individual, tailor-made compartment, thus minimizing the possibility of side-reactions, such as aggregation, disulfide cross-linking, etc. Water–AOT–isooctane is one of the most intensively studied reverse micellar systems for protein solubilization. With this in mind, we applied it for solubilization and refolding of TcGAL from inclusion bodies.

Addition of inclusion bodies suspension to the water–AOT–isooctane micellar system (up to final protein concentration of 1–2 mg/ml) yielded a slightly turbid solution, indicating that the protein is still mis-folded and partially aggregated. Incubation with a mixture of reduced and oxidized glutathione (GSH/GSSG 3:1) [31] during 10–15 min and subsequent saturation with FAD solubilized the protein completely and a fully transparent solution was obtained. This micellar enzyme

Table 1
(Partial) purification of soluble TcGAL expressed in *E. coli*. The activity was determined using 1 mM D-arabinono-1,4-lactone and 50 μM cytochrome *c* as substrates, in potassium pyrophosphate, pH 8.8 ($I = 25$ mM).

Step	Protein	Activity	Specific activity	Yield
	mg	U	U $\mathrm{mg^{-1}}$	%
Cell extract	782	177	0.23	100
Q-Sepharose	469	234	0.50	132
Hydroxyapatite	57	186	3.26	105
Phenyl sepharose	16	19	1.19	11

system was used in subsequent experiments for kinetic and spectral characterization.

3.4. Catalytic properties of refolded TcGAL

It has been shown for a number of different enzymes that they are optimally active in RM at a w_0 where the inner radius of the micelle is equal to that of the entrapped protein: $r_{enzyme} = r_{micelle}$. It is believed that under these conditions the surfactant shell works like a molecular matrix, re-adjusting and supporting the native protein conformation [17]. In case of enzymes existing in different oligomeric forms the activity profiles show several optima, where each of these optima reflects different oligomeric forms of the enzyme [17].

To quantify and optimize the yield of active enzyme, the catalytic activity of solubilized and refolded TcGAL in the RM system was measured as a function of the hydration degree (w_0) of the micelles. As a control in these experiments, the well-studied homologous enzyme, AtGALDH [12] was used.

The activity of TcGAL in the reaction with D-arabinono-1,4-lactone and 1,4-benzo-quinone in dependence of w_0 is presented in Fig 3A. Two optima of TcGAL activity are observed at w_0 values of 22 and 27, which can be assigned to the monomeric and dimeric forms of TcGAL, respectively. From 3D-modeling experiments we could judge that TcGAL as well as AtGALDH have a non-spherical shape with subunit dimensions of approximately $47 \times 60 \times 73$ Å [23]. For proteins with a non-spherical shape the inner radius of the micelles, r_m, is equal to the maximal protein radius (half the length of the longest dimension of the protein) r_p. Thus, the r_p value for each oligomeric form can be estimated with the position of the activity optimum (w_0) using the empirical equation [17]:

$$r_p = r_m(\text{Å}) = 1.5w_0 + 4 \quad (3)$$

From this equation and the data in Fig. 3A it follows that TcGAL has a $r_p = 37$ Å at $w_0 = 22$ and $r_p = 46$ Å at $w_0 = 28$ (Table 2). This indeed suggests the accommodation of monomeric and dimeric TcGAL in the AOT RM at hydration degrees of 22 and 28, respectively. The activity level of monomeric TcGAL is slightly higher (by 20%) compared to the dimer; the K_m values for D-arabinono-1,4-lactone are similar, 0.22 ± 0.02 mM for $w_0 = 20$ and 0.25 ± 0.02 mM for $w_0 = 27$, respectively.

AtGALDH showed a similar activity profile in the RM system. The activity optima of AtGALDH are observed at w_0 values of 23 and 28, indicating the formation of monomeric and dimeric species as observed with TcGAL. In contrast to TcGAL, the activity of AtGALDH at $w_0 = 20$ is 25% lower than at $w_0 = 28$. The K_m values of AtGALDH for L-galactono-1,4-lactone are in the range of 0.20–0.22 mM and practically do not change in the range of w_0 from 18 to 35.

In aqueous solution, pH 7.2, AtGALDH shows a $k_{cat} = 100 \pm 10\ s^{-1}$ and $K_m = 0.18 \pm 0.02$ mM with L-galactono-1,4-lactone as the variable substrate and 1,4-benzoquinone as electron acceptor, in good agreement with previous results [12]. Thus, the activity level of AtGALDH in reverse micelles at optimal hydration degrees ($k_{cat} = 84 \pm 10\ s^{-1}$ and $113 \pm 10\ s^{-1}$ at $w_0 = 23$ and 28, correspondingly) and the Michaelis constant for L-galactono-1,4-lactone are of the same order compared to aqueous solution. In other words, solubilization of AtGALDH in reverse micelles does not significantly change the catalytic properties of the enzyme.

