DESIGN OF ANTIPARASITIC DRUGS

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A Comparison of Two Independently Determined Structures of Trypanothione Reductase from *Crithidia fasciculata*

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Abstract

The enzyme trypanothione reductase (TR) is unique to trypanosomes and leishmania parasites, the causal agents of several important medical and veterinary tropical diseases. TR helps regulate the intracellular reducing environment of the parasite and it has been identified as a target for developing novel chemotherapeutic agents by structure-aided drug design. For this purpose it is essential to have confidence in the structural detail of the molecular target. Two independent studies of *Crithidia fasciculata* TR at medium resolution, in different space groups have afforded an opportunity to assess the reliability of the models. We summarize the important methodological details of each analysis and present a comparison of the geometry, thermal parameters and three-dimensional structure of the models. Particular attention has been paid to the disulfide substrate-binding site which is the area of most interest with respect to enzyme inhibition. The comparison has shown that the structures agree closely with Cα atoms superposing with an r.m.s. of less than 0.5 Å. The consistency of the models gives a high level of confidence that they are suitable for computer-aided drug design. The conformation of many side chains in the active site, in particular the catalytic residues, are well conserved in both structures. However, the comparison indicates a difference in the conformation of Trp21 and Met113 which together form a hydrophobic patch on the rim of the active-site cleft and interact with the spermidine moiety of the substrate. Consideration of the electron-density maps together with the structural comparison indicates that there is some conformational flexibility in this region of the active site. This heterogeneity may be used in the recognition of the substrate by the enzyme and should be considered when mapping out the size, shape and chemical properties of the active site.

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1. Introduction

Parasitic flagellated protozoa of the order Kinetoplastida, suborder Trypanosomatina which includes the genera *Leishmania* and *Trypanosoma*, are responsible for a range of diseases in tropical and subtropical regions of the world (World Health Organization, 1991). Infection with *T. brucei* spp causes African sleeping sickness in humans and nagana in cattle. In South and Central America an estimated 16 to 18 million people are infected with *T. cruzi* resulting in Chagas' disease. New cases of this American trypanosomiasis are appearing in the United States of America *via* blood transfusions from infected blood supplies (Kirchhoff, 1993). More widespread, and affecting an even greater number of people, are infections from *Leishmania* species which occur in the Americas, Africa and extending from the Middle East through to Asia.

Treatments for these diseases are unsatisfactory. They often involve the use of highly toxic drugs to which some of the parasites have developed resistance in any case. Advances in the molecular sciences are having a significant influence in many areas of medicine through rational drug design or drug identification (Hol, 1986; Verlinde & Hol, 1994). The molecular biology and biochemistry of trypanosomes are being studied (Fairlamb, 1989) and suitable targets for chemotherapy being identified. One such target involves trypanothione [N^1, N^8-bis(glutathionyl)spermidine] metabolism and the enzyme trypanothione reductase (TR). TR and its

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substrate are unique to trypanosomes and they perform redox regulation in an analogous fashion to the operation of glutathione and glutathione reductase (GR) in mammals (Fairlamb, Blackburn, Ulrich, Chait & Cerami, 1985; Fairlamb & Cerami, 1992). TR (Fig. 1) is a homodimeric enzyme with a subunit molecular mass of about 52 kDa and catalyzes the transfer of electrons to oxidized trypanothione *via* a prosthetic FAD group and redox-active cysteine disulfide (Shames, Fairlamb, Cerami & Walsh, 1986; Ghisla & Massey, 1989). The structure contains about 30% α-helix, 30% β-sheet and 40% loops, turns and extended structure. Each subunit consists of four domains: the FAD-binding domain, the NADPH-binding domain, the interface domain and the central domain. The disulfide-binding site is seen as a cleft, of about 20 Å in width by 15 Å deep, at the subunit interface, and is composed of residues from central and FAD-binding domains of one subunit and the interface domain of the other. A full description is given by Bailey, Fairlamb & Hunter (1994).

The critical observation suggesting that TR is worthy of study as a target for chemotherapy is that it is specific for trypanothione disulfide and will not process glutathione disulfide, conversely human GR is specific for glutathione disulfide and will not process trypanothione disulfide (Shames *et al.*, 1986). Crystallographic analyses of TR and comparison with the high-resolution structure of human GR with which it shares about 30% sequence identity (Kuriyan *et al.*, 1991; Hunter *et al.*, 1992; Bailey, Smith, Fairlamb & Hunter, 1993) have helped to determine the structural basis for this discrimination. The differences between the enzyme substrates, in terms of size and chemical properties such as their overall charges (−2 for glutathione disulfide and +1 for trypanothione disulfide) have been shown to be important factors in the enzyme–substrate complementarity.

