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Overexpression of *Crithidia fasciculata* Trypanothione Reductase and Crystallization Using a Novel Geometry

BY COREY L. STRICKLAND, ROBERT PUCHALSKI, SAVVAS N. SAVVIDES AND P. ANDREW KARPLUS

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca NY 14853, *USA*

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Abstract

TR1, a previously cloned gene for *Crithidia fasciculata* trypanothione reductase (TR), has been overexpressed in *Escherichia coli* strain SG5 to produce about 20 mg enzyme l^{-1} of culture. Since natural *C. fasciculata* TR is heterogeneous, this expression system provides an important source of homogeneous *C. fasciculata* TR for use in structural studies and drug design. Steady-state kinetic constants of the purified recombinant enzyme are $K_m = 56\ \mu M$ and $k_{cat} = 10\ 500\ min^{-1}$. Four crystal forms of TR1 were grown using this preparation. Synchrotron radiation was crucial to discover the high level of order present in crystal form IV, which diffracts to about 1.4 Å resolution. To optimize growth and handling of form IV crystals, a novel crystallization setup called the 'plug drop' was developed.

The protozoan parasites of the trypanosomatid family cause several well known diseases, such as African sleeping sickness, Oriental sore and South American Chagas disease, which combined affect tens of millions of people (Gibbons, 1992). Treatments for these diseases have met with little success and are generally plagued by serious side effects (Walsh, Bradley & Nadeau, 1991). Analysis of the trypanosomatid parasites has revealed that they are sensitive to oxidative stress (Docampo & Moreno, 1984). It is postulated that an absence of catalase/peroxidase hemoproteins makes the parasites almost totally dependent on a unique oxidation detoxification system, which is based on trypanothione (TS_2), a conjugate of glutathione with spermidine (Fairlamb, Blackburn, Ulrich, Chait & Cerami, 1985). Several of the currently available drugs are thought to work by overloading this system through a systematic increase in oxidative stress (Walsh, Bradley & Nadeau, 1991).

Trypanothione reductase (TR) is an important enzyme in the trypanothione-dependent pathway (Shames, Fairlamb, Cerami & Walsh, 1986). TR uses NADPH to reduce oxidized trypanothione which builds up as a result of oxidative stress. The analogous enzyme in mammals is glutathione reductase (GR), which uses NADPH to reduce oxidized glutathione. Human GR and TR show a high degree of specificity for their respective substrates (Shames, Fairlamb, Cerami & Walsh, 1986), which is partly due to electrostatic effects as trypanothione has a +1 charge while glutathione has a −2 charge. Thus, it seems likely that selective inhibitors could be designed to inhibit the parasitic TR and not affect the host GR.

Efforts to take advantage of this enzyme as a drug target have led to its isolation and/or cloning from several species of trypanosomes including *T. cruzi* (Sullivan & Walsh, 1991), *T. brucei* (Fairlamb, Carter, Cunningham & Smith, 1992), *T. congolense* (Shames, Kimmel, Peoples, Agabian & Walsh, 1988; Sullivan, Shames & Walsh, 1989) and *C. fasciculata* (Field, Cerami & Henderson, 1991; Shames, Fairlamb, Cerami & Walsh, 1986; Aboagye-Kwarteng, Smith & Fairlamb, 1992). Comparison of the sequences of the various TR's has shown that they are at least 68% identical with each other indicating that their structures will be extremely similar, especially at the active site. For *C. fasciculata* TR, sequence heterogeneity has been found by comparing gene sequences from different strains of *C. fasciculata* (Aboagye-Kwarteng, Smith & Fairlamb, 1992; Field, Cerami & Henderson, 1991).

The crystal structure of *T. cruzi* TR has been solved at 3.3 Å resolution (Lantwin, Schlichting, Kabsch, Pai & Krauth-Siegel, 1994) and the *C. fasciculata* TR has been determined at 2.4 Å (Kuriyan, Kong, Krishna, Sweet, Murgolo, Field, Cerami & Henderson, 1991) and independently near 2.8 Å resolution, both with and without a substrate analogue (Hunter, Bailey, Habash, Harrop, Helliwell, Aboagye-Kwarteng, Smith & Fairlamb, 1992; Bailey, Fairlamb & Hunter, 1994; Bailey, Smith, Fairlamb & Hunter, 1993). These structures provide initial information with which structure-based drug design can be carried out to develop inhibitors that are not toxic to the host. However, the iterative process of structure-based drug design demands a readily available supply of homogeneous protein and, ideally, high-resolution structural information. TR from *T. congolense* (Sullivan, Shames & Walsh, 1989) and *T. cruzi* (Sullivan & Walsh, 1991) have already been overexpressed in *E. coli*. Here we report the overexpression and kinetic characterization of a single isozyme of *C. fasciculata* TR, and the growth of single crystals that diffract to about 1.4 Å resolution.