Refolded TcGAL is less active than AtGALDH. Refolding TcGAL from inclusion bodies yielded approximately 15–20% of the enzymatic activity as compared to the activity of AtGALDH (Fig. 3). Interestingly, TcGAL is also active with molecular oxygen (aerated buffer) in the absence of alternative electron acceptors. In the reaction with molecular oxygen, a similar profile of TcGAL activity as a function of w_0 of the RM was obtained: two activity optima at the hydration degrees, $w_0 = 22$ and $w_0 = 27$ are observed. The specific activity with molecular oxygen is 2–3 times lower than that in the presence of 1,4-benzoquinone (4.4 ± 0.4 and 3.75 ± 0.4 U mg^{-1} versus 11 ± 1 and 9 ± 1 U mg^{-1} at $w_0 = 22$ and $w_0 = 27$, respectively), while the K_m value for D-arabinono-1,4-lactone is comparable to the reaction in the presence of 1,4-benzoquinone (0.2 ± 0.02 mM at both $w_0 = 22$ and $w_0 = 27$). As a reference system for the oxidase activity of TcGAL in the reverse micelles the A113G variant of AtGALDH was used. In contrast to wild-type AtGALDH this mutant protein shows high oxidase activity, while the dehydrogenase activity is retained [23]. In the micellar system, the A113G variant showed two activity optima at the hydration degrees $w_0 = 22$ and $w_0 = 26$, both in the reaction with 1,4-benzoquinone and with molecular oxygen. Analogous to wild-type AtGALDH, these activity optima can be assigned to the monomeric and dimeric form of the enzyme, respectively. Using Eq. (3), the estimated dimensions of the A113G variant ($r_p = 37$ Å for the monomer and $r_p = 43$ Å for the dimer) are close to those found for TcGAL ($r_p = 37$ Å for the monomer and $r_p = 44.5$ Å for the dimer).

In the reaction with 1,4-benzoquinone as electron acceptor, the activity of the dimeric form of A113G is about 1.5 fold higher compared to the monomer, similar to wild-type AtGALDH (Table 3). As found for TcGAL, the specific activity of the A113G variant in the reaction with molecular oxygen was about 2.5–3 times lower than with 1,4-benzoquinone (10.3 ± 1 and 13.2 ± 1 U mg^{-1} versus 29.5 ± 2 and 37.5 ± 2 U mg^{-1} at $w_0 = 22$ and $w_0 = 26$, respectively) (Table 3). The reaction rates in micellar system at the optimal hydration degree ($w_0 = 26$) are comparable with those measured in solution both in the reaction with 1,4-benzoquinone and with molecular oxygen. Thus, one

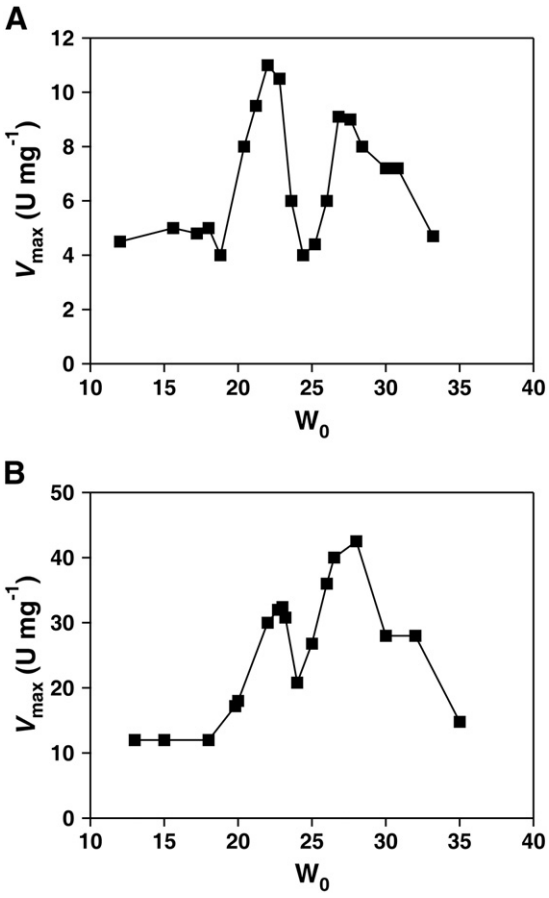

Fig. 3. Catalytic activity of TcGAL and AtGALDH in AOT reverse micelles. (A) Activity of TcGAL with D-arabinono-1,4-lactone; (B) Activity of AtGALDH with L-galactono-1,4-lactone. The maximal activity was determined as a function of the hydration degree (w_0) of the reverse micelles. Experimental conditions: AOT (0.1 M), 1,4-benzoquinone (2.3 mM), 25 mM sodium phosphate, pH 7.2 and varying concentrations of lactone substrate. Kinetic parameters have maximal error values of 20%.

