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Crystallization and preliminary crystallographic analysis of trypanothione reductase from *Trypanosoma cruzi*, the causative agent of Chagas' disease

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Trypanothione reductase from *Trypanosoma cruzi* is the most promising target molecule for the rational design of a specific drug against Chagas' disease. The recombinant protein was purified in a single chromatographic step and crystallized. Two crystal forms suitable for X-ray diffraction analysis were obtained. Tetragonal crystals (a=b=87.4 Å, c=152.3 Å) were grown from 30% polyethylene glycol (average M_r = 8,000) in the presence of 0.2% β-*n*-octylglucoside (space group either $P4_2$ with one dimer or $P4_222$ with one monomer in the asymmetric unit). Monoclinic crystals (space group P2, a=136.3 Å, b=91.1 Å, c=126.0 Å, β=94°) were grown from 1.2 M sodium citrate in the presence of 2% octanoyl-*N*-methyl-glucamide. They contain two dimers of the enzyme in the asymmetric unit; both crystal forms diffract to 3 Å resolution.

Trypanothione reductase; Drug design; Chagas' disease; Flavoprotein; Protein crystallography; *Trypanosoma cruzi*

1. INTRODUCTION

The thiol metabolism of nearly all organisms is based on the glutathione/glutathione reductase system [1]. The flavoenzyme glutathione reductase keeps the [GSH]/[GSSG] ratio at a high level and thus provides reducing equivalents for processes such as the reduction of disulfides, the detoxication of peroxides and the synthesis of DNA precursors via the glutaredoxin system [2].

Trypanosomes and leishmanias, the causative agents of conditions such as African sleeping sickness, Chagas' disease, oriental sore, Kala-azar and the Nagana cattle disease, show a metabolic peculiarity. These parasitic protozoa do not possess glutathione reductase. Rather, their glutathione metabolism is closely connected to that of polyamines, the main thiols being N^1,N^8-bis(glutathionyl)spermidine (trypanothione, $T(SH)_2$ [3] and monoglutathionylspermidine. These conjugates are kept in the thiol state by the flavoenzyme trypanothione reductase (TR) (TS_2+NADPH+H^+ → $T(SH)_2$+$NADP^+$). Trypanothione reductase has been isolated from the insect parasite *Crithidia fasciculata* [4] and from *Trypanosoma cruzi*, the causative agent of Chagas' disease [5]. The trypanothione reductase genes of *T. congolense* [6] and *T. cruzi* [7] have been cloned and expressed in *Escherichia coli*. Although all Trypanosomatidae possess a trypanothione metabolism they are not equally sensitive to the compounds interfering with this pathway. Difluoromethylornithine, an inhibitor of spermidine synthesis which is successfully used as a drug against African sleeping sickness, is not effective against Chagas' disease [8]. On the other hand, *Trypanosoma cruzi* is very sensitive to oxidative stress as indicated by the fact that the only drugs available, nifurtimox and benznidazole, are redox-cyclers [9]. Therefore trypanothione reductase is a most attractive target molecule for the development of a specific drug against Chagas' disease.

T. cruzi trypanothione reductase is structurally and mechanistically related to human glutathione reductase [5,10,11]. The crucial difference between parasite and host enzyme is their mutually exclusive specificity for the respective disulfide substrate. Comparative kinetic studies in solution show that a selective inhibition of *T. cruzi* trypanothione reductase should be possible [12,13]. For human glutathione reductase the 3-dimensional structure [14,15], the stereochemistry of catalysis

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Abbreviations: GSH, glutathione; GSSG glutathione disulfide; TR, trypanothione reductase; TS_2, trypanothione disulfide; $T(SH)_2$, trypanothione.

[16,17] and the binding mode of several drugs are known [10]. Structural information on trypanothione reductase is available for the *C. fasciculata* enzyme [18,19]. This TR shows 69% sequence identity with the isofunctional enzyme of *T. cruzi*. Since even minor amino acid substitutions can strongly influence the binding of inhibitors the authentic enzyme is the candidate of choice for drug design against *T. cruzi*.