Much of the biochemical characterization of TR has been carried out on the enzyme isolated from the insect parasite *Crithidia fasciculata*. This trypanosomatid is non-pathogenic to humans and one of the easier to culture *in vitro*. This TR shares approximately 70% sequence homology with the enzymes from pathogenic species. Key residues are conserved (Hunter *et al.*, 1992). The TR from *C. fasciculata* has been studied independently in two crystal forms. A monoclinic form at Rockefeller University, NY (Kuriyan *et al.*, 1990, 1991), Protein Data Bank (PDB) accession code 2TPR (Bernstein *et al.*, 1977) and a tetragonal form at the University of Manchester, England (Bailey *et al.*, 1994, PDB accession code 1TYT).

The availability of two crystal structures offers the opportunity for a critical comparison to assess the reliability of the models. We assume that consistency can be taken to indicate correctness. The experimental details are given and compared for each analysis, the models are assessed in terms of agreement with known stereochemical parameters and fit to the electron-density maps and overlapped on each other to obtain root-mean-square (r.m.s.) deviations. The disulfide substrate-binding site represents the most important part of the molecule from the point of view of structure-aided drug design and we pay particular attention to this part of the molecule. The TR monoclinic structure will be referred to as TRM and the tetragonal structure, TRT. The TRT

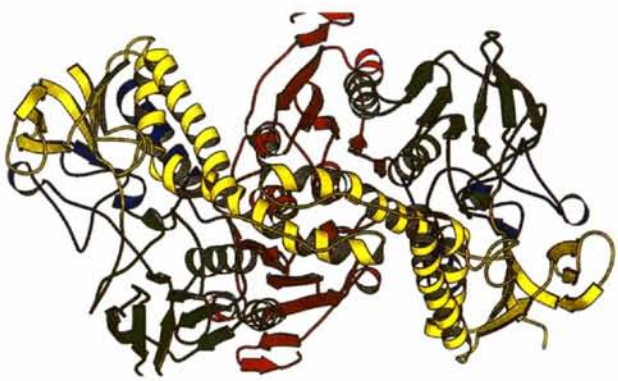
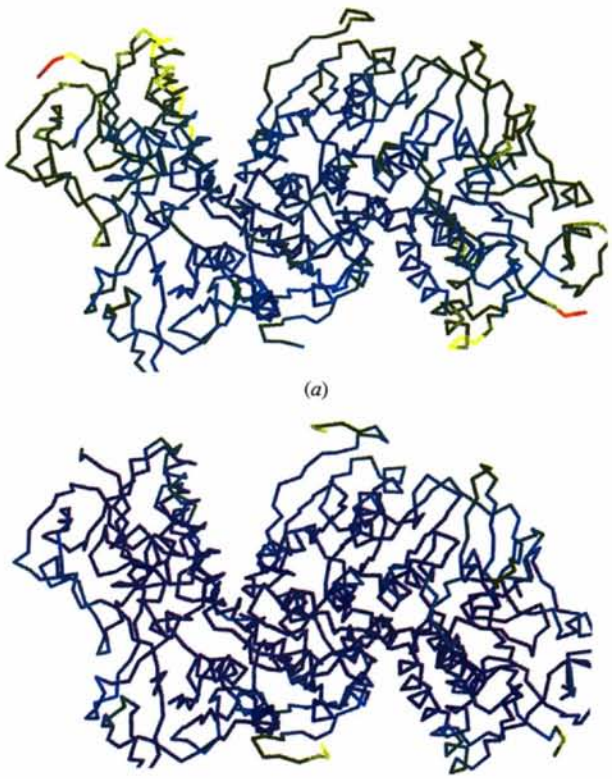


Fig. 1. Ribbon diagram of the tetragonal model of trypanothione reductase from *Crithidia fasciculata*. (FAD-binding domain, yellow; NADPH-binding domain, green; interface domain, red; central domain, blue). Figs. 1, 4 and 6 were obtained using *MOLSCRIPT* (Kraulis, 1991).



(*a*)

(*b*)

Fig. 2. Cα traces of both structures coloured according to the real-space fit to electron density (red = 0.60, blue = 0.95). (*a*) TRM (*b*) TRT. Figs. 2, 3, 4, 6 and 8 were derived from *O* (Jones *et al.*, 1991).