Table 2
Comparison of the inner radius of the micelle (r_m) calculated from the position of the activity optima ($w_{0,opt.}$) of TcGAL in water–AOT–isooctane reverse micelles and the maximal radius (r_p) of dimeric and monomeric forms of TcGAL obtained from 3D-homology modeling [23].

TcGAL oligomeric form	a·b·c, Å	r_p, Å	$w_{0,opt.}$	r_m, Å ($r_m = 1.5w_0 + 4$)	Enzyme state in RM
Monomer	47×60×73	36.5	22	37	
Dimer	94×60×73	47	28	46	

a*b*c—is the geometrical size of the enzyme; r_p—is half the length of longest dimension of the ellipsoid.

can conclude that TcGAL and the A113G variant of AtGALDH have similar properties in the RM system.

No TcGAL activity could be detected (neither in the reaction with 1,4-benzoquinone nor with molecular oxygen) when the enzyme was refolded in the presence of FMN. Thus, refolding of TcGAL inclusion bodies in reverse micelles only yields active protein in the presence of FAD and not FMN.

3.5. Circular dichroism

The structural properties of AtGALDH and TcGAL were addressed by CD spectroscopy. The CD spectra of AtGALDH in aqueous solution (Fig. 4A) and TcGAL in RM (Fig. 4B) have very similar profiles with two negative extremes of nearly equal intensity at 210 and 222 nm. Indeed, quantitative analysis of the CD spectra showed that the secondary structure of both enzymes is similar and characterized by a high α-helix content (Table 4). Together with the activity studies this clearly demonstrates the formation of native-like TcGAL structure upon refolding in RM.

4. Discussion

In this paper we have studied the substrate, cofactor and electron acceptor specificity of the recently discovered enzyme TcGAL [5].

Table 3
Kinetic parameters of TcGAL determined in the reverse micellar system (RM) of AOT in isooctane at optimal hydration degrees ($w_0 = 22$ and 27) corresponding to the functioning of the monomeric and dimeric form of the enzyme, as compared to the same parameters determined for AtGALDH in aqueous buffer solution and in AOT RM at optimal hydration degrees ($w_0 = 23$ and 28). Kinetic parameters of the A113G variant of AtGALDH in AOT RM at optimal hydration degrees ($w_0 = 22$ and 26) are presented as a reference for the oxygen reactivity of TcGAL. Experimental conditions: AOT (0.1 M), 1,4-benzoquinone (2.3 mM), 25 mM sodium phosphate, pH 7.2 and varying concentrations of lactone substrate.

Enzyme	Substrates[a]	Activity (U mg^{-1})		K_m (mM)	
		M[b]	D[b]	M[b]	D[b]
TcGAL (RM)	D-AL/BQ	11.0 ± 1	9.1 ± 1	0.22 ± 0.02	0.25 ± 0.02
TcGAL (RM)	D-AL/O_2	4.4 ± 0.4	3.8 ± 0.4	0.20 ± 0.02	0.20 ± 0.02
AtGALDH	L-GAL/BQ	38.6 ± 2	-	0.18 ± 0.02	-
AtGALDH (RM)	L-GAL/BQ	32.4 ± 2	42.4 ± 2	0.20 ± 0.02	0.20 ± 0.02
A113G (RM)	L-GAL/BQ	29.5 ± 2	37.5 ± 2	0.25 ± 0.02	0.22 ± 0.02
A113G (RM)	L-GAL/O_2	10.3 ± 1	13.2 ± 1	0.20 ± 0.02	0.18 ± 0.02

[a] L-GAL, L-galactono-1,4-lactone; D-AL, D-arabino-1,4-lactone; BQ, 1,4-benzoquinone.
[b] Enzyme oligomeric form: M—monomer and D—dimer.

TcGAL completes ascorbate biosynthesis in *T. cruzi* parasites, which are unable to scavenge ascorbate from their environment.

Recombinant expression of TcGAL from the pBAD-TcGAL vector yielded a mixture of active soluble protein and inactive inclusion bodies. Purification of soluble TcGAL was hampered by considerable loss of activity. Possibly, the native enzyme requires disulfide bridges, which are unlikely to be formed in the reducing environment of the *E. coli* cytoplasm [32]. Doubly tagged TcGAL was also expressed at very low levels in *E. coli* [5]. Unfortunately, no information was provided about the solubility properties of this recombinant protein.

