Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8769–8773, October 1991
Biochemistry

Engineering the substrate specificity of glutathione reductase toward that of trypanothione reduction

(trypanosomes/molecular modeling/drug design)

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ABSTRACT **Glutathione reductase (EC 1.6.4.2; CAS registry number 9001-48-3) and trypanothione reductase (CAS registry number 102210-35-5), which are related flavoprotein disulfide oxidoreductases, have marked specificities for glutathione and trypanothione, respectively. A combination of primary sequence alignments and molecular modeling, together with the high-resolution crystal structure of human glutathione reductase, identified certain residues as potentially being responsible for substrate discrimination. Site-directed mutagenesis of *Escherichia coli* glutathione reductase was used to test these predictions. The mutation of Asn-21 to Arg demonstrated that this single change was insufficient to generate the greater discrimination against trypanothione shown by human glutathione reductase compared with the *E. coli* enzyme. However, the mutation of Ala-18, Asn-21, and Arg-22 to the amino acid residues (Glu, Trp, and Asn, respectively) in corresponding positions in *Trypanosoma congolense* trypanothione reductase confirmed that this region of polypeptide chain is intimately involved in substrate recognition. It led to a mutant form of *E. coli* glutathione reductase that possessed essentially no activity with glutathione but that was able to catalyze trypanothione reduction with a k_{cat}/K_m value that was 10% of that measured for natural trypanothione reductases. These results should be of considerable importance in the design of trypanocidal drugs targeted at the differences between glutathione and trypanothione metabolism in trypanosomatids and their hosts.**

The selective binding of substrate to enzyme is a fundamental feature of enzyme-catalyzed reactions. The molecular basis of substrate recognition has recently become accessible to study by the methods of site-directed mutagenesis. Several attempts have been made to manipulate the specificity of particular enzymes, though the results have not always fulfilled the original intention—e.g., with trypsin (1) and aspartate aminotransferase (2). Limited success, sometimes unexpected, at redefining or broadening the substrate specificities of enzymes has been observed with cytochrome $P450_{coh}$ (3), α-lytic proteinase (4), and hexose 1-phosphate uridylyltransferase (5). Most notably perhaps, in *Bacillus stearothermophilus* lactate dehydrogenase, the change of Gln-102 to Arg converted the enzyme into a malate dehydrogenase (6); in *Escherichia coli* glutathione reductase, the coenzyme specificity was switched from NADP(H) to NAD(H) by the cumulative effect of seven site-directed changes (7).

Glutathione reductase (GR; EC 1.6.4.2; CAS registry number 9001-48-3) is the enzyme that, within most cells, is responsible for maintaining glutathione (GSSG) in its reduced state (GSH). This is an important component of the cell's defense against oxidative stress; GSH also plays a crucial part in the biosynthesis of the deoxyribonucleotide precursors of DNA (8). However, the trypanosomatid parasites responsible for African sleeping sickness, leishmaniasis, and South American Chagas disease possess, in addition to GSSG, a dithiol, N^1,N^8-(bis)glutathionyl spermidine, given the trivial name trypanothione [$T(S)_2$] (9) (Fig. 1). In these organisms, $T(S)_2$ plays an important role in the maintenance of reduced thiols and is itself maintained in its reduced state by the enzyme trypanothione reductase (TR; CAS registry number 102210-35-5) (10, 11). GR and TR are members of a family of enzymes, the flavoprotein disulfide oxidoreductases, and possess similar kinetic properties. However, they have almost mutually exclusive substrate specificities (10–13). The gene encoding TR in *Trypanosoma congolense* has been isolated and overexpressed (14, 15), and alignment of the inferred amino acid sequence with the primary structures of human (16) and *E. coli* (17) GRs has shown 41% and 38% sequence identity, respectively. This level of identity suggests that the three-dimensional structure of TR will be similar to that of GR, for which excellent high-resolution crystal structures are available in both the absence and presence of bound substrates (18–21).