Table 1. *A comparison of experimental procedures used in two structure determinations of trypanothione reductase from Crithidia fasciculata*

	Tetragonal	Monoclinic
Data collection		
Source	Sealed tube, Cu $K\alpha$ target Station 9.6, SRS, Daresbury	NSLS, Brookhaven
Wavelength (Å)	1.5418 and 0.895	1.1
Detectors	Xentronics area detector Photographic film and FAST area detector	FAST area detector
Temperature (K)	283	Not reported
Results		
Space group	$P4_1$	$P2_1$
Unit-cell dimensions (Å, °)	$a = 128.9$	$a = 60.0$
		$b = 161.8$
	$c = 92.3$	$c = 61.5$
		$\beta = 104.1$
Percentage solvent	65	54
Resolution range (Å)	8.0–2.6	10.0–2.4
Unique reflections	35000 $F > \sigma F$	33134 $F > 2\sigma F$
Completeness (%)	84.5	76
Structure solution and refinement		
Method	Molecular replacement	Molecular replacement
Model	Human GR	Human GR
Atoms used in calculations	Main chain, Cβ and FAD	All protein and FAD
Refinement protocol	Restrained least-squares Simulated annealing	Restrained least-squares Simulated annealing
Final R factor (%)	16.1	19.1 (6.0–2.4 Å)
R.m.s. deviations from ideality		
Bonds (Å)	0.017	0.011
Angles (°)	3.35	2.7

sequence starts with a methionine at the N terminus (Met1). This is not in the TRM model and hence there is a difference in residue numbering of 1 between the structures. (*e.g.* Ser2 in the TRT structure is equivalent to Ser1 in the TRM structure). The tetragonal numbering will be used throughout.

2. Comparison of experimental methods

2.1. *Crystallization*

The first point to address is why two different crystal forms occurred. *C. fasciculata* TR is present as isoforms (Kuriyan *et al.*, 1991; Field, Cerami & Henderson, 1992). A comparison of the amino-acid sequences of TRT and TRM shows three differences. These are Asp297(TRT) = Glu(TRM), Phe454(TRT) = Val(TRM) and Gln480(TRT) = Asp(TRM). These residues are not involved in contact with symmetry-related molecules. It seems likely that the slightly different crystallization conditions used in each study are responsible for the different crystal forms.

The crystallization conditions for TRM are 10 mg ml^{-1} enzyme solution in 10 m*M* Hepes buffer, 0.5 m*M* EDTA and 5%(*w*/*v*) sodium azide (pH 7.0), equilibrated by vapour diffusion with 22%(*w*/*v*) polyethylene glycol 8000, 0.1 *M* ammonium sulfate, pH 7.2 at room temperature. (Kuriyan *et al.*, 1990). For TRT: 18 mg ml^{-1} enzyme solution in 0.1 *M* phosphate buffer and 50%(*w*/*v*) saturated ammonium sulfate (pH 7.0), equilibrated by vapour diffusion with 80% saturated ammonium sulfate at 278 K. (Hunter *et al.*, 1990).

External loops in the FAD- and NADPH-binding domains are involved in forming the lattice contacts for both crystal forms, although the detail is different. Details of the crystal packing in TRT are presented by Bailey *et al.* (1994).

2.2. *Data collection, structure solution and refinement*

Table 1 presents a summary of the experimental procedures used in the structure determinations for each form. The differences in these procedures test to some extent how the two paths have converged to produce similar results.

The diffraction data quality is very similar for each form as judged by the resolution and number of reflections. Both structures were solved by molecular replacement (MR) using models based on the structure of human GR (Karplus & Schulz, 1987, 1989). For TRM the complete protein structure plus FAD provided the search model and the first model for refinement. For TRT only main-chain atoms and FAD were used. In the latter case this was a strategic decision to reduce model bias in the conformation of side chains. The TRT refinement