2. MATERIALS AND METHODS

2.1. *Protein purification*

Recombinant *T. cruzi* trypanothione reductase was produced in glutathione reductase-deficient *E. coli* SG 5 cells [20] using the overproducing expression vector pIBITczTR. The cells were grown and induced as described [6]. The enzyme was prepared according to Sullivan and Walsh [7] but with several modifications. The harvested bacterial cells were disrupted by lysozyme digestion [21] which results in higher enzyme yields than sonication. 100g cells were suspended at 25°C in 250 ml 50 mM potassium phosphate, 1 mM EDTA, 60 μM FAD, 2 mM dithiothreitol, 100 μM phenyl-methane-sulfonylfluoride, pH 7.0, in the presence of 0.2 mg/ml lysozyme. After stirring for 5 min, 5 ml of a 4% sodium deoxycholate solution and 2.5 mg DNase I were added, the suspension was stirred for 30 min at 25°C, and centrifuged. The supernatant was kept on ice, the cell pellet was resuspended in buffer, stirred for another 30 min at 25°C and cleared by centrifugation. The extracts were combined, and $(NH_4)_2SO_4$ was added to 70% saturation. After standing for 16 h at 4°C and centrifugation, the protein pellet was dissolved in 10 mM potassium phosphate, 1 mM EDTA, pH 7.0 and dialyzed for 5 h at 25°C. The protein solution was then applied to a 2'5'ADP-Sepharose column (2 × 14 cm) previously equilibrated with 50 mM potassium phosphate, 1 mM EDTA, pH 7.0. The column was washed with 4 vol. of 50 mM potassium phosphate, 1 mM EDTA, pH 7.0 and then equilibrated with 25 mM potassium phosphate, 1 mM EDTA, pH 7.0. Trypanothione reductase was eluted with 300 μM NADPH in 25 mM potassium phosphate, 1 mM EDTA, pH 7.0, precipitated with $(NH_4)_2SO_4$ and stored at 4°C.

2.2. *Crystallization*

The precipitate was dialysed against 20 mM Tris-HCl, 1 mM EDTA, pH 8.0, 500 mM sodium citrate, and adjusted to a protein concentration of 10–15 mg/ml. Octanoyl-*N*-methylglucamide was added to a final concentration of 1–2%. 10 μl droplets of the protein solution were allowed to equilibrate by vapor diffusion with 1 ml 1.2 M sodium citrate. Crystals grew within 1–2 weeks at 20°C (Fig. 1A). Another crystal form of the enzyme prepared according to Sullivan et al. [6] was obtained from polyethylene glycol (PEG). Starting with 9% (w/v) PEG (average M_r 8,000), 0.2% β-*n*-octylglucoside in 100 mM Tris-HCl, 1 mM EDTA, pH 8.0 in the droplet and with 30% PEG 8000 in the reservoir, small yellow diamond-shaped crystals appeared within 3 days at 20°C (Fig. 1B). In some cases tetragonal yellow rods were obtained. All crystals of recombinant *T cruzi* TR grew only at 20°C and not at 4°C.

2.3. *Crystallographic methods*

X-ray diffraction data were collected on an electronic area detector (Siemens/Nicolet, Madison, WI) at 4°C with a crystal-to-detector distance of 13 cm. X-rays (CuK_α) were generated by a GX-18 rotating anode (Elliot/Enraf-Nonius, Delft) and focused by Franks double mirror optics. The anode was operated at 35 kV and 50 mA. An oscillation camera (Supper, Natick, Mass.) with a vertical rotation axis was used to move the crystal. Camera and detector were controlled by a simplified version of a program of Blum et al. [22]. Complete sets of corrected intensity data to 3 Å resolution were extracted from the raw data frames by the program XDS [23,24]. Space group symmetry was automatically determined by an extension of the XDS program (Kabsch, unpublished).