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Expression and characterization of TR1 and TR5

The genes for TR1 and TR5 were inserted into the pET-3xc expression plasmid under the control of a T7 polymerase promoter (Fig. 1) resulting in the two plasmids pET-TR1 and pET-TR5. The genes for TR1 and TR5 differ in several nucleotide positions (Field, Cerami & Henderson, 1991), but these did not affect the construction of pET-TR1 or pET-TR5. The substitution of a G at position 1435 in the gene for TR1 for a C in the gene for TR5 results in a Glu at residue 478 in TR1 and a Gln in TR5. The other nucleotide differences are silent at the protein level. Using this system, active TR is expressed at about 2% of the soluble protein, allowing the purification of about 170 mg of TR1 from a 10 l culture. TR5 was also expressed at similar levels, but our initial preparation from 10 l of cells only yielded about 20 mg. Initial crystallization attempts using TR5 were not successful, so the purification was not optimized.

Steady-state kinetic constants for recombinant TR1 are $K_m = 56\ \mu M$ and $k_{cat} = 10\ 500\ min^{-1}$ (see Fig. 1 legend). The K_m is comparable to the value of 53 μM K_m for the natural *C. fasciculata* TR, however the k_{cat} is about 30% of the previously reported k_{cat} of 31 000 min^{-1} for natural *C. fasciculata* TR (Shames, Fairlamb, Cerami & Walsh, 1986). We have reproduced a k_{cat} near 10 000 min^{-1} for two different preparations of TR1 and, in fact, natural *C. fasciculata* TR purified in our laboratory has a k_{cat} of only 8500 min^{-1}. The cause of this discrepancy is unknown, but one possibility for the decreased k_{cat} is that heavy metals or other compounds present during the purification inactivated a portion of the TR1. Such a mechanism has been invoked to explain fluctuations in the observed k_{cat} for various human GR preparations (Nordhoff, Bücheler, Werner & Schirmer, 1993). However, recombinant TR from *T. congolense* and *T. cruzi* have k_{cat} values that are similar to their natural counterparts (Sullivan, Shames & Walsh, 1989; Sullivan & Walsh, 1991), so a low k_{cat} cannot be a general feature of *E. coli* expression of TR. It is interesting that the k_{cat} observed for TR1 is of similar magnitude to the values of 9200 and 14 200 min^{-1} reported for natural TR from *T. congolense* (Sullivan, Shames & Walsh, 1989) and *T. cruzi* (Krauth-Siegel, Enders, Henderson, Fairlamb & Schirmer, 1987). In this respect, the value of k_{cat} for natural *C. fasciculata* TR would appear to be the anomalous one, but it has been independently measured by two laboratories using different strains of *C. fasciculata* (El-Waer, Douglas, Smith & Fairlamb, 1991; Shames, Fairlamb, Cerami & Walsh, 1986).

Crystallization of recombinant TR

Initial attempts to crystallize TR1 using the conditions described for natural *C. fasciculata* TR (Kuriyan, Wong, Guenther, Murgolo, Cerami & Henderson, 1990) gave small crystals that were not of diffraction quality. The inability to reproduce the previous crystals may be related to the heterogeneity known to be present in the TR from different strains of *C. fasciculata* (Aboagye-Kwarteng, Smith & Fairlamb, 1992) or possibly due to subtle differences in purity of the natural and recombinant enzymes. When this initial attempt at crystallization failed, we used the 'fast screen' method (Jancarik & Kim, 1991) to screen for crystallization conditions both of TR1