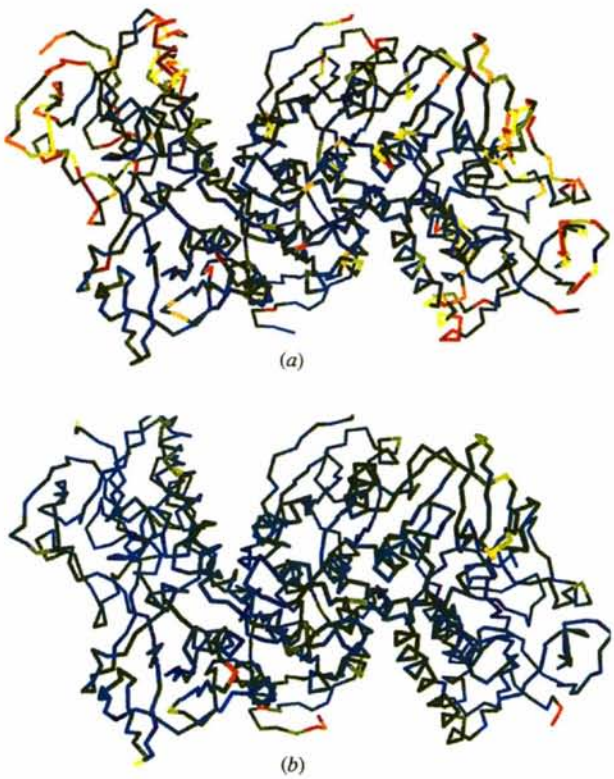


Fig. 3. Cα traces of both structures in a colour range according to the isotropic thermal parameters (blue, typically less than 30 Å^2; red, greater than 80 Å^2). (*a*) TRM (*b*) TRT.

was initiated with the *TNT* program (Tronrud, Ten Eyck & Matthews, 1987) then used *X-PLOR* (Brünger, 1990). The TRM structure was refined only with *X-PLOR*. Both refinements utilized restrained least-squares and simulated-annealing protocols during refinement. Non-crystallographic symmetry restraints were employed at an intermediate stage of the refinements and then removed towards the end of this part of the anlaysis.

3. Assessment of errors

3.1. *Coordinate error, real-space fit and thermal parameters*

In considering errors associated with each structure and for comparison purposes we have looked at the agreement of the models with the diffraction data and the thermal parameters.

The method of Luzzati (Luzzati, 1952; plots not shown) suggests internal estimates of the overall coordinate error for both structures to be between 0.2 and 0.3 Å. It is perhaps more informative to consider the real-space fit to electron density as calculated with *O* (Jones, Zou, Cowan & Kjeldgaard, 1991) in conjunction with the isotropic thermal parameters.

The real-space fit of each structure to its respective electron density is shown in Fig. 2. The option RS_FIT scores each residue as the fit of the atoms to the peaks in the electron density where the atoms have been assigned and scores them on a scale of −1 (bad) to 1 (good), giving a useful guide to the success or otherwise of map interpretation. The most satisfactory regions are in the bulk of the protein. However, the fit of the TRM structure (Fig. 2*a*) as judged by the RS_FIT scores is, overall, not as good as the TRT structure (Fig. 2*b*). The values of 0.60 and 0.95 for the RS_FIT represent the upper and lower bounds of fit calculated for all residues in both structures. A contribution to the variance in scale of RS_FIT must reside in the thermal parameters associated with each structure.