3. RESULTS AND DISCUSSION

3.1. *Purification of trypanothione reductase*

T. cruzi trypanothione reductase was purified from recombinant *E. coli* cells by affinity chromatography on 2'5'ADP-Sepharose. Elution by NADPH instead of NADP [6] yields pure trypanothione reductase in a single chromatographic step. Routinely 5 to 7 mg enzyme were purified from a 2-liter culture. Starting out with a 30-liter fermenter culture, the same protocol was followed but the dialyzed extract was chromatographed on 2'5'ADP-Sepharose in several portions.

3.2. *Crystallization of the enzyme*

The crystallization behaviour of TR is influenced by the preparation procedure. The enzyme purified as described above crystallizes in the presence of 20 mM but not of 100 mM Tris. In contrast, 100 mM Tris was successfully used as a buffer to crystallize the enzyme prepared according to [6]. Trypanothione reductase isolated from *Trypanosoma cruzi* epimastigotes yielded microcrystals using $(NH_4)_2SO_4$ as precipitant [5] whereas the recombinant enzyme crystallizes from citrate (sodium, potassium, ammonium and lithium salts) and from polyethylene glycol under hanging and sitting drop conditions. Crystals suited for X-ray diffraction analysis were obtained under two different conditions. Monoclinic rods grew from 1.2 M sodium citrate, 20 mM Tris, 1 mM EDTA, pH 8.0, 2% octanoyl-*N*-methylglucamide (Fig. 1). Tetragonal crystals of 200 μm × 200 μm × 800 μm were obtained from 30% PEG-8000, 100 mM Tris, 1 mM EDTA, pH 8.0, 0.2% β-*n*-octylglucoside.

Surprisingly, the *T. cruzi* enzyme crystallized so far only in the presence of non-ionic detergents. *T. congolense* TR formed crystals also in the absence of detergents but addition of 0.1% β-*n*-octylglucoside dramatically improved the crystals in size and shape (Krauth-Siegel, unpublished).

3.3. *Crystallographic analysis*

The crystals grown from polyethylene glycol were found to be tetragonal with unit cell dimensions $a=b=87.4$ Å and $c=152.3$ Å, by precession photographs.

The autoindexing using the XDS program indicated that this crystal belonged to the primitive tetragonal lattice system, and systematic absences of reflection intensities along the *c*-axis allowed only $P4_222$ or $P4_2$ as possible space groups. Assuming 1 monomer or 1 dimer or trypanothione reductase per asymmetric unit for $P4_222$ and $P4_2$, respectively, the specific volume V_m is 2.9 $Å^3$/Da. This corresponds to a crystal solvent content of about 57%, which is close to the average value for crystals of a 100 kDa protein. The *R*-factor for the symmetry-related reflection intensities for both space groups were comparable, the merging *R*-factors being 13.2%