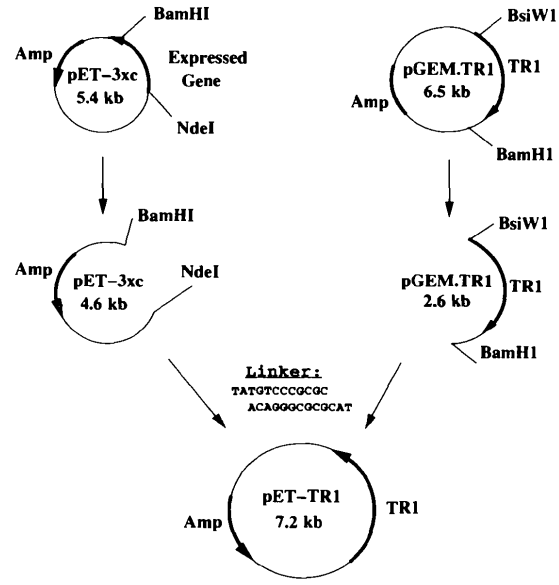

Fig. 1. Construction of the overexpression vector for *C. fasciculata* TR. The pET-3xc plasmid was cut with *Bam*HI and *Nde*I and the 4.6 kb fragment isolated. The pGEM.TR1 (Field, Cerami & Henderson, 1991) plasmid was cut with *Bsi*WI and *Bam*HI and the 2.6 kb fragment was isolated. These two pieces plus a linker, to replace the first ten nucleotides that were removed by the restriction cuts, were ligated together. The resulting plasmid is pET-TR1. pET-TR5 was constructed in an analogous manner. pET-3xc was obtained from Novogen and the two unphosphorylated oligonucleotide linkers were made by the Cornell Biotechnology Analysis and Synthesis Facility. pGD1-2 (Tabor & Richardson, 1985), a plasmid containing the T7 polymerase gene repressed by a heat-sensitive λ repressor (cI857), and the *E. coli* strain SG5 (Greer & Perham, 1986), a GR deletion strain, were a gift from the laboratory of C. Walsh (Havard University, Boston, MA). pGEM.TR5 and pGEM.TR1 (Field, Cerami & Henderson, 1991), plasmids containing the gene for the TR1 and TR5 isozymes of *C. fasciculata* TR, were a gift of H. Field (Rockefeller University, New York, NY). All DNA manipulations were carried out according to Maniatis (Maniatis, Fritsch & Sambrook, 1982), except fragment isolation was performed according to Errington (Errington, 1990). Trypanothione reductase was then overexpressed (Sullivan, Shames & Walsh, 1989) and purified (Shames, Fairlamb, Cerami & Walsh, 1986). The concentration of pure TR was determined by the absorbance at 466 nm using $\varepsilon_{466} = 11.3\ mM^{-1}\ cm^{-1}$. TR kinetic assays (Shames, Fairlamb, Cerami & Walsh, 1986) were performed at 295 K with trypanothione concentrations of 12, 24, 50, 100, 200 and 400 μM. The rates for the kinetic experiments were fitted by non-linear least squares to the Michaelis–Menten equation using the program *KaleidaGraph* (Abelbeck Software, 1993) for the Macintosh giving a K_m of 56±3 μM and a k_{cat} of 10 500±360 min^{-1}.

Table 1. *Crystal forms of recombinant TR*1

All crystallization conditions were carried out at room temperature and initially determined by the hanging-drop method. Space groups were determined using simulated precession photos on a San Diego Multiwire Systems X-ray area detector using a Rigaku-RU200 rotating-anode generator. This setup was used to determine the diffraction limits for forms I, II and III. The diffraction limit for form IV was determined at CHESS (Fig. 3). The number of molecules per asymmetric unit has been confirmed by molecular replacement.

			Unit-cell parameters						
Form	Protein status	Space group	a (Å)	b (Å)	c (Å)	β	Resolution limit (Å)	Dimers per asymmetric unit	Percent solvent
I*	Native	$P2_1$	124.0	161.7	58.5	93.7	2.1	2	53
II†	Native	$P2_12_12_1$	107.0	168.8	58.7	90.0	1.9	1	50
III†	Native	$P2_12_12_1$	105.9	169.6	60.0	90.0	1.9	1	53
IV‡	Native	$P2_1$	58.54	161.7	61.2	98.8	1.4	1	53

* Reservoir conditions: 6% PEG 4000, 0.1 *M* sodium acetate (pH 4.65). Crystals were grown from 20 µl hanging drops, with the initial drop containing 10 µl of 10 mg ml^{-1} TR1 and 10 µl of reservoir. Crystals appeared in several days and have a rectangular shape with size around 200 × 300 × 600 µm.