The structural gene for GR from *E. coli* has been isolated (17) and overexpressed (22, 23), and the structure of human GR is a good model for protein engineering experiments on *E. coli* GR (24–26). We have now tested the roles proposed for several amino acids in the binding of substrate by GR and, on this basis, have engineered the specificity of the enzyme away from GSSG and toward $T(S)_2$. A preliminary account of some of this work has appeared (27). Comparable experiments on TR in which TR has been mutated to cause it to acquire activity with GSSG as substrate have been described (28).

MATERIALS AND METHODS

Materials. Complex bacteriological media were from Difco and all media were prepared as described by Maniatis *et al.* (29). Ethidium bromide, NADPH, and GSSG were from Sigma. Oxidized $T(S)_2$ was purchased from Bachem. Ultrapure agarose and CsCl were from Bethesda Research Lab-

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Abbreviations: GSSG, glutathione; $T(S)_2$, trypanothione; GR, glutathione reductase; TR, trypanothione reductase.

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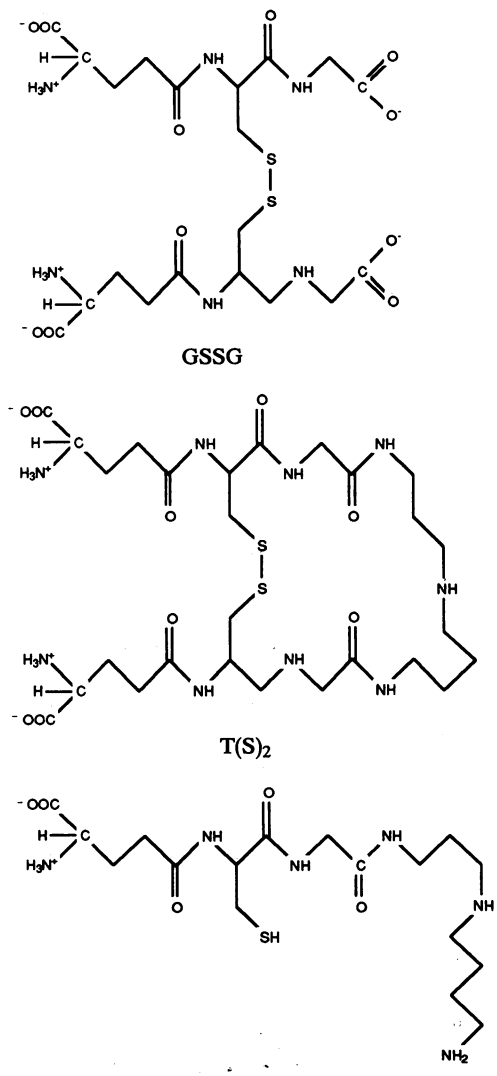

FIG. 1. Structures of GSSG, $T(S)_2$, and glutathionyl spermidine.

oratories. All other chemicals were of analytical grade wherever possible. Glass-distilled water was used throughout.

Restriction enzymes *Hin*dIII and *Eco*RI were purchased from Pharmacia. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. T4 DNA ligase and T4 polynucleotide kinase were from Amersham. *E. coli* TG1 [K12, Δ(*lac-pro*), *supE, thi, hsdD5/F' traD36, proA*$^+$*B*$^+$, *lacI*^{q}, *lacZ*ΔM15] was provided by Amersham. Strains SG5 [F$^-$ Δ(*his-gnd*) Δ*gor* Δ*lac araD* Str^R] and NS3 [Δ(*his-gnd*) Δ*gor* Δ*lac araD* Str^R *pro*/F' *proA*$^+$*B*$^+$, *lacI*^{q}, *lacZ*ΔM15] have been described (17, 23).

Site-Directed Mutagenesis and DNA Sequencing. Site-directed mutagenesis was carried out on a derivative of M13 containing the noncoding strand of the *gor* gene (K19gor3'δEcoRI) (23). The mutagenic oligonucleotides 5'-GGC AGC GGC GGT ATC GAA TCC ATC TGG AAC GCG GCT ATG TAC GGC CA-3' (A18E, N21W, R22N), 5'-CAT ACT TCC TAT GAA GAT GTG CTC GGT GAT AAT AAC GTT GAT GTA-3' (N101D, K105D), 5'-TGC AGC GGG TGC ACG TCT CTC TGA A-3' (R319A), and 5'-GCC TCC ATC CGT CGC GCG GCT ATG TA-3' (N21R) were annealed to single-stranded template and mutants were isolated using the phosphorothioate method (30) as marketed by Amersham. Putative mutants were screened directly by dideoxynucleotide DNA sequencing (31, 32) using the T7 system purchased from Pharmacia. The whole of any mutated gene was resequenced (25) to ensure that spurious mutations had not been introduced during the mutagenesis reactions.