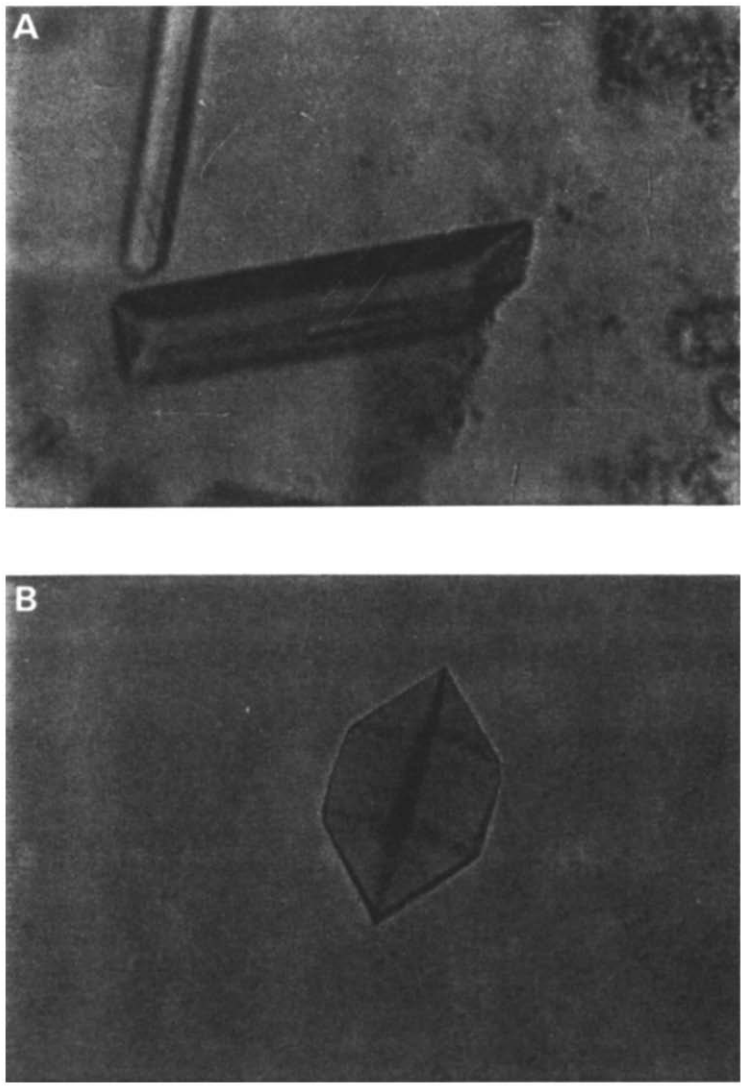

Fig. 1. Crystals of *T. cruzi* trypanothione reductase. (A) Monoclinic yellow crystals (200 μm × 200 μm × 1,000 μm) raised in the presence of sodium citrate. (B) Diamond-shaped yellow crystals (100 μm × 100 μm × 150 μm) were grown using polyethylene glycol as a precipitant.

for $P4_222$ (for 32,740 unique reflections) and 9.8% for $P4_2$ (for 22,024 unique reflections), respectively.

The monoclinic crystals obtained from sodium citrate were found to have space group symmetry $P2_1$ with cell dimensions of a=136.3 Å, b=91.1 Å, c=126.0 Å and β=94°. The crystals diffract to beyond 3 Å resolution and are stable in the X-ray beam for 5–6 days. In a total of 81,913 observations 45,259 unique reflections were recorded. The merging R-factor for the symmetry-related reflection intensities was 12.9%. Assuming two dimers of trypanothione reductase per asymmetric unit, the specific volume V_m is 3.9 Å^3/Da. Four dimers in the asymmetric unit would result in a calculated V_m of 1.95 Å^3/Da. These values are at the extremes of the range normally observed for protein crystals [25]. Self-rotation and cross-rotation function calculations are consistent with two dimers in the asymmetric unit. Work is in progress to solve this structure by molecular replacement using the data of the *Crithidia fasciculata* enzyme which were kindly made available to us by Dr. J. Kuriyan [18].

For functional and pharmacological purposes the sodium citrate-Tris system appears to be most promising. Kinetic studies in the presence of 1.2 M sodium citrate, 20 mM Tris, 1 mM EDTA, pH 8.0 yielded a K_m value for trypanothione disulfide of 60 μM which can be regarded as normal; the specific TR activity in this system was >25% when compared with standard assay conditions [12]. In contrast, when e.g. 1 M ammonium sulfate is added to a TR assay containing 100 mM HEPES, 1 mM EDTA, pH 7.0, the activity decreases by more than 95%.

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REFERENCES

[1] Meister, A., in: Glutathione, Chemical, Biochemical, and Medical Aspects, Part A (D. Dolphin, R. Poulson and O. Avramovic, Eds.), Wiley, New York, 1989, pp. 1–48.