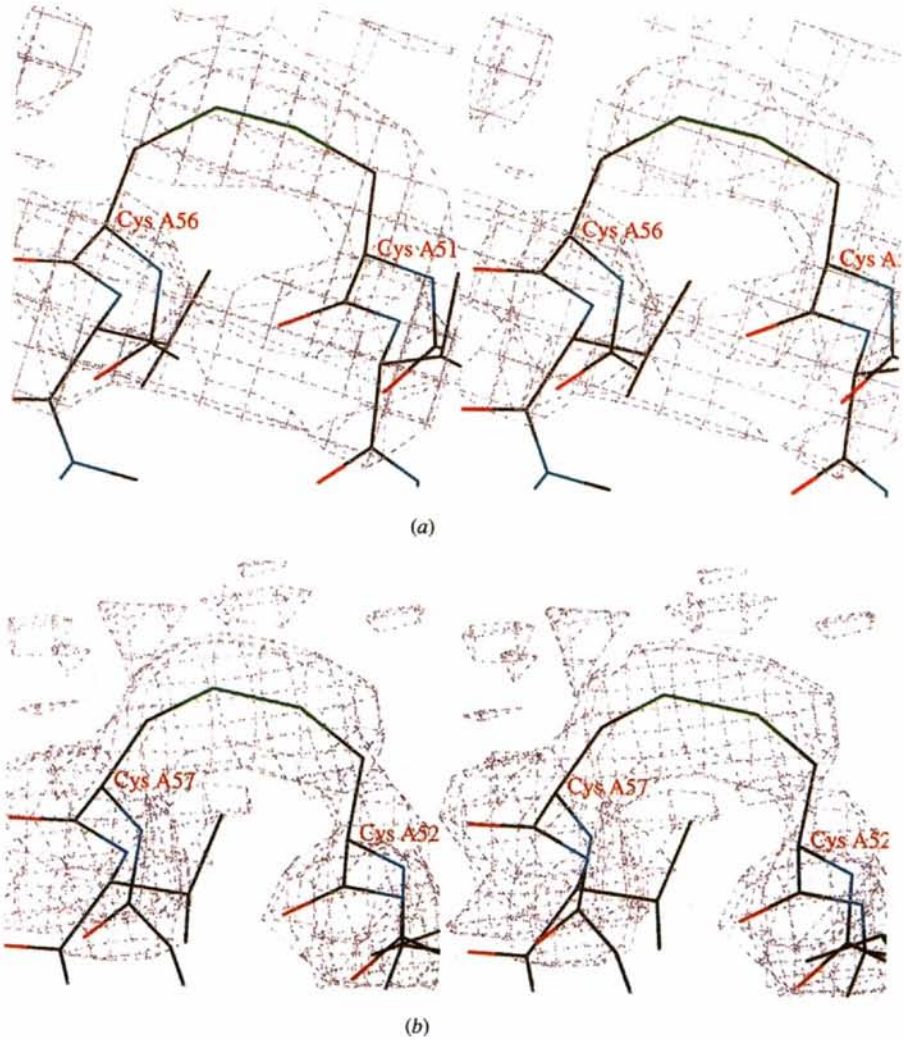


Fig. 4. A stereoview of the active-site disulfide from subunit *A* superimposed with the electron density ($2F_o - F_c$; thistle-coloured chicken wire) at the 1.0σ level. (*a*) TRM (*b*) TRT.

Inspection of the isotropic thermal parameters of the two structures shows that the TRM structure is scaled at two or three times greater than the TRT structure. Contributions to this difference may be due to the temperature at which data was collected, scaling or a refinement artefact, or it may just be a property of each crystal form. What is significant is that the trends in thermal parameters are consistent between the two structures (Fig. 3). 'Cold' regions (where atoms have lower thermal parameters) are found within the bulk of the enzyme, especially at the bottom of the disulfide substrate-binding site cleft. As detailed by Bailey *et al.* (1994) this indicates that this area is consistently rigid. 'Hot' sections, with the higher thermal parameters are located in surface loops, C- or N-terminal residues and at the rim of the active-site cleft. As expected the correlation between the real-space fit and the thermal parameters is good.

3.2. *Stereochemical quality*

The stereochemical quality of a structure is a useful guide to its reliability. The program *PROCHECK* (Morris, MacArthur, Hutchinson & Thornton, 1991; Laskowski, MacArthur, Moss & Thornton, 1993) is particularly useful for checking the geometry of protein structures. The strict nature of any restraints in the refinement procedure must, however, be taken into account. A comparison of stereochemical details of two similar structures also offers information on the correctness of any statistical outliers.

Ramachandran plots (Ramachandran & Sasisekharan, 1968; not shown) for each model are satisfactory for an enzyme structure at medium resolution. The TRT structure contains 88.4% of residues in the most favoured regions, and 11.2% in additionally allowed regions while the TRM structure has 89.2% in most allowed regions and 9.4% additionally allowed. An important point to note is that Tyr45 is in a disallowed region in both structures. This strain is explained by specific hydrogen bonds in a tight loop (Bailey *et al.*, 1994). Tyr45 is at the end of a nine-residue insert compared to the starting model of human GR. In each analysis this section was newly built onto the model and it is encouraging that each structure presents the same feature.

Analysis of the side-chain torsional (χ_1 and χ_2) angles shows that the TRT structure has 10.3% of residues greater than 2.5 standard deviations from the ideal, the TRM structure having 5.8%. These residues are almost exclusively those with long side chains (Lys, Arg, Asn, *etc.*). However, Cys57 of the active-site disulfide of both structures, is also shown to be over 2.5 standard deviations from the ideal. This cysteine forms a charge-transfer complex with the isoalloxazine of the FAD in the two-electron reduced form of the enzyme. As previously observed in the analogous enzyme GR, the disulfide bond between Cys52 and Cys57 is left-handed (Fig. 4; Karplus & Schulz, 1989; Thornton, 1981). It has been postulated by Karplus & Schulz (1989) that this strained conformation exists such that during catalysis, when the disulfide is broken in reaction with substrate, it forms a more relaxed disulfide enzyme–substrate complex. The study by Bailey *et al.* (1993) on the tetragonal form of *C. fasciculata* TR in complex with one of the physiological substrates, N^1-glutathionylspermidine disulfide is in agreement with this idea. Confidence in the strained conformation of this mechanistically active residue is enhanced again by its observation in each structure, and in each structure the fit to the electron density is good. The sequence alignment of 23 members of the flavoprotein-disulfide oxidoreductase class of enzymes reveals that Gly56 and Pro59 are invariant (Aboagye-Kwarteng, Smith & Fairlamb, 1992). It has been suggested that these residues cause a local distortion in a stretch of helix carrying the catalytically active