† Reservoir conditions: 20–22% PEG 4000, 0.1 *M* HEPES (pH 6.0). Crystals were grown from 20 µl hanging drops, with the initial drop containing 10 µl of 12.0 mg ml^{-1} TR1 plus 50 m*M* dithiothreitol and 10 µl of reservoir. Crystals appeared in 2–3 weeks and have a rectangular shape with a size about 300 × 300 × 600 µm.

‡ Reservoir conditions: 21% PEG 8000, 0.1 *M* sodium citrate (pH 4.9). Crystals were grown from 40 µl plug drops, with the initial drop containing 24 µl 10.0 mg ml^{-1} TR1 and 16 µl of reservoir. Crystals appeared in several days and have a rectangular shape with size around 150 × 150 × 300 µm.

alone and in the presence of 0.6 m*M* trypanothione. The results were nearly identical with or without trypanothione, with wells 37 (0.1 *M* acetate, pH 5.6, and 8% PEG 4000) and 40 (0.1 *M* citrate, pH 5.6, 20% 2-propanol and 20% PEG 4000) yielding crystalline material. Through optimization of these conditions, we have been able to obtain four crystal forms of TR1 (Table 1). Optimized crystal form I growth conditions remained very similar to those of well 37 of the fast screen. Crystal forms II and III grow under identical conditions and were obtained through systemic variation of condition number 37 at higher pH values. Crystal forms I, II and III are grown using 20 µl hanging drops. Crystal form IV was obtained through systemic variation of condition 40. Initially form IV crystals grew best with 0.6 m*M* trypanothione present, but after refinement of the conditions they grow identically with or without trypanothione. Form IV appears to be isomorphous to the crystals of natural TR that were originally reported for natural *C. fasciculata* TR (Kuriyan, Wong, Guenther, Murgolo, Cerami & Henderson, 1990), however form IV crystals grow from 21% PEG 8000 at pH 4.9 while natural *C. fasciculata* crystals grow from 22% PEG 8000 and 0.1 *M* ammonium sulfate at pH 7.2. Crystals of form IV grow more reproducibly in sandwich drops (Jones, Ward & Perozzo, 1988) than in hanging drops, however the crystals grow attached so strongly to the glass coverslip that removal of the crystals without damage is difficult.

To overcome this problem, plug drops (Fig. 2) were developed and used to grow form IV crystals. In this geometry, crystals fall to an air/fluid interface as they grow and do not adhere to a coverslip. This geometry has the further advantage that the crystals tend to nucleate at the top of the drop which equilibrates the fastest and then the growing nuclei fall to bottom of the drop, which equilibrates slower and has a lower level of supersaturation which can lead to more controlled growth (Feher & Kam, 1985). Two other advantages of the plug drops are that large volumes can be used so that larger crystals can be grown (Fox & Karplus, 1993), and that they can be set up in the common Linbro plates that are used routinely for hanging-drop crystallization.

As seen in Table 1, the unit cells of the different crystal forms are all closely related. Each of the four crystal forms has $b \simeq 160$, $c \simeq 60$ and $a \simeq 60$ Å or 110 Å depending on the total number of dimers in the unit cell. Crystals of forms I, II and III have $a \simeq 110$ Å and contain four dimers in the unit cell, while form IV crystals have $a \simeq 60$ Å and have two dimers in the unit cell. It is interesting that none of the crystal forms has a monomer in the asymmetric unit, suggesting that the dimer may be inherently asymmetric.

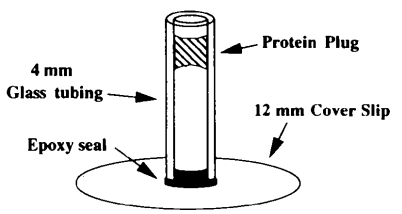

Fig. 2. Plug drop crystallization design. A 1 cm length of glass tubing with an internal diameter of 4 mm was siliconized and then epoxyed onto a 12 mm diameter glass coverslip. 40 µl of the protein solution to be crystallized (in this case made by mixing 24 µl of 10 mg ml^{-1} TR stock with 16 µl of the reservoir against which the plug would be equilibrated) was placed in the epoxied glass tubing. The unit was placed into a well of a Linbro tissue-culture plate and surrounded with 500 µl of reservoir solution, and then the well was sealed. Although the Linbro plate is convenient, the plug-drop unit could be placed in any sealed container.

