Glutathione Reductase Turned into Trypanothione Reductase: Structural Analysis of an Engineered Change in Substrate Specificity[†,‡]

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ABSTRACT: *Trypanosoma* and *Leishmania*, pathogens responsible for diseases such as African sleeping sickness, Chagas' heart disease, or Oriental sore, are two of the very few genera that do not use the ubiquitous glutathione/glutathione reductase system to keep a stable cellular redox balance. Instead, they rely on trypanothione and trypanothione reductase to protect them from oxidative stress. Trypanothione reductase (TR) and the corresponding host enzyme, human red blood cell glutathione reductase (GR), belong to the same flavoprotein family. Despite their closely related three-dimensional structures and although their natural substrates share the common structural glutathione core, the two enzymes are mutually exclusive with respect to their disulfide substrates. This makes the parasite enzyme a potential target for antitrypanosomal drug design. While a large body of structural data on GR complexes is available, information on TR−ligand interactions is very limited. When the two amino acid changes Ala34Glu and Arg37Trp are introduced into human GR, the resulting mutant enzyme (GRTR) prefers trypanothione 700-fold over its original substrate, effectively converting a GR into a TR [Bradley, M., Bücheler, U. S., & Walsh, C. T. (1991) *Biochemistry 30*, 6124−6127]. The crystal structure of GRTR has been determined at 2.3 Å resolution and refined to a crystallographic *R* factor of 20.9%. We have taken advantage of the ease with which ligand complexes can be produced in GR crystals, a property that extends to the isomorphous GRTR crystals, and have produced and analyzed crystals of GRTR complexes with glutathione, trypanothione, glutathionylspermidine and of a true catalytic intermediate, the mixed disulfide between trypanothione and the enzyme. The corresponding molecular structures have been characterized at resolutions between 2.3 and 2.8 Å with *R* factors ranging from 17.1 to 19.7%. The results indicate that the Ala34Glu mutation causes steric hindrance leading to a large displacement of the side chain of Arg347. This movement combined with the change in charge introduced by the mutations modifies the binding cavity, forcing glutathione to adopt a nonproductive binding mode and permitting trypanothione and to a certain degree also the weak substrate glutathionylspermidine to assume a productive mode.

Glutathione reductase (GR; E. C. 1.6.4.2.)[1] is a ubiquitous flavoprotein responsible for maintaining a high ratio of reduced to oxidized glutathione in the cells of most organisms. Its function is important for protection against oxidative stress and in the production of deoxyribonucleotides (Meister & Anderson; 1983; Schirmer, et al., 1989). Parasitic *Trypanosoma* and *Leishmania*, pathogens responsible for diseases such as African sleeping sickness, Chagas' heart disease, or Oriental sore (Markell & Voge, 1981), are two of the very few genera that do not rely on the glutathione/glutathione reductase system to keep a stable redox balance. Instead, they utilize trypanothione, a covalent conjugate of glutathione and spermidine (^1N,^8N-bis(glutathionyl)spermidine; TS_2; Figure 1), and trypanothione reductase (TR) to maintain a reducing cellular environment (Fairlamb et al., 1985; Fairlamb & Cerami, 1985; Shames et al., 1986; Krauth-Siegel et al., 1987). TR is absolutely essential for these parasites as they are extremely sensitive to oxidative stress, and TR is the only enzyme providing reducing equivalents (Fairlamb & Cerami, 1992). GR and TR are both members of the large and well-characterized protein family of FAD-dependent NAD(P)H oxidoreductases [for overviews, see Pai (1991), Petsko (1991), and Williams

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[1] Abbreviations: GR, glutathione reductase; GRTR, double mutant (A34E/R37W) of human glutathione reductase; GSSG, glutathione disulfide; TS_2, trypanothione disulfide; Gsp_{ox}, glutathionyl spermidine disulfide; rms, root mean square; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid.

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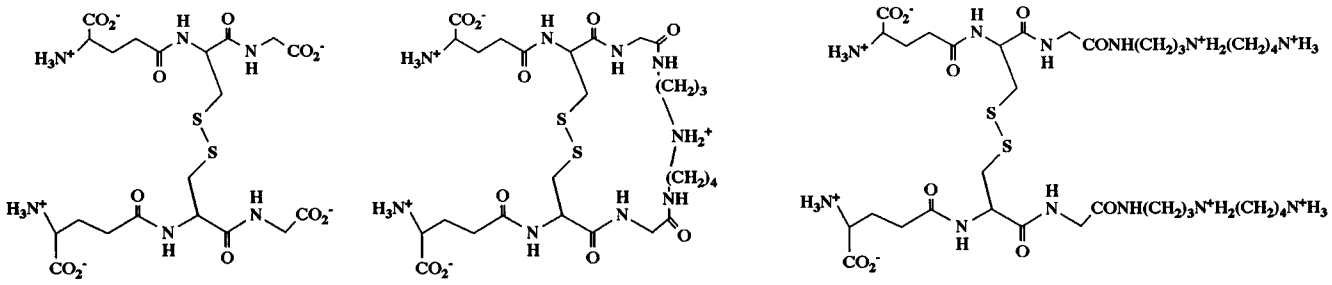

FIGURE 1: Chemical structures of glutathione disulfide (GSSG), trypanothione disulfide (TS_2), and glutathionylspermidine disulfide (Gsp_{ox}). All three of these compounds share the same molecular structure at the γ-glutamic acid ends. They differ, however, at the glycine ends of the common glutathione core. Here, Gsp_{ox} has spermidine chains attached to each of the two glycines while in TS_2 one spermidine links the ends. Note that this spermidine bridge makes even reduced trypanothione still unimolecular.

(1992)] and share close structural and mechanistic similarities, e.g., a 41% sequence identity between *Trypanosoma congolense* TR and human GR. Nevertheless, TRs and the host enzyme, human red blood cell GR, are mutually exclusive with respect to their disulfide substrates (Shames et al., 1986; 1988). Human GR, for instance, has a 9000-fold preference for glutathione over trypanothione based on V_{max}/K_m values (Bradley et al., 1991).

Both GR and TR are dimeric molecules whose subunits fold into four domains, the FAD-binding, the NADPH-binding, the central, and the interface domains. Their NADPH-binding sites and the mechanisms underlying their reductive half-reactions are very similar (Pai & Schulz, 1983; Pai et al., 1988; Lantwin et al., 1994). The nucleotides are separated from the disulfide substrates through the isoalloxazine ring of FAD, a geometry characteristic of all FAD-dependent NAD(P)H oxidoreductases (Pai, 1991; Petsko, 1991; Williams, 1992). This paper focusses on the binding sites of the respective disulfide substrates, which are formed by residues of the FAD, NADPH, and central domain of one monomer and the interface domain of the other monomer. Both enzymes posses a redox-active disulfide, which is involved in the formation of an enzyme−substrate mixed disulfide and the chemical mechanisms of their respective oxidative half-reactions must be very similar (Pai & Schulz, 1983; Williams, 1992). Although the disulfide-binding site in TR in general rather closely resembles that for GSSG in GR, it is much wider in the outer region in TR than it is in GR due to different orientations of two helices in the FAD domains (Kuriyan et al., 1991). A detailed comparison of the disulfide parts of the two active sites of GR and TR has been presented by Lantwin et al. (1994).

The structures of GRs from *Escherichia coli* (Mittl & Schulz, 1994) and from human red blood cells have been determined (Schulz et al., 1978); that of the human enzyme has been solved to a resolution of 1.54 Å (Karplus & Schulz, 1987). Extensive analyses of its complexes with its natural substrate GSSG and with a variety of different glutathione analogues have been described (Karplus & Schulz, 1989; Janes & Schulz, 1990; Karplus et al., 1989). Crystal structures of TRs from *Crithidia fasciculata* (Kuriyan et al., 1991; Hunter et al., 1992) and from *Trypanosoma cruzi* have been solved (Lantwin et al., 1994). Until now, however, attempts to bind trypanothione to crystals of TR have been unsuccessful despite the efforts of a number of laboratories. Only the crystal structures of TR complexed with the alternative substrate glutathionyl spermidine (Bailey et al., 1993) and with the weak but selective inhibitor mepacrine (Jacoby et al., 1996) have been reported.