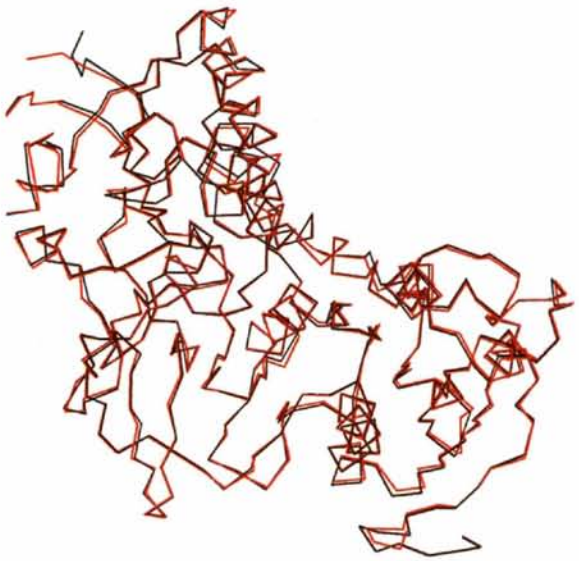


Fig. 5. Subunit *A* of TRM (in red) superimposed on subunit *A* of the TRT structure (black).

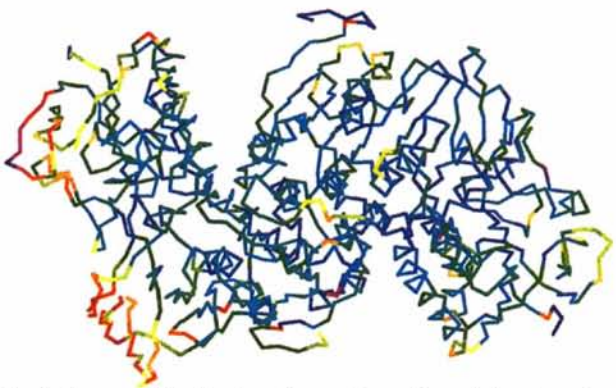


Fig. 6. Cα trace of TRT coloured according to the deviation from the TRM structure. Blue represents an r.m.s. typically less than 0.1 Å, red greater than 0.3 Å.

disulfide thus presenting the correct conformation for catalysis (Mattevi, Schierbeek & Hol, 1991).

4. Structural comparison

4.1. *Overall and subunit structure*

In Table 2 we present r.m.s. deviations determined by a least-squares superposition of the two structures. These values are derived from comparisons of subunit atoms. So TRT subunits *A* and *B* are superimposed on each other and then individually on TRM subunits *A* and *B*. The r.m.s. deviations give an indication of the compound effect of random errors and of genuine conformational differences. Cα atoms superpose in the range 0.29–0.44 Å. The Cα deviations are of the order of the expected internal estimates of errors in the structures based on the Luzzati statistics. Deviations between superimposed subunits from the same structure are not appreciably different from those from different struc-

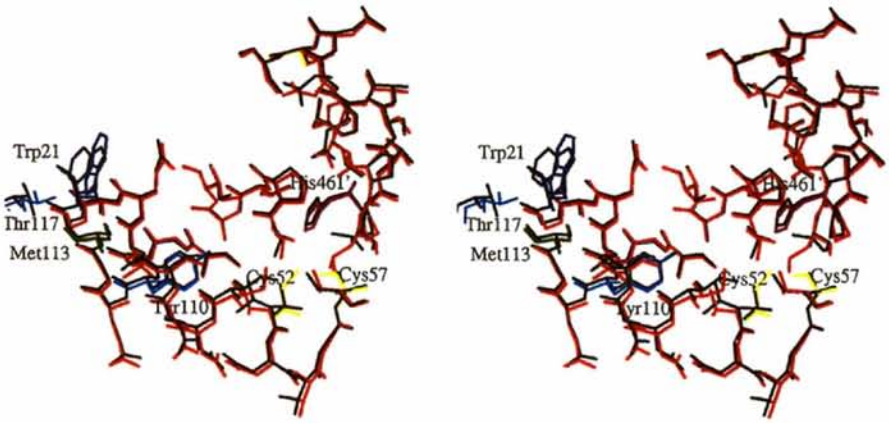


Fig. 7. A stereoview into the active-site clefts of the two trypanothione reductase structures superimposed. TRM atoms are drawn in black, TRT in red with selected residues coloured. Cys52 and 57 are yellow, Tyr110 and Thr117, cyan; His461, purple, Met113, green; Trp21, blue.