To obtain correctly folded active protein, TcGAL inclusion bodies were solubilized and refolded using an AOT-isooctane RM system. The important advantage of this approach is that upon solubilization each enzyme molecule is accommodated in an "individual compartment." The possibility to "isolate" each protein molecule minimizes side processes of protein folding, such as aggregation and intermolecular disulfide bond formation. The abovementioned suggestion that native TcGAL requires disulfide bridges is supported by the fact that refolding of TcGAL in reverse micelles requires the presence of a redox system consisting of a mixture of reduced and oxidized glutathione (GSH: GSSG). The refolded TcGAL protein shows native-like secondary structure with an α-helix and β-sheet content very similar to that of AtGALDH in aqueous solution (Table 4). The high α-helix content of TcGAL is consistent with the structural properties of other VAO-family members [9,33].

As previously found for a number of enzymes, the activity in the RM system can be much higher than in aqueous solution, even if the enzyme is correctly folded in solution [17]. As a rule, this superactivity effect can be observed for membranotropic/membrane-dependent

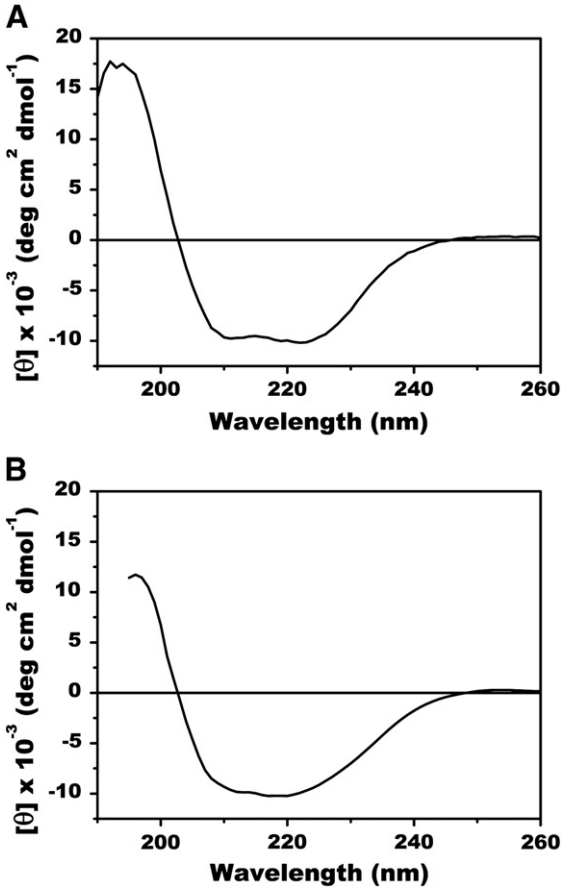

Fig. 4. Circular dichroism spectra of AtGALDH and TcGAL. (A) AtGALDH in 25 mM sodium phosphate, pH 7.2; (B) TcGAL in AOT reverse micelles. Experimental conditions: AOT (0.04 M), 10 mM sodium phosphate, pH 7.2, 20 °C. Protein concentrations were 0.05–0.1 mg/ml.

Table 4
Secondary structure parameters determined from CD experiments for TcGAL inclusion bodies refolded in AOT reversed micelles. The same parameters determined for AtGALDH in aqueous solution are presented as a reference. Experimental conditions: 0.04 M AOT, 10 mM sodium phosphate, pH 7.2. Protein concentrations were 0.05–0.1 mg/ml.

Secondary structure element	TcGAL in micelles	AtGALDH in buffer
	%	%
α-helix	33.1	32.4
β-sheets (antiparallel)	8.6	8.1
β-sheets (parallel)	8.9	9.1
β-turn	16.6	16.8
loops	34.0	35.4

Note: Standard errors for the determination of secondary structure parameters (obtained from 3 independent experiments) did not exceed 10%.

enzymes. The molecular mechanism of enzyme regulation by the micellar matrix depends on enzyme specificity as well as the system composition; see for example Ref. [29]. Normally, the surfactant shell works like a molecular matrix, re-adjusting and supporting the optimal enzyme conformation. The RM approach used in the current work clearly improves the possibility of obtaining active TcGAL in its proper microenvironment. This new application reinforces the interest of the use of the RM system.