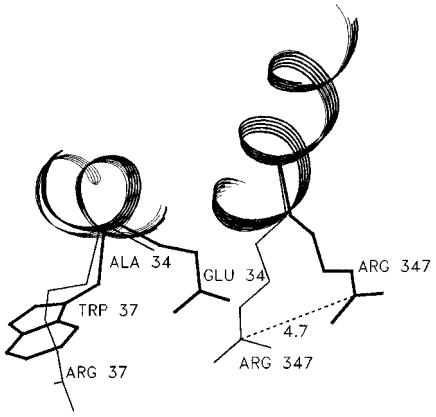

FIGURE 2: Comparison of GRTR (bold) and human GR in the region of the mutations. Note that the Ala34Glu mutation would put the head group of Glu34 only 1.7 Å from the head group of Arg347. This close contact causes a 4.7 Å shift of Arg347 away from the active site. Figure prepared using Setor (Evans, 1993).

On the basis of the high-resolution structures available for GR and its substrate complexes, active site mutations were constructed with the aim to change the specificity of GR from the natural substrate glutathione to trypanothione (Bradley et al., 1991; Henderson et al., 1991) or that of TR to glutathione (Sullivan et al., 1991). The double mutant Ala34Glu/Arg37Trp of human GR (GRTR) showed a ratio of k_{cat}/K_m of 9000 min^{-1} M^{-1} with glutathione as substrate, 14 000-fold lower than native human GR. The mutated protein preferred trypanothione 700-fold over glutathione as a disulfide substrate (Bradley et al., 1991). Here we describe the crystal structures of this double mutant of human GR together with its glutathione, trypanothione, and glutathionyl spermidine complexes. In addition, the mixed disulfide between trypanothione and GRTR, an intermediate of the new catalytic reaction, is also characterized. Figure 2 shows a close-up of the part of the active site that is effected by the changes. The importance of TR for survival of the pathogens and the uniqueness of its substrate make it a target for antitrypanosomal drug design. A better understanding of the structural basis for the respective substrate specificities of human GR and TR will be crucial in designing possible leads for inhibitors specifically targeting TR to fight the devastating diseases caused by these parasitic protozoa.

EXPERIMENTAL PROCEDURES

Materials

1-*n*-octyl-β-D-glucopyranoside and glutathione disulfide (GSSG) were purchased from Sigma, oxidized trypanothione

disulfide (TS_2) from Bachem (Switzerland). Originally, glutathionyl spermidine disulfide (Gsp_{ox}) was isolated from stationary phase *E. coli* cells according to Tabor and Tabor (1975) and later purchased from Bachem. All other materials and chemicals were of the highest grade commercially available.

Methods

Preparation of the Proteins. The Ala34Glu/Arg37Trp mutant of human glutathione reductase (GRTR) encoded on plasmid pUB302, recombinant human GR, and recombinant *T. cruzi* TR (Sullivan & Walsh, 1991) were all overexpressed in GR-deficient *E. coli* SG5 cells (Greer & Perham, 1986), according to Bücheler et al. (1990), and purified as previously described (Bradley et al., 1991). *E. coli* GR was kindly provided by Prof. Charles H. Williams, Jr. (Ann Arbor, MI).

Kinetic Measurements. The pH dependence of enzymatic reactions was measured between pH 5.0 and 8.0 using 100 mM sodium phosphate, 50 mM sodium citrate, and 1 mM EDTA as buffer and between pH 6.5 and 8.5 in 100 mM HEPES and 1 mM EDTA. In addition, both buffer systems contained 150 mM KCl in order to minimize the effects of ionic strength. Conductivity varied between 29 and 35 mS in the phosphate/citrate system and between 18 and 23 mS in the HEPES buffer. Kinetics of human GR, GRTR, and *E. coli* GR were measured in potassium phosphate buffer, pH 6.9, $I = 0.3$ μ, 200 mM KCl, and 1 mM EDTA. The standard assay contained 100 μM NADPH and 1 mM GSSG (Worthington & Rosemeyer, 1976). In the case of *E. coli* GR, the assays were initiated by the addition of enzyme because of the strong inhibition of the bacterial enzyme by NADPH (Arscott et al., 1989). In all other cases, assays were started by the addition of GSSG. The activity of *T. cruzi* TR was measured as described (Jockers-Scherübl et al., 1989). Due to the high cost and very limited supply of Gsp_{ox}, the kinetics of the mutant protein with this substrate were determined in a total volume of 90 μL using microcuvettes in a Beckman DU 65 spectrophotometer. All other kinetics were measured in 1 mL standard cuvettes in a Hitachi 150−20 spectrophotometer.

Crystallization. A solution of 10 mg/mL of GRTR was dialyzed extensively at 4 °C against 2 × 200 mL 100 mM potassium phosphate, pH 8.0, containing 0.01% sodium azide and approximately 2 mg of FAD. The third batch of dialysis buffer had ammonium sulfate added to a final concentration of 0.16 M, and dialysis was continued for another 24−48 h. The dialyzed solution was then used as the protein stock for growing crystals by the hanging-drop vapor diffusion method (McPherson, 1985). The reservoir contained 1 mL 0.57−0.90 M ammonium sulfate in 100 mM potassium phosphate, pH 8.0. Five microliters of the protein solution were mixed with 5 μL of the reservoir solution, and 1-*n*-octyl-β-D-glucopyranoside was added to a final concentration of 0.5% (w/v).

Preparation of Crystalline Enzyme−Substrate Complexes. To prepare crystalline enzyme−substrate complexes, crystals were soaked at room temperature in artificial mother liquor (1.23 M ammonium sulfate, 100 mM potassium phosphate) containing either a saturating concentration of glutathione disulfide, 80 mM trypanothione disulfide, or 42 mM glutathionyl spermidine disulfide titrated to the proper pH with KOH. All soaking experiments were performed both at pH 8.0 and near the pH optimum of the respective enzymatic reaction. The mixed disulfide between trypanothione and the enzyme was prepared in artificial mother liquor at pH 6.5 containing 100 mM trypanothione and 0.5% (w/v) β-mercaptoethanol. The latter crystal was mounted in a capillary filled with the soaking solution, held in place by cotton fibers, and allowed to stand for 1 h before starting data collection. The characteristic change of color from yellow to orange was monitored as an indication for the formation of the reaction intermediate.

Table 1: pH Optima for the Disulfide Reductase Reactions Catalyzed by Human GR, GRTR and *T. cruzi* TR[a]

disulfide substrate	human GR	GRTR (A34E, R37W)	*T. cruzi* TR
GSSG	7.0 (A) 6.8[b] 7.3[c]	5.6[c]	
TS_2	7.4[c]	6.5[c]	7.75[d] (A, B)
Gsp_{ox}		6.75[d] (A, B)	7.25−7.5[d] (A, B)

[a] The pH profiles were recorded in steps of 0.25 pH units in (A) 100 mM sodium phosphate, 50 mM sodium citrate, 150 mM KCl, and 1 mM EDTA between pH 5 and 8 and (B) 100 mM Hepes, 150 mM KCl, and 1 mM EDTA between pH 6.5 and 8.5. The NADPH concentration was 100 μM and disulfide substrates were 1 mM GSSG, 250 μM TS_2, and 100 μM Gsp_{ox}, respectively. [b] Worthington and Rosemeyer (1976). [c] Bradley et al. (1991). [d] The pH optima were relatively broad; at pH values 0.25 units away from the actual peak, the activity decreased by only 10% or less.