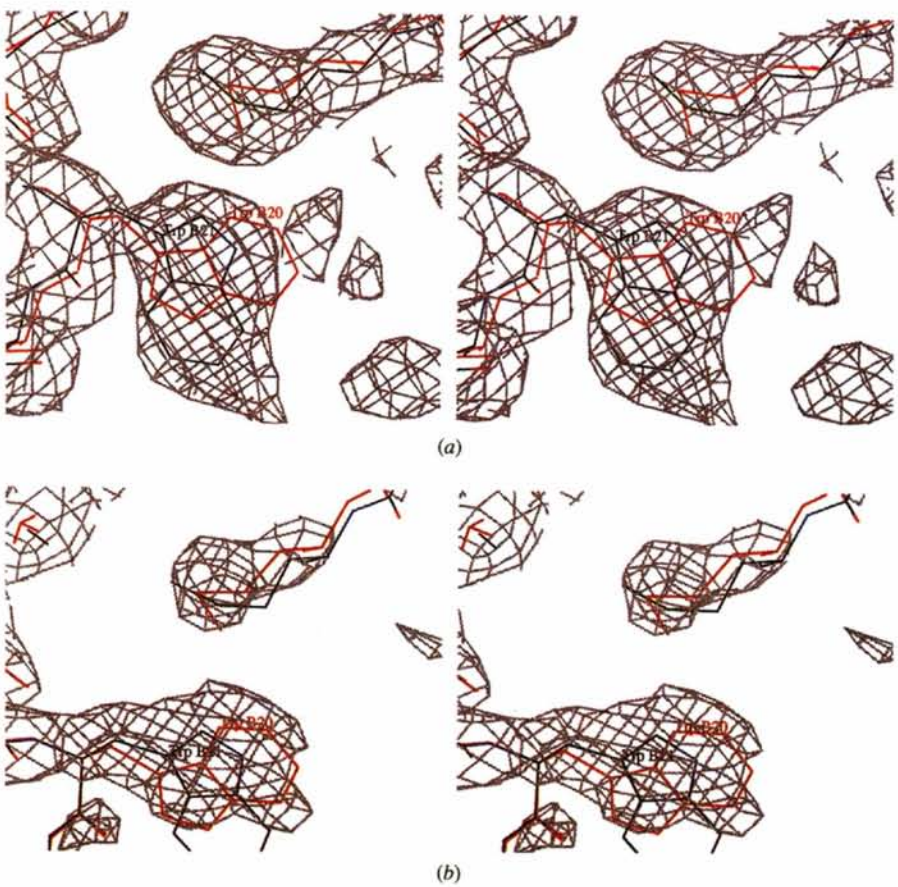


Fig. 8. Trp21 and Met113 of both the TRM and TRT structures superimposed on the electron density (1.2σ) for (*a*) TRM (*b*) TRT. The TRM coordinates are red; TRT, black and multicoloured.

Table 2. *Root-mean-square deviations* (Å) *obtained from a series of least-squares superpositions using sets of atoms from the subunits of trypanothione reductase TRT and TRM structures*

	Atom set used	Tetragonal *A*	Tetragonal *B*	Monoclinic *A*
Tetragonal *B*	All	0.706	—	—
	Cα	0.328	—	—
	Side-chain	1.069	—	—
Monoclinic *A*	All	1.024	1.025	—
	Cα	0.375	0.296	—
	Side-chain	1.498	1.518	—
Monoclinic *B*	All	1.036	1.017	0.948
	Cα	0.415	0.442	0.415
	Side-chain	1.505	1.465	1.376

tures. For the side-chain atoms common to TRM and TRT and for side chains of just TRM subunits the r.m.s. deviations are about 1.5 Å. However, comparing side-chain atoms for TRT subunits the value is significantly lower, 1.0 Å, a value more in line with previous comparative studies of independently refined protein structures (Wlodawer, Borkakoti, Moss & Howlin, 1986). Visual checks identified numerous examples where side chains in TRM were positioned in different orientations in each subunit and where functional groups at the end of side chains were reversed *i.e.* histidines and glutamines. In some cases there were clear indications that some orientations were incorrect and that switching functional groups would result in the identification of sensible hydrogen-bonding patterns. These discrepancies force the r.m.s. deviations to a higher value than is real. During the refinement of TRT (Bailey *et al.*, 1994) particular attention was paid to hydrogen-bonding patterns in helping to position side-chain functional groups and in comparing and checking subunits. We consider that the all-atom r.m.s. deviation for TRT subunits is the more appropriate value.