All the crystal forms diffract to at least 2.0 Å resolution, with form IV diffracting to near 1.4 Å at the Cornell High Energy Synchrotron Source (Fig. 3). Because of the small size of form IV crystals, the intense synchrotron source was crucial for seeing the intrinsic limit of diffraction of this crystal form. This emphasizes that reported limits of diffraction must be regarded as a function of crystal size and X-ray source, and that forms I, II and III may also diffract further than the values reported in Table 1. Such high-resolution diffraction has rarely been seen for crystals of proteins this size (near 500 amino acids). Interestingly the TR homologue, glutathione reductase, which has been analyzed at 1.54 Å resolution (Karplus & Shulz, 1987) provides the other known example, and comparison of these two structures should prove valuable.

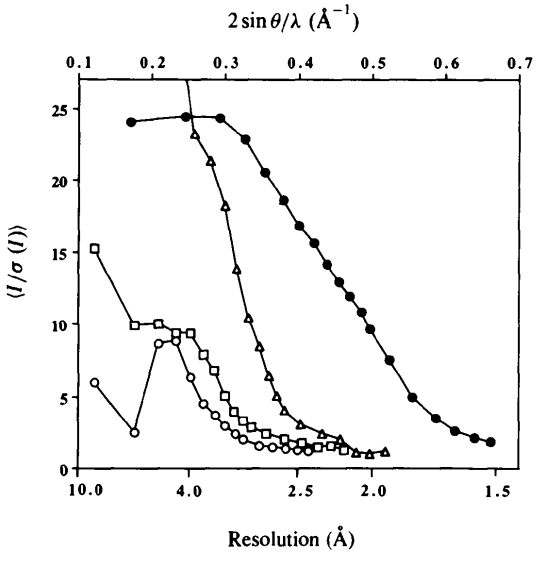

Fig. 3. Diffraction strength as a function of resolution for TR crystal forms. Diffraction strength is given by $\langle I/\sigma(I)\rangle$ over all reflections in a given resolution range, where I is the integrated intensity and $\sigma(I)$ is the standard error of the intensity measurement. The curves shown are based on laboratory diffraction data for TR form I (□), form II (△) and form IV (○) and synchrotron data for form IV (●). Typical crystal sizes used are indicated in Table 1 and the statistics are based on relatively complete data sets. The laboratory diffraction data were collected at room temperature with a single San Diego Multiwire Systems detector on a Rigaku RU-200 rotating-anode X-ray generator (Cu $K\alpha$ radiation; 0.5 × 5.0 mm focal spot; running at 50 kV, 150 mA; graphite monochromator). Parameters for the laboratory data: crystal-to-detector distance = 790 mm through a helium path; frame width = 0.10° in ω; rate = 60 s frame^{-1}; 0.280 mm collimator. The synchrontron data were collected at beam -line F1 at the Cornell High Energy Synchrotron Source using λ = 0.91 Å radiation and a 0.1 mm collimator. The crystal was flash frozen in a rayon loop (Teng, 1990; David Rogers, personal communication) and 90 contiguous 60 s, 2° oscillation images were recorded on 8 × 10″ 20.3 × 25.4 cm Fuji image plates using a crystal-to-detector distance of 189 mm through air. The data was processed with *DENZO* and *SCALEPACK* (Otwinowski, 1993).

The structure of the protein in each crystal form has been solved by molecular replacement using *X-PLOR* (Brünger, 1990) with coordinates of the 2.4 Å resolution structure of natural *C. fasciculata* TR (Kuriyan, Kong, Krishna, Sweet, Murgolo, Field, Cerami & Henderson, 1991) as the search model. Consistent with the result that crystal form IV can also grow in the absence of trypanothione, the crystals do not appear to contain bound trypanothione in the active site. High-resolution analyses of these crystal forms may provide insight into the reasons for the polymorphic nature of TR crystals and will provide structural details useful for structure-based drug design.

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