Collection of Diffraction Data and Structure Solution. Crystals were mounted in thin-walled glass capillaries (Supper, Natwick, MA). X-ray diffraction data were collected at 4 °C on an X1000 multiwire area detector (Siemens, Madison, WI) using Cu K_α radiation from rotating anode sources (GX-18, Elliot/Enraf-Nonius, Delft, NL; 0.1 × 1 mm^2 focal spot, 35 kV, 50 mA or RU200H, Rigaku, Japan; 0.2 × 2 mm^2 focal spot, 37 kV, 70 mA). Radiation was Ni-filtered and focused using Franks mirror optics. For data reduction, we used an updated version of the program package XDS (Kabsch, 1988a,b). Due to the high degree of isomorphism between the crystals of human GR and GRTR, the coordinate set of native human GR (3GRS) obtained from the Protein Data Bank was used without modification for the initial phasing of the diffraction pattern of the mutated protein.

Model Building and Refinement. The necessary limited rebuilding of the atomic model and the manual fitting of the ligands was done using the graphics programs TURBO (Jones, 1978) and O (Jones, 1991). The present models have been refined against all reflections with $I/\sigma I > 0.1$ of their respective data sets using X-PLOR standard protocols (Brünger et al., 1987) and the improved stereochemical parameter set (Engh & Huber, 1987). Given the limited resolution of our data, greater weight was put on the geometry, and water molecules were fitted only if they could make specific contacts with the substrates or with important residues in the active site and if their temperature factors were less than 35.0 Å^2 after refinement.

RESULTS

pH Optima of Human GR, GRTR, and TR for Their Disulfide Substrates. The pH optima of GRTR for the reduction of GSSG, Gsp_{ox}, and TS_2 were rather broad and generally lower than those shown by native human GR and by *T. cruzi* TR for their respective substrates (Table 1). GRTR's very low residual activity with glutathione disulfide

Table 2: Kinetic Constants for Human GR, GRTR, *E. coli* GR, and *T. cruzi* TR

	human GR	GRTR	*E. coli* GR	*T. cruzi* TR
GSSG				
K_m (mM)	0.065[a]	>50[b]	0.061[c]	ns
k_{cat} (min^{-1})	12 600		44 000	
k_{cat}/K_m (M^{-1} min^{-1})	1.9×10^8	9×10^3	7.2×10^8	
TS_2				
K_m (mM)	ns[e]	0.5	2[c]	0.018[d]
k_{cat} (min^{-1})		1250	6100	
k_{cat}/K_m (M^{-1} min^{-1})		2.5×10^6	3×10^6	2.8×10^8
Gsp_{ox}				
K_m (mM)	ns	>25	>25	0.022[d]
k_{cat} (min^{-1})		1335	500	
k_{cat}/K_m (M^{-1} min^{-1})		5.3×10^4	2×10^4	1.6×10^8

[a] Worthington and Rosemeyer (1976). [b] Bradley et al. (1991). [c] Henderson et al. (1991). [d] Jockers-Scherübl et al. (1989). [e] ns = the compound is not a substrate of the enzyme. Unless cited otherwise, the kinetic constants were determined in potassium phosphate buffer $I = 0.3$ μ, 200 mM KCl, 1 mM EDTA, pH 6.9 (Worthington & Rosemeyer, 1976).

(Bradley et al., 1991) and the remarkably low optimum pH of 5.6 for this reaction clearly demonstrate the consequences of the modification of charges introduced at the active site of the mutant protein. As expected, pH optima for the reduction of the glutathionyl spermidine conjugates did not change dramatically and are 1 pH unit higher than those with GSSG.

Kinetic Constants of Human GR, GRTR, E. coli GR, and T. cruzi TR for Disulfide Substrates. The kinetic constants of GRTR were determined in GR assay buffer (potassium phosphate, $I = 0.3$ μ, 200 mM KCl, 1 mM EDTA, pH 6.9). This buffer has been chosen since comparison of several HEPES and phosphate buffer systems indicated that the mutant protein retained the preference for phosphate buffer and the presence of a neutral salt shown by human GR (Worthington & Rosemeyer, 1976). Under these conditions, TS_2 is a 50-fold better substrate than Gsp_{ox}, with this preference being entirely due to better binding of TS_2. The turnover numbers with both glutathionyl spermidine conjugates are nearly identical (Table 2). The k_{cat}/K_m ratio of 2.5×10^6 M^{-1} min^{-1} for TS_2 agrees well with that reported by Bradley et al. (1991) (6×10^6 M^{-1} min^{-1}) for another buffer system at pH 6.5 that yielded a 4-fold lower K_M value for TS_2 and a concomitantly slightly lower k_{cat} value.

Crystallization. The mutant glutathione reductase crystallized isomorphously with human GR; its solubility, however, was drastically reduced. Therefore, GRTR was crystallized over reservoir solutions containing 18% saturated ammonium sulfate instead of 30% as in the case of human GR. Although GRTR did crystallize at the same pH as human GR (pH 7.0), the size of the crystals could be increased by changing to pH 8.0. Adding 0.5% (w/v) 1-*n*-octyl-β-D-glucopyranoside to the protein solution also improved crystal quality, resulting in larger and more compact crystals. At room temperature, crystals reached their full size (ca. $0.3 \times 0.2 \times 0.08$ mm^3) within 2 days. They belong to the monoclinic space group B2 (chosen to stay consistent with the original GR X-ray analysis; Schulz et al., 1975) with cell constants of $a = 120.3$ Å, $b = 85.2$ Å, $c = 63.6$ Å, $\gamma = 58.7°$ and contain one protein monomer per asymmetric unit.

Structure Solution and Refinement. As both human GR and GRTR crystallized in an identical fashion, the PDB entry 3GRS could be directly used for phasing the diffraction patterns obtained from liganded and unliganded GRTR crystals. The overall structure of the protein did not change at all. The electron density in the active site was consistent with the changes in amino acid side chains introduced by the mutations. Difference Fourier analyses confirmed the presence of the ligands, and the additional electron density was used to guide the construction of atomic models of the bound ligands.

Table 3: Summary of Soaking Conditions and X-ray Statistics[a]

soak	GRTR	GSSG	TS_2	TRMD	Gsp_{ox}
substrate (mM)	NA	420	80	100	42
soak time (h)	NA	24	55	1[b]	3
resolution (Å)	2.3	2.7	2.5	2.8	2.4
number of observered reflections	33 199	30 986	29 533	19 274	29 646
number of unique reflections	20 765	14 065	17 797	12 975	16 124
% completeness	85.4	91.7	92.1	86.6	84.8
R_{sym} %	11.0	11.7	6.9	10.5	5.6
R_{dif} %	NA	18.6	11.0	19.0	11.6

[a] Abbreviations are as follows: GSSG = glutathione disulfide; TS_2 = trypanothione disulfide; TRMD = trypanothione mixed disulfide; Gsp_{ox} = glutathionylspermidine disulfide. Unless specified otherwise, all soaking experiments were performed in 0.9 M ammonium sulfate, 0.2 M potassium phosphate, pH 6.0−6.5, at room temperature. $R_{sym} = \sum_{i,hkl}(|I(i,hkl) - \langle I(i,hkl)\rangle|/\sum_{i,hkl}\langle I(hkl)\rangle$, $R_{dif} = \sum|F_{soak} - F_{nat}|/\sum F_{nat}$. [b] The mixed disulfide data were collected from a crystal mounted in a sealed capillary filled with 200 mM KH_2PO_4, pH 6.0, 1 M ammonium sulfate, 100 mM trypanothione, and 1% β-mercaptoethanol. The crystal was allowed to sit in this solution for 1 h prior to data collection and remained in the solution for the duration of data collection.