Figs. 5 and 6 show an overlap of one subunit from each structure and the TRT structure Cα trace coloured on the basis of deviation from the TRM structure, respectively. The disulfide substrate-binding site cleft is especially consistent. The loop with the highest deviations (coloured red, located at the bottom left) corresponds to a region in which one subunit of the TRM structure was built with an eight-peptide poly-Ala chain as the electron density was diffuse.

Overlaps of the FAD molecules were also calculated. The r.m.s. range was 0.14–0.27 Å. There are no significant differences to discuss, the r.m.s. values being comparable to the error in coordinates.

4.2. *The disulfide substrate-binding site*

For the purposes of structure-based drug design, there must be high confidence in the quality of the active-site model. Care must be taken to ensure that the molecular target is well characterized and accurate. With respect to trypanothione reductase we have paid particular attention to comparing the active sites. As mentioned above, the r.m.s. deviation between the active sites of TRM and TRT are low with respect to the rest of the enzyme. Fig. 7 shows some of the residues important for catalysis (Cys52, Cys57, His461′). A high consistency is observed, including Cys57 which, as has already been discussed, has disfavoured χ_1 and χ_2 angles.

A notable difference is observed, however, between the residues Trp21 and Met113 which adopt different conformations in each structure. These two residues have been predicted to aid docking and binding of substrates by providing a hydrophobic patch which would interact with the mainly aliphatic spermidine moiety of the substrate trypanothione (Smith, Mills, Thornton & Fairlamb, 1991). These interactions have subsequently been observed in the crystal structure of TR complexed with N^1-glutathionylspermidine disulfide (Bailey *et al.*, 1993). These residues are far removed from crystal lattice contacts and, at first sight, it seems unlikely that they should have different conformations. A value judgement must be made on whether any one conformation is correct or if there is more than one conformation. This problem needs to be addressed since any alterations in the conformations of the active-site residues could influenze the size, shape and electrostatic properties of the active-site target. We went back to inspect the electron-density maps to investigate this further. Fig. 8 shows both models of this region superimposed on the corresponding electron-density maps. Since the dimer contains two such regions we inspected both, we also made used of omit maps during this appraisal of density. One example is given for each structure in Fig. 8. The side-chain torsional angles for the TRM structure are over 2 standard deviations from the ideal. The TRT structure has the Trp $N^{\varepsilon 1}$ in a position to form a hydrogen bond with the side-chain hydroxyl group of Thr117 (O^{γ}) on an adjacent helix. The distances are 3.18 and 3.17 Å for subunits *A* and *B*, respectively. The RSR_rotamer option in *O* (real-space refinement of each χ_1 rotamer against omit electron density, Jones & Liljas, 1984) offers the less common conformation of the TRT structue as a better alternative to the current model of the TRM structure in both subunits, suggesting that while a dual occupancy probably exists, a major conformation may be that of the TRT structure. A dual conformation of Met113 also seems likely from the density, and this may be correlated to the conformation of Trp21. Whilst the results of the structure analyses of TR's indicate that the substrate-binding cleft is a well defined rigid part of the molecule (Bailey *et al.*, 1994) Trp21 and Met113 are located out near the rim of the cleft in a segment of the molecule which, as has been discussed above and shown in Fig. 3, is reasonably mobile. We speculate that some flexible component of the disulfide-binding site may be necessary for the enzyme to bind the natural substrates. The rotation of side chains at the rim of the active site may assist by opening up the cleft giving the substrate

more ready access to slip down to a reactive part of the enzyme. The flexibility of the Trp/Met patch may be reduced once the substrate is in place.

5. Concluding remarks

Despite some differences in the experimental methods used, the overall conclusion from this comparison is that consistent structural results have been achieved. Ambiguous assignments of functional groups, different interpretation of similar density and bias inherited from initial models may all contribute to some of the differences observed in the models. The critical part of the enzyme with respect to drug targeting, namely the disulfide substrate-binding active site has been well characterized. Differences in this area are consistent with slight variance in interpretation of electron density. The comparison has highlighted the possibility of multiple conformations for Trp21 and Met113, important residues that interact directly with the natural substrates. The comparison has provided confidence that the TR model is of a reliability suitable for computer-assisted drug design. As Kuriyan *et al.* (1991) indicated, the comparison of different TR structures will provide a consensus template for use in drug design. This has now been achieved.

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