[2] Schirmer, R.H. and Schulz, G.E., (1987) in: Pyridine Nucleotide Coenzymes, Part B, Coenzymes and Cofactors, vol. 2 (D. Dolphin, R. Poulson and O. Avramovic, Eds.), Wiley, New York, 1987, pp. 333–379.

[3] Fairlamb, A.H., Blackburn, P., Ulrich, P., Chait, B.T. and Cerami, A. (1985) Science 227, 1485–1487.

[4] Shames, S.L., Fairlamb, A.H., Cerami, A. and Walsh, C.T. (1986) Biochemistry 25, 3519–3526.

[5] Krauth-Siegel, R.L., Enders, B., Henderson, G.B., Fairlamb, A.H. and Schirmer, R.H. (1987) Eur. J. Biochem. 164, 123–128.

[6] Sullivan, F.X., Shames, S.L. and Walsh, C.T. (1989) Biochemistry 28, 4986–4992.

[7] Sullivan, F.X. and Walsh, C.T. (1991) Mol. Biochem. Parasitol. 44, 145–147.

[8] Fairlamb, A.H. (1989) Parasitology 99, S93–112.

[9] Docampo, R. and Stoppani, A.O.M. (1979) Arch. Biochem. Biophys. 197, 317–321.

[10] Schirmer, R.H., Krauth-Siegel, R.L. and Schulz, G.E., in: Glutathione, Chemical, Biochemical and Medical Aspects, Part A (D. Dolphin, R. Poulson and O. Avramovic, Eds.), Wiley, New York, 1989, pp. 553–596.

[11] Williams, C.H., Jr., in: Chemistry and Biochemistry of Flavoenzymes, vol. 3 (F. Müller, Ed.), CRC Press, Boca Raton, 1992, pp. 121–211.

[12] Jockers-Scherübl, M.C., Schirmer, R.H. and Krauth-Siegel, R.L. (1989) Eur. J. Biochem. 180, 267–272.

[13] Krauth-Siegel, R.L., Lohrer, H., Bücheler, U.S. and Schirmer, R.H., in: Biochemical Protozoology (G. Coombs and M. North, Eds.), Taylor and Francis, London, 1991, pp. 493–505.

[14] Schulz, G.E., Schirmer, R.H., Sachsenheimer, W. and Pai, E.F. (1978) Nature 273, 120–124.

[15] Karplus, P.A. and Schulz, G.E. (1987) J. Mol. Biol. 195, 701–729.

[16] Pai, E.F. and Schulz, G.E. (1983) J. Biol. Chem. 258, 1752–1757.

[17] Karplus, P.A. and Schulz, G.E. (1989) J. Mol. Biol. 210, 163–180.

[18] Kuriyan, J., Kong, X.P., Krishna, T.S.R., Sweet, R.M., Murgolo, N.J., Field, H., Cerami, A. and Henderson, G.B. (1991) Proc. Natl. Acad. Sci. USA 88, 8764–8768.

[19] Hunter, W.N., Bailey, S., Habash, J., Harrop, S.J., Helliwell, J.R., Aboagye-Kwarteng, T., Smith, K. and Fairlamb, A.H. (1992) J. Mol. Biol. 227, 322–333.

[20] Greer, S. and Perham, R.N. (1986) Biochemistry 25, 2736–2742.

[21] Leberman, R., Antonsson, B., Giovanelli, R., Guariguata, R., Schumann, R. and Wittinghofer, A. (1980) Anal. Biochem. 104, 29–36.

[22] Blum, M., Metcalf, P. and Wiley, D.C. (1987) J. Appl. Crystallogr. 20, 235–247.

[23] Kabsch, W. (1988) J. Appl. Crystallogr. 21, 67–71.

[24] Kabsch, W. (1988) J. Appl. Crystallogr. 21, 916–924.

[25] Matthews, B.W. (1968) J. Mol. Biol. 33, 491–497.