A 0.1 σ cut-off was applied to all diffraction data sets. As expected given the close relationship with GR, the refinement proceeded very smoothly and, except for changing the side chains of the two mutated amino acids, only minor manual rebuilding was required. At present, the molecular structure of GRTR has been determined at 2.3 Å resolution and refined to a crystallographic *R* factor of 20.9%. The structures of its complexes vary between 2.3 and 2.8 Å resolution and their refinement resulted in *R* factors ranging from 17.1 to 19.7%. The rms deviations for bond lengths are between 0.010 and 0.018 Å, and the rms deviations for bond angles vary from 1.59 to 2.00°; all these values are well within accepted limits. A limited number of water molecules has been assigned only when they were thought to interact with ligands at the active site and if the corresponding *B* factors stayed lower than 35 $Å^2$. A summary of soaking conditions and data collection statistics for GRTR and its complexes is shown in Table 3; the corresponding refinement statistics are given in Table 4.

Structure of GRTR. The structures of native human glutathione reductase (human GR) and of the mutant glutathione reductase (GRTR) are isomorphous. In human GR crystals, residues 1−17 are disordered and therefore not visible in the electron density map (Schulz et al., 1978; Karplus & Schulz, 1987). In the maps of GRTR, Ala17 could be fitted into the electron density with confidence, the other 16 N-terminal residues, however, are still not resolved. The domain organization of GRTR is the same as the one found in GR, the FAD-binding, NADPH-binding, central, and interface domains (Schulz et al., 1978). The tertiary and quaternary structures of GRTR are identical to those of the parent protein human GR; this includes the presence of an intersubunit disulfide bridge between Cys90 and its symmetry mate in the second subunit (Pai & Schulz, 1983).

Table 4: Summary of Refinement Statistics[a]

soak	GRTR	GSSG	TS_2	TRMD	Gsp_{ox}
resolution range of refinement (Å)	10.00−2.3	10.00−2.7	10.00−2.5	10.00−2.8	10.00−2.3
number of reflections used in refinement[b]	17 144	12 177	15 872	10 245	16 124
R_{crys} %	20.9	18.9	17.1	17.1	19.7
rmsd bond lengths	0.014	0.013	0.014	0.012	0.012
rmsd bond angles	1.82	1.78	2.01	1.79	1.79

[a] Abbreviations are the same as in Table 3. $R_{crys} = \sum|F_{obs} - F_{calc}|/\sum F_{obs}$. [b] Only reflections with $I > 0.1\sigma$ I were used in the analysis.

The framework for electron transfer is unchanged, the binding sites for the substrates NADPH and disulfide are on opposite sides of the prosthetic group FAD. Side chains from both subunits contribute to the disulfide binding site. It is in this cleft, where the only three out of 478 residues are located which showed conformations significantly different from those observed for equivalent amino acids in GR (Karplus & Schulz, 1989). Two of these represent the sites of mutations (Ala34Glu, Arg37Trp); the third one is Arg347, whose guanidinium group is moved by approximately 5 Å away from its original position through steric interaction with the side chain of the newly introduced Glu34 (Figure 2). While the mobility of the mutated residues was about the same as that of the native residues, the *B* factors for Arg347 in GRTR were clearly higher than in GR (22.2 $Å^2$ *vs* 10.2 $Å^2$).

Formation of GRTR Complexes. For the GRTR complexes with GSSG and TS_2 as well as for the mixed disulfide between trypanothione and the enzyme, the quality of those parts of the electron density maps representing the ligands was improved when diffraction data were collected at pH 6.5 instead of pH 8.0. This seemed to be caused by an increase in occupancy of the ligands and by increased stability of the complexes at pH 6.5 for both the oxidized trypanothione soaks and the mixed disulfide. In contrast to this, the glutathionyl spermidine soak, although the least well-defined structure of all the soaks, gave slightly better results at pH 8.0.

The crystals soaked with glutathione disulfide were stable for at least several days, whereas crystals soaked with trypanothione disulfide at pH 8.0 lost their diffracting power after ca. 8 h. At pH 6.5, this effect was less dramatic, although data collected after a soaking time of 72 h were of poorer quality than those obtained after 48 h of soaking. Crystals did appear to be more stable in the presence of oxidized glutathionyl spermidine than they were in the presence of trypanothione. No clear correlation, however, between crystal stability and any single substrate parameter could be established.

The orange-colored, 2-electron-reduced form of GRTR (EH_2) can be produced by soaking the crystals in β-mercaptoethanol. In the absence of substrate, EH_2 is remarkably stable against reoxidation by air, again analogous to human GR (Pai & Schulz, 1983). When, however, GRTR was soaked with β-mercaptoethanol in the presence of reduced glutathione (GSH), bleaching of the crystals indicated that either the 4-electron reduced state of the enzyme (EH_4) was formed or the flavin was lost from the protein. The same result was obtained either with reduced glutathione alone or with a mixture of β-mercaptoethanol and GSSG. A summary of soaking and data collection statistics is shown in Table 3.

The most dramatic effect of variation of pH was seen on the formation of the mixed disulfide between trypanothione and the enzyme. Again, this species was produced in the crystals slightly modifying a method originally established for human GR and GSSG (Pai & Schulz, 1983). At pH 8.0, the presence of β-mercaptoethanol led to the reduction of the crystalline enzyme as indicated by the well-known yellow-orange color change. Crystallographic analysis later confirmed that the redox-active Cys58−Cys63 disulfide bridge had opened leading to a charge-transfer complex but that formation of a mixed disulfide had not taken place. At pH 6.5, however, close to the pH optimum for the enzymatic back reaction, the mixed disulfide was readily formed. It should be noted that in both cases the intersubunit disulfide bridge was still intact. These results mirror our experience with the crystal chemistry of GR (Pai & Schulz, 1983).

Structures of GRTR Complexes. The binding of disulfide substrates to GRTR will be described applying nomenclature designed to distinguish the two identical halves of the symmetric GSSG molecule, which become nonequivalent upon binding to the asymmetric active sites of human GR or GRTR. The Roman numeral I indicates the mixed-disulfide-forming half; the first leaving group is marked by II (Karplus et al., 1989). GR and GRTR are both catalytically active only as dimers. The active sites of the enzymes are built up by residues 30−37 (the region of GRTR mutations), residues 59−60, 110−117, and 339−347 of the subunit encompassing the corresponding redox-active disulfide and residues 406′ and 467′−476′ of the second, symmetry-related subunit.

GSSG. γ-Glu-II of GSSG is interacting with residues of the second subunit and, as has been found in human GR, is the most important anchor for the binding of all substrate molecules to GRTR (Figure 3A). While the two carboxyl groups at the ends of GS-II occupy their established positions, the entire GSSG molecule is rotated by 180° around an axis connecting these two groups. This leads to the expulsion of GS-I, the other half of GSSG, from the active site. This part of the molecule shows higher mobility and protrudes into solution (Figure 3A,B) but still makes contacts, albeit weak ones, to the carboxyl group of Gly-I and to the side chains of Arg109 and Lys414′. In addition to this dominant position, the electron density map of the GSSG complex contains very weak features that correspond to the conformation adopted by GSSG when bound to human GR.