Both TcGAL and AtGALDH show two activity optima in RM, depending on w_0, corresponding to the monomeric and dimeric forms of the enzymes. It is, however, believed that both enzymes function as monomers, since for AtGALDH no dimer formation was observed in aqueous solution [12]. Apparently, the micellar system in this case can be considered as a specific compartment, which supports the formation of dimer molecules. One can speculate that in the living cells both monomeric and dimeric forms of TcGAL and AtGALDH may function depending on the enzyme microenvironment, such as the membrane-associated state.

In line with previous reports [5,6], we found that TcGAL can oxidize both L-galactono-1,4-lactone and D-arabinono-1,4-lactone, with a slight preference for the latter substrate. The preference of TcGAL and TbALO for D-arabinono-1,4-lactone discriminate the trypanosomal enzymes from AtGALDH, which shows a clear preference for L-galactono-1,4-lactone [34]. Despite the preference of TcGAL and TbALO for D-arabinono-1,4-lactone, genome analysis suggests that ascorbate biosynthesis in trypanosomes proceeds via L-galactose and L-galactono-1,4-lactone [6]. Logan et al. found unusually high k_{cat} values for the TcGAL activity with L-galactono-1,4-lactone (673 s^{-1}) and D-arabinono-1,4-lactone (649 s^{-1}) [5]. Given the reported experimental procedures, we conclude that these k_{cat} values cannot be real.

Previously, TcGAL was annotated as a galactonolactone oxidase [5], but the activity with molecular oxygen was never demonstrated. We found that, besides cytochrome *c* and 1,4-benzoquinone, TcGAL can also use molecular oxygen as electron acceptor. The reactivity with molecular oxygen is in accordance with the fact that TcGAL lacks a recently identified gatekeeper residue that prevents dehydrogenases of the VAO-family to act as oxidases [23]. This gatekeeper residue is situated near the C4a locus on the flavin *re*-side, opposite of the substrate binding site, and likely prevents oxygen access to the reduced flavin [35]. Replacement of the corresponding residue in AtGALDH (Ala113) into a Gly results in a catalytically competent oxidase [23].

TcGAL has been reported to contain non-covalently bound FMN as redox-active cofactor [5]. The binding of FMN is unexpected since TcGAL has a VAO fold with a cofactor binding domain that contains residues specifically interacting with the adenine and pyrophosphate parts of FAD [9]. However, the trichloroacetic acid precipitation used to release the flavin cofactor promotes the acid catalyzed hydrolysis of FAD [36]. Indeed, here we could show that soluble recombinant TcGAL employs a non-covalently bound FAD as redox active cofactor and that the refolding of TcGAL inclusion bodies in reversed micelles only yields active protein in the presence of FAD and not FMN. Recently, also for another VAO-family member, the glycopeptide hexose oxidase Dbv29, an FMN cofactor was identified [37]. The method used to release the cofactor is, however, not clear.

The C-terminal HWXK motif is conserved in ascorbate-synthesizing and some closely related enzymes including alditol oxidase (AldO) [34]. From the crystal structure of AldO [38] it becomes evident that only the His and Lys residues of this motif are located close enough to the flavin to directly interact. The Lys residue, however, is part of the active site of AldO, and hydrogen bonds with the C1-OH of the substrate, the site of oxidation. A similar important role in the active site seems to be reserved for Lys450 in TcGAL, since replacing it with glycine renders the protein inactive [5]. The His residue could be involved in flavin binding, but since both GALDH and AldO contain FAD rather than FMN, this motif is not specific for FMN association. The lack of activity and flavin binding observed in the W448G mutant [5] could be due to incorrect folding of the protein. Logan et al. assume that the non-conservative mutations do not affect protein folding, but no experimental evidence is given.

5. Conclusions

In the present work we have successfully applied and further advanced a recently suggested approach [23] for solubilization and refolding of inclusion body protein using a reverse micelles system. Thus, using an AOT–isooctane reverse micelles system we were able to obtain untagged TcGAL in active form and investigate its secondary structure and catalytic properties. In contrast to an earlier report [5], we found that native TcGAL employs a non-covalently bound FAD as redox active cofactor, in agreement with its amino acid sequence. Recombinant TcGAL oxidizes both L-galactono-1,4-lactone and D-arabinono-1,4-lactone using different electron acceptors, including molecular oxygen.

6. Outlook

Up to now, the exploitation of the ascorbate synthesizing pathway in general and TcGAL in particular in *T. cruzi* as a potential drug target for Chagas' disease is greatly hampered by the lack of a crystal structure for the group of aldonolactone oxidoreductases. The results obtained in this study and our previous work on the homologous plant enzyme GALDH provide a good framework for further structure–function relationship studies aimed at identifying important residues involved in catalysis and eventually in solving the crystal structure and finding active site inhibitors.

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