TS_2. A view of oxidized trypanothione (TS_2) as it is bound in the active site of GRTR is shown in Figure 4, panels A and B. There is clear density accounting for the entire ligand except for 17C1′T and 17C2′T, the central carbon atoms of the spermidine chain. The overall topology of TS_2 is very similar to that of GSSG bound to human GR (Karplus et al., 1989). The two sulfur atoms of TS_2 are very close to the position the GSSG disulfide assumes in its complex with human GR. They fit nicely into stronger density with a bond length of 2.1 Å compared to 2.0 Å for the protein's redox-active disulfide. The proximal sulfur of TS_2, which will form

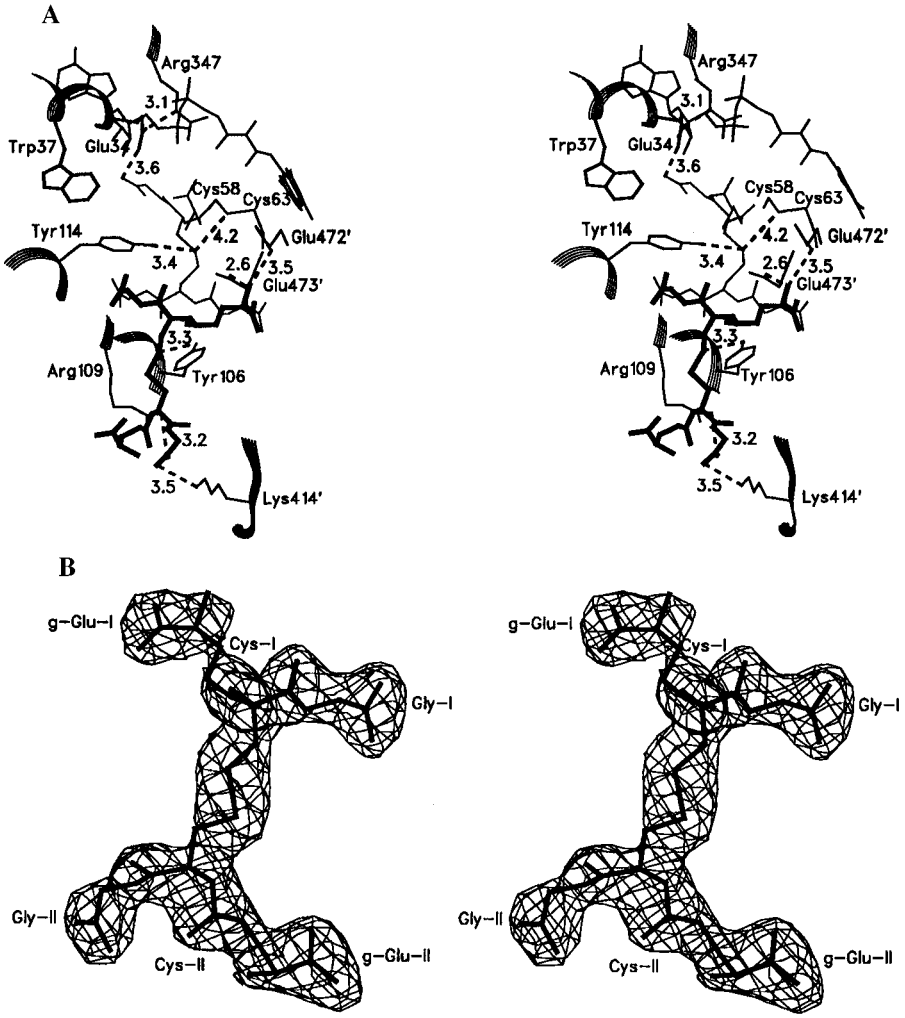

FIGURE 3: (A) Convergent stereo representation of GSSG bound to GRTR (bold) and GSSG bound to human GR. GS-I and GS-II denote the two halves of the glutathione disulfide molecule. (B) The same complex with electron density corresponding to an $F_o - F_c$ map contoured at a 1.5 σ level. All atoms of glutathione were removed from structure factor calculation. Figures prepared using Setor (Evans, 1993).

the mixed disulfide with Sγ of Cys58 in the catalytic turnover, is at a distance of 4.0 Å from its reaction partner. The carbonyl of γ-Glu-I is stabilized in its position by hydrogen bonding to Glu 472′ via a water molecule (wat480). Both Gly-II and Gly-I of TS_2 are found close to the positions they adopt in the GR·GSSG complex (Figure 4C).

The spermidine chain squeezes through a narrow opening surrounded by Trp37, the carboxyl group of γ-Glu-I, wat485, and the carboxyl group of Glu34. Another factor in stabilizing parts of the spermidine chain is a 3.3 Å hydrogen bond from Glu34 to the amide linkage between spermidine and Gly-I. Gly-I also makes a hydrogen bond between its carbonyl oxygen and the backbone amide of Glu34, similar to the hydrogen bond formed in the human GR·GSSG complex between the carboxyl group of Gly-I and the backbone amide of Ala34. In addition, the side chain of Ile113 contacts part of Gly-II. Trp37 is in the same position as in the ligand-free form of GRTR. Its ring is perpendicular to the direction of the spermidine chain sterically interfering with its binding. The chain assumes at least two conformations but can still be traced into continuous, albeit low, electron density. In one of these conformations, the secondary ammonium ion hydrogen bonds to water485 (wat485) located in the center of the "ring" formed by the TS_2 molecule (Figure 4A). This water molecule also interacts with the carbonyl oxygen of Cys-II of TS_2 and the carboxyl group of γ-Glu-I. In the other conformation, the spermidine chain runs closer to the center of the ring, forcing out wat485 and allowing the secondary ammonium ion to interact directly with the two oxygens mentioned above.

Mixed Disulfide between GRTR and Trypanothione. There is no electron density that would indicate the presence of TS_2; therefore, all the trypanothione must be involved in formation of the mixed disulfide, in which the sulfur of Cys58 is now linked with that of Cys-I (Figure 5, panels A and B). The location of this disulfide is essentially the same as in the mixed disulfide between glutathione and human GR. γ-Glu-II, Cys-II, and Gly-II are well-defined in the map and occupy positions comparable to those in the TS_2 complex. Figure 5A shows that, in general, the trypanothione moiety is bound similarly to trypanothione disulfide, with several notable changes. As found in all enzyme substrate complexes described here, the γ-glutamyl group of γ-Glu-II is fixed through interactions with the residues of the second subunit (region 406′ and 467′−476′). It is, however, slightly more mobile.

One of the most pronounced movements in the active site of GRTR observed upon formation of the mixed disulfide is a 90° rotation of the side chain of Trp37 that now lies parallel to the spermidine chain, allowing close van der Waals contacts to the polyamine (see Figure 5, panels A and B). The position of the spermidine chain is unequivocal

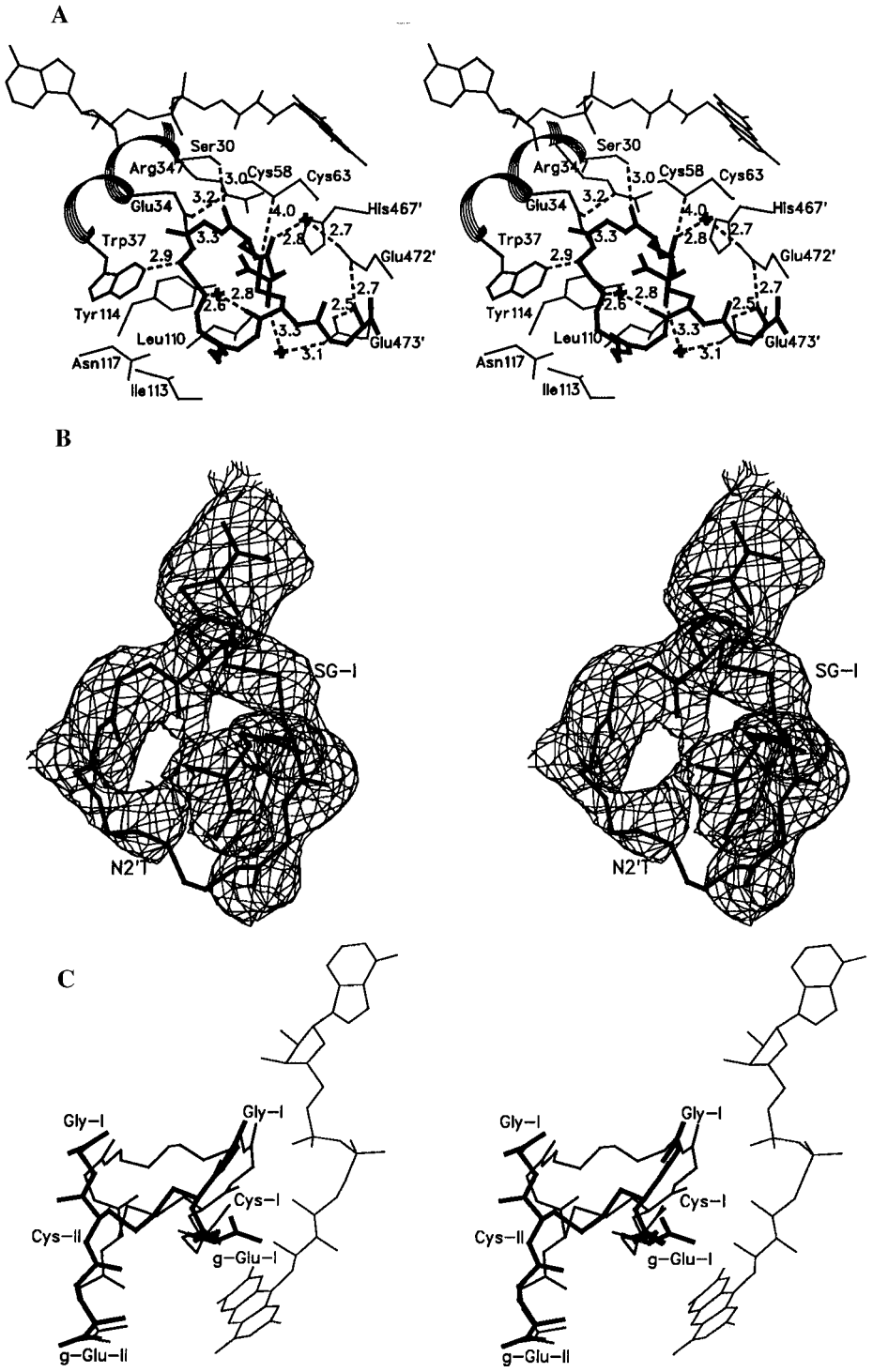

FIGURE 4: (A) Convergent stereo representation of trypanothione disulfide (TS_2) bound to GRTR. (B) The same complex with electron density corresponding to an $F_o - F_c$ map contoured at a 2.0 σ level. All atoms of trypanothione were removed from structure factor calculation. (C) Comparison of TS_2 bound to GRTR and GSSG bound to human GR (bold). Figures prepared using Setor (Evans, 1993).

where it makes a hydrogen bond to Glu34, which is also found in the GRTR·TS_2 complex. It is also well-defined in the region where new opportunities for van der Waals contacts with Trp37 exist, but there are still parts of the chain that are mobile (Figure 5, panels A and B).

Gsp_{ox}. This compound shows the lowest occupancy of all of the ligands in this study. The best-defined portion of the molecule is the disulfide, which is represented by reasonable electron density. The closest distance between a sulfur of Gsp_{ox} and the enzyme's redox-active disulfide is 6.8 Å. As one moves away from the S−S bond, the density gets patchy. The only other part of Gsp_{ox} that allows at least an attempt at tracing is one of the two spermidine chains. It appears, however, to have at least three conformations with one of them somewhat favored. There is continuous density extending from Gly-I wrapping around the surface of the enzyme molecule in generally the same direction as in the TS_2 complex. It should be noted that γ-Glu-II, which is normally well-defined and tightly bound, is rather mobile with its orientation rotated by 90°. γ-Glu-I and the spermidine chain attached to Gly-II are not visible at all. Due to the equivocal placement of parts of the Gsp_{ox} molecule, only the electron density is shown in Figure 6 with one probable orientation overlaid.

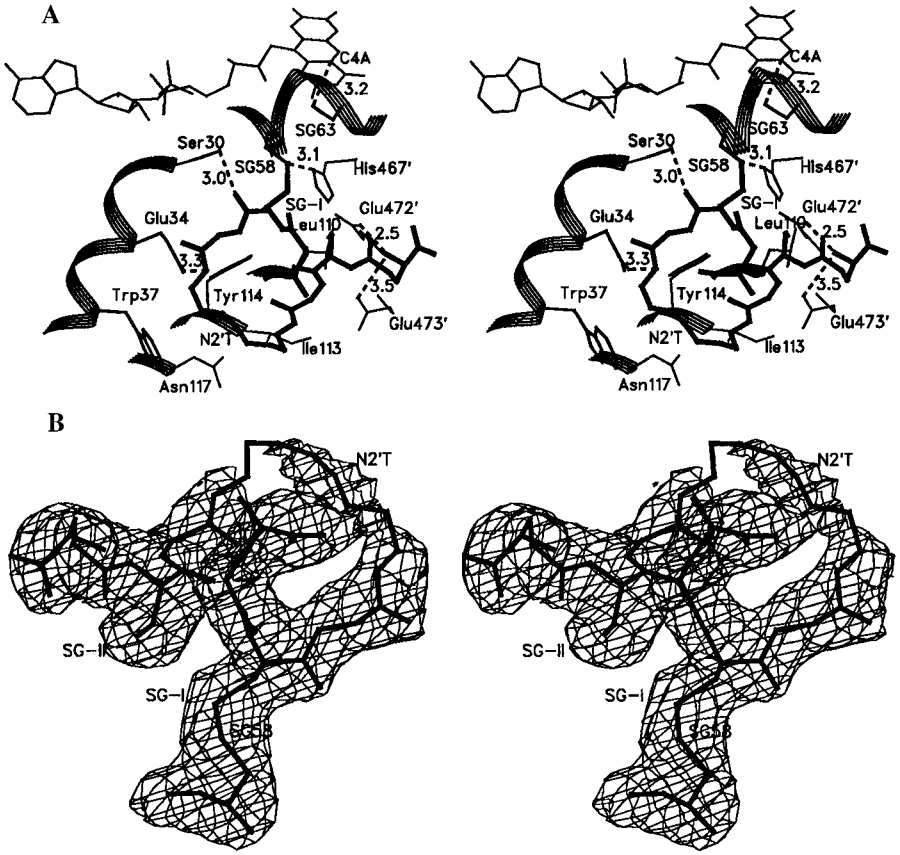

FIGURE 5: (A) Convergent stereo representation of the mixed disulfide between trypanothione and GRTR. (B) The reaction intermediate with electron density corresponding to an $F_o - F_c$ map contoured at a 2.5 σ level. All atoms of trypanothione and Cys58 for the protein were removed from structure factor calculation. SG-I and SG-II mark the positions of the trypanothione sulfurs; SG58 denotes the sulfur of Cys58. Figures prepared using Setor (Evans, 1993).

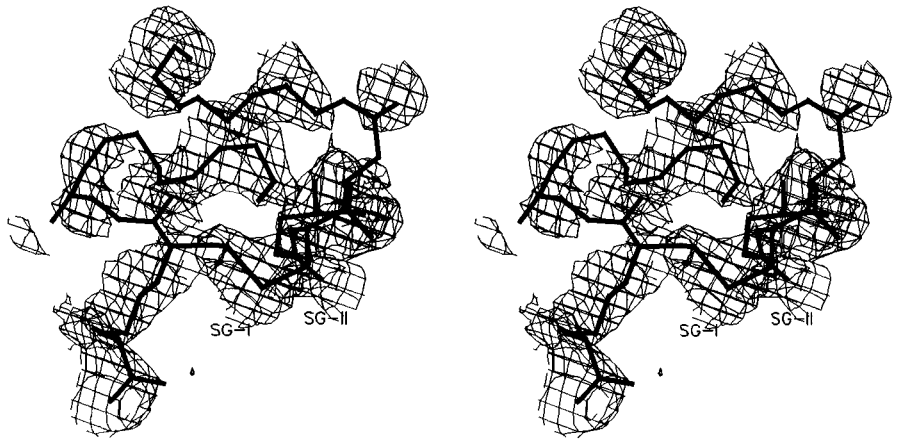

FIGURE 6: Convergent stereo representation of glutathionylspermidine disulfide bound to GRTR with electron density corresponding to an $F_o - F_c$ map contoured at a 1.75 σ level. All atoms of glutathionylspermidine disulfide were removed from structure factor calculation. Note the many discontinuities of the electron density indicating rather low occupancy. Figures prepared using Setor (Evans, 1993).

DISCUSSION

pH Profiles and Kinetic Data. The combination of relatively low catalytic activities of GRTR and high K_m values, especially for Gsp_{ox}, required large amounts of material for the determination of the pH profiles of GRTR. The pH optimum of GRTR for GSSG is about 1 unit lower than that of native human GR (Table 2), consistent with the interpretation that a negatively charged residue at position 34 interferes with GSSG binding. The pH optimum for the reduction of the glutathionyl spermidine conjugates by GRTR is 1 pH unit higher than for the reduction of GSSG reflecting the direct involvement of Glu34 in substrate binding, an interaction also found in trypanothione reductase (Bailey et al., 1993).

GRTR's catalytic efficiency with TS_2 as substrate is identical to that of *E. coli* GR (see Table 2), an enzyme which is less stringent in its disulfide substrate requirements than its human counterpart (Henderson et al., 1991). The ability of the bacterial enzyme to utilize TS_2 as a substrate may be partly due to having an asparagine residue instead of Arg37. Site-directed mutagenesis of *E. coli* GR reversing this change reduced k_{cat}/K_m for TS_2 by a factor of 2.5 (Henderson et al., 1991). The K_m of GRTR for TS_2 is 4 times lower than that of *E. coli* GR, again supporting the contribution of Glu34 to TS_2 binding.

Structure of GRTR. A superposition of the structures of human GR and GRTR depicting the area of the mutations Ala34Glu and Arg37Trp is shown in Figure 1. Apart from the changes at the sites of mutation themselves there are no significant structural differences, with one exception. The side chain of Arg347 is displaced through steric hindrance with the longer side chain of Glu34. The guanidinium group moves close to 5 Å from its position toward the bulk solvent. As far as its ability to bind to possible disulfide substrates is concerned, this forced movement has essentially the same effect as removing the arginine altogether leading to a limited opening of the active site. This interpretation is consistent with the kinetic results that show that GRTR behaves very similar to a triple mutant, in which Arg347 was additionally replaced by Ala347 (Bradley et al., 1991). GRTR's slightly higher k_{cat} value for TS_2 (700 min^{-1} *vs* 500 min^{-1}) could be explained by a stabilizing influence exerted by the bulky Arg347 on the newly introduced glutamic acid residue.

It is important to also note the differences between the substrates GSSG and TS_2. Topologically and chemically, GSSG and TS_2 are identical except for the presence of the spermidine in TS_2 bridging the two glycine residues. This additional chain might well require more space in the active site than human GR is able to provide; consistent with kinetic results, experiments to bind TS_2 to human GR or to form the mixed disulfide between them were not successful (Pai, E. F., unpublished results). Two crucial mutations transforming GR into GRTR seem to change the active site of the mutant enzyme enough to enable the binding and catalytic conversion of TS_2. The spermidine chains of all ligands tested in this work, however, are still the most poorly defined parts in the corresponding electron density maps.

The other major difference between GSSG and TS_2, also caused by the addition of a spermidine, is the switch in the overall charge of the substrates. GSSG contains a total of four carboxyl groups and two free amino groups, whereas TS_2 has only two carboxyl groups, two free amino groups, and three additional amino groups, two of which form amide bonds. The net charge of GSSG is -2, that of TS_2 is $+1$. The spermidine bridge also adds a large hydrophobic part to TS_2 that is not found in GSSG.

The amino acid changes that create GRTR modify the overall charge of the active site (overall positive in human GR) by effectively removing two arginine residues (Arg37 and Arg347) and adding a glutamate residue shifting the overall charge toward more negative values. There are also more specific effects evident. Replacing Arg37 by a tryptophan removes a positive charge, slightly opens up the active site and provides a hydrophobic binding environment for the spermidine moiety of TS_2. The glutamic acid introduced in position 34 interacts with the amide bond formed between one of the spermidine's amino groups and Gly-I, replacing the anchoring function played by Arg37 holding the free carboxylate of Gly-I in GSSG. The effect on its neighbor Arg347 has already been described above.

GRTR·GSSG Complex. The k_{cat}/K_m value for GSSG as substrate decreases from 1.2×10^8 M^{-1} $min^{-1}$for human GR to 9×10^3 M^{-1} min^{-1} for GRTR, an approximately 14000-fold decrease (Bradley et al., 1991). The interpretation for this dramatic effect is evident from the structure of the GSSG complex of GRTR. As can be seen in Figure 3A, the binding of GSSG is completely disrupted by the mutations with the exception of the binding of γ-Glu II to the other subunit. The positively charged residues required for binding to the other free carboxyl groups of GSSG are no longer present. The movement of Arg347, 5 Å out of the active site, abolishes the last important contact accessible to GSSG in a catalytically competent conformation (Figure 3, panels A and B). Figure 3A illustrates the contacts that have been broken in GRTR. In human GR, Tyr114 makes a close contact to the disulfide of GSSG. It is interesting that, although an identical contact is not possible in the mutant enzyme, one of the GSSG sulfurs is now found in hydrogen-bonding distance from Tyr106 (Figure 3A).

In the GRTR·GSSG structure, there is extremely weak electron density for portions of GSSG close to the conformation adopted by GSSG when bound to human GR. Since GSSG is still a substrate for GRTR, although a very poor one, this weak density most probably represents the low percentage of molecules adopting a catalytically competent conformation.

GRTR·TS_2 Complex. A view of TS_2 as it is bound in the active site is shown in Figure 4, panels A and B. The overall topology of trypanothione binding is very similar to that of GSSG to human GR (Karplus et al., 1989). Again, binding of γ-Glu-II to residues of the other subunit (region 406′ and 467′−476′) is conserved and the tightest interaction in this complex.

A superposition of GSSG bound to human GR and TS_2 bound to GRTR is shown in Figure 4C. When compared to human GR·GSSG, the direction of the disulfide bond of TS_2 is slightly shifted. The sulfur of Cys-I, however, that will react with Sγ of Cys58 is in a nearly identical position to that in human GR·GSSG at a distance of 4.2 Å from its reaction partner. It appears that the carbonyl of γ-Glu-I is fixed by a hydrogen bond to wat480, which in turn interacts with Glu 472′. This replaces the direct protein contacts found in human GR. Both Gly-II and Gly-I of TS_2 lie close in position to the corresponding residues of GSSG in human GR.

It should be noted that, although the spermidine chain is asymmetric, the limited resolution of our analyses and the observed mobility of parts of this chain do not allow the unambiguous determination of its orientation. Apart from the obviously beneficial changes of charge introduced by the mutations they also provide stabilization to the spermidine chain via hydrogen bonds in the case of Glu34 or hydrophobic interactions in the case of Trp37. Ile113 in human GR is equivalent to Ser109 in TR and it is likely that a mutation here would allow a serine residue to hydrogen bond to the carbonyl oxygen of Gly-I and further stabilize the complex. Asn117 in human GR is equivalent to Met113 in TR, which has been proposed to play an important role in binding of the spermidine chain to TR (Bailey et al., 1993; Jacoby et al., 1996). Tyr114 hydrogen bonds with the TS_2 disulfide bridge as well as with several carbonyl oxygens of TS_2 and wat485 to further stabilize the complex. There is an equivalent tyrosine residue (Tyr110) in TR which may perform a similar function and may be important to trypanothione binding to native TR. Most of the contacts made to the spermidine chain, however, are weak and may not significantly contribute to the binding energy of TS_2.

There are a number of tightly bound water molecules stabilizing the conformation of TS_2 in the active site of GRTR. γ-Glu-I, whose corresponding GSSG-binding contacts have been disrupted by the mutations, is stabilized by a complexed water that links its carbonyl oxygen to the carboxyl group of Glu 472′ of the second subunit. Another

water molecule bound together with TS_2 at the active site links γ-Glu-I and Glu473′, stabilizing γ-Glu-I, which makes none of the contacts found with human GR. These contacts, to a certain degree, compensate for the loss of contacts no longer possible in the mutant enzyme.

Mixed Disulfide between GRTR and Trypanothione. Analysis of the mixed disulfide between trypanothione and GRTR was expected to yield further insight into the mode of trypanothione binding. At the properly lowered pH, all the trypanothione bound is involved in formation of the mixed disulfide; there is no indication of the presence of TS_2 (Figure 5, panels A and B). The structure of this covalent intermediate of catalysis is similar to that of the mixed disulfide between glutathione and human GR (Pai & Schulz, 1983; Karplus et al., 1989). The fact that most of the substrate−enzyme contacts that have been identified as important for catalysis are conserved, especially the sulfur−sulfur distances, proton shuttle involving the catalytic His467′, and even the Tyr114 disulfide interactions, enables the formation of this true reaction intermediate in the crystalline state strongly corroborating the kinetic analyses. The results of this study confirm that GRTR uses essentially the same mechanism, including equivalent reaction intermediates, in reducing TS_2 that GR applies when splitting GSSG into two GSH molecules. The differences in the substrate specificity for the most part are therefore due to differences in substrate binding, an interesting aspect of which is represented by the "induced fit" movement undergone by Trp37 upon the formation of the mixed disulfide adopting a position that has been described for the equivalent residue in the *Crithidia fasciculata* TR·Gsp_{ox} complex (Bailey et al., 1993). As TS_2 alone does not show this structural change, one has to assume that it is induced by the formation of the substrate−enzyme disulfide bond.

GRTR·Gsp_{ox} Complex. Glutathionyl spermidine disulfide is a very poor substrate for GRTR (Table 2), and the reasons for this are evident from the crystal structure of its GRTR complex. This substrate has the lowest occupancy of all of the substrates in this study. The disulfide bond is too far from the enzyme's redox-active cysteine to undergo sulfur−sulfur interchange. Contrary to GSSG, Gsp_{ox} does not seem to be able to flip periodically into a conformation more amenable to catalysis. Most probably, this is caused by the added bulkiness of the second spermidine chain. Even γ-Glu-I, the most important anchor in all the other ligand complexes, has rotated by 90° and is rather mobile in this structure.

Comparison of GRTR with TR. It is evident from this study and from previous crystallographic work on the glutathionyl spermidine disulfide complex of *C. fasciculata* TR (Bailey et al., 1993), which in the absence of any structural information on a TR·TS_2 complex will have to serve as a substitute, as well as from many site-directed mutagenesis studies (Henderson et al., 1991; Bradley et al., 1991; Sulllivan et al., 1991), that the size and charge of the active sites of both GR and TR are key to their mutually exclusive substrate specificities. As there is nearly 85% sequence homology between GR and TR in the region of the active site, it was of particular interest in the present study to elucidate how GRTR, differing in only two amino acids from GR, can very effectively discriminate against GSSG and is even able to process TS_2 catalytically.

The active site arrangement of GRTR is not too different from that of TR. The "stacking" of Trp37 (the equivalent residue in TR is Trp21) that we observed in the mixed disulfide structure has also been found in the TR·Gsp_{ox} complex. This structure shows an equivalent hydrogen bond between Glu18 of TR (the equivalent of Glu34 in GRTR) and the amide linking Gly-I to the spermidine chain. The introduction of Glu34 with the concomitant formation of a hydrogen bond helps to stabilize TS_2 in a catalytically competent conformation. This mutation also pushes Arg347 out of the active site, removing a positive charge and breaking a contact important for GSSG binding. In addition, the two mutations alter the overall charge of the active site, enhancing the binding of a largely positive substrate, like trypanothione.

The mutually exclusive substrate specificities have been further examined in kinetic and modeling studies done by Faerman et al. (1996), where they examined the role of active site charge versus inhibitor charge in the binding of a series of chloropromazine inhibitors to both GR and TR. They found that human GR has a negative and *C. fasciculata* TR has a positive electrostatic potential at their active sites, compatible with the charges of their respective substrates or inhibitors. Clearly, the overall charges of the active sites and their substrates play a crucial role in their substrate specificities.

As is to be expected, there are several important contacts between Gsp_{ox} and native TR, which are not found in GRTR complexes. One is made by Met113 in TR, which corresponds to Asn117 in GRTR. It has been proposed that this residue makes a hydrogen bond to the secondary amine of the spermidine chain and van der Waals contacts to other parts of the chain (Bailey et al., 1993). A similar interaction was also observed in the mepacrine·TR complex, between Met113 and N10 of the mepacrine ring (Jacoby et al., 1996), suggesting that Met113 is an important contact for binding of the spermidine chain in general and the secondary ammonium ion specifically. In the GRTR·TS_2 complex, Asn117 is only 4.5 Å from one of the locations of the spermidine chain identified in the electron density map, and would provide an interesting site for further mutational studies to test the contributions of methionine−spermidine interactions to substrate binding.

In TR, Asn22 acts to stabilize and orient Glu18 for hydrogen bonding to the spermidine chain. In GRTR, the residue analogous to Glu18 in TR is Glu34. GRTR is catalytically slightly more efficient than the Glu34−Trp37−Arg347 triple mutant. We propose that this is caused by Arg347, although its head group has been pushed out of the active site; the remainder of its side chain may still provide some stabilization for the other active site residues, especially for Glu34. One could attempt to mimic the effect exerted by Asn22 in TR by changing either Arg347 or Arg38, both of which lie close to Glu34, to asparagine residues to provide additional stabilization for Glu34.

Another point that has been stressed by Bailey et al. (1993) and is supported by this study, is the rigidity of the active site and the fact that the substrate adapts its conformation to the environment of the active site. In all the structures presented here, the active site remained relatively unchanged upon substrate binding. The one notable exception is the movement of Trp37 in the mixed disulfide structure.

Conclusion. GRTR was designed based on comparisons between the structures of the human GR·GSSG complex and that of TR (Bradley et al., 1991). This was accompanied by a number of predictions as to the specific roles of these

selected residues (Glu34, Trp37, and Arg347) would play in TR. One of them was, that Glu34 would make a hydrogen bond to the secondary amine of the spermidine chain. In our structures and also in the TR·Gsp_{ox} complex (Bailey et al., 1993), a hydrogen bond between Glu34 and the amide linking Gly-I and the spermidine chain is found instead. We do see evidence for a specific van der Waals interaction between Trp37 and the hydrophobic region of the spermidine chain, which is consistent with the results of Bailey et al. (1993) and the prediction of Bradley et al. (1991). Finally, the importance of removing Arg347 is now clear from the structures presented here. This arginine side chain must be either removed completely or at least oriented away from the active site to eliminate the effect of its positive charge on the binding of TS_2 and to allow more space for the binding of substrate.

As no structural information on how TR enzymes interact with their proper substrate TS_2 is available to date, we undertook the work described here with the intention to contribute to our understanding of this important interaction. It is still very puzzling to think that crystalline GR will bind a multitude of substrates, inhibitors, and other ligands; that GRTR, an artificially created TR, allows the binding of several ligands; but that for TR, only two complexes have been structurally characterized, the ones with Gsp_{ox} (Bailey et al., 1993) and with mepacrine (Jacoby et al., 1996). Nevertheless, the structural basis for the narrow substrate specificities, shown by GR, TR, and even GRTR, in spite of the similarities between their substrates and their protein sequences, are beginning to become clearer. The structures of the GRTR mutant presented here will hopefully support ongoing efforts toward structure-based drug design aimed at the treatment of diseases like trypanosomiasis and leishmanias.

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