Plasmid Construction. Plasmid or bacteriophage replicative form DNA was prepared by CsCl density gradient centrifugation (29). For the purpose of screening, plasmids were prepared on a miniscale by using the alkaline lysis method (29). Restriction endonuclease digestion of DNA was carried out as recommended by the enzyme suppliers. The mutant genes were isolated by digesting bacteriophage replicative form DNA with *Eco*RI and *Hin*dIII and the *gor* gene fragment was subcloned into the expression vector pKK223-3 digested with the same enzymes (22, 23).

Growth of Cells and Purification of GR. Wild-type and mutant GRs were purified from the *gor*-deletion *E. coli* NS3, transformed with the appropriate expression plasmid, as described (23).

Measurement of Kinetic Parameters. The wild-type and mutant GRs were assayed in the direction of GSSG reduction at 30°C in 0.1 M potassium phosphate (pH 7.5) at a fixed NADPH concentration of 100 μM and various concentrations of either GSSG or $T(S)_2$. The kinetic parameters were estimated by regression analysis (33) as described in Scrutton *et al.* (7) and in Tables 1 and 3.

RESULTS

The crystallographic analysis of GSSG bound to human GR has identified a number of amino acid residues likely to be important for binding the substrate, leading to the conclusion that the substrate specificity is largely determined by interactions with side chains rather than with the main protein chain (21). A comparison of the amino acid sequence of TR from *T. congolense* (14, 15) with the sequences of the GRs from human erythrocytes (16), *E. coli* (17), and *Pseudomonas aeruginosa* (34) suggests that changes in some of these residues in the parasite enzyme confer on it the specificity for the altered substrate $T(S)_2$. However, work in this area has been hampered by the lack of a crystal structure for TR, which has been solved (35). We therefore used a combination of sequence alignments and molecular modeling to suggest which residues are likely to be most important in substrate discrimination and have tested these predictions by site-directed mutagenesis of *E. coli* GR. Following Karplus *et al.* (21), we designate the two halves of GSSG as GS-I and GS-II, where GS-I is the half that forms a transient disulfide link to a cysteine residue of the enzyme during catalysis (18).

Molecular Modeling of the TR Active Site. An initial model for the active site of TR was constructed using the crystal structure coordinates of human GR as a template for the amino acid sequence of *T. congolense* TR. The conformation of $T(S)_2$ in the active site was generated from coordinates defining GSSG bound to human GR, which were kindly supplied by G. E. Schulz (University of Freiburg). This model highlighted the close similarity in the active site regions of the two proteins: almost all the changed residues are located in a region adjacent to the spermidine moiety of $T(S)_2$. The most notable differences are three arginine residues at positions 37, 38, and 347 in human GR that are replaced with neutral residues in *T. congolense* TR, and three acidic residues in TR, Glu-18, Asp-112, and Asp-116 (equivalent to Ala-34, Asn-116, and Lys-120 in human GR), with no counterparts in the latter enzyme (Table 1). Arg-37 in human GR has been clearly shown by crystallographic analysis to form an important ionic interaction with the α-carboxylate group of the glycine residue of bound GS-I; similarly, Arg-347 interacts with the α-carboxyl group of the γ-glutamyl residue of GS-I (21). It seemed that the acidic residues in TR might

Table 1. Sequences of substrate specificity regions of GR and TR

Region	Sequence
N-terminal	
GR	
E. coli	IGGGSGGIASINRAAMYG-QKCALIEAK---------ELGGTC
Human	IGGGSGGLASARRAAELG-ARAAVVESH---------KLGGTC
Ps. aeruginosa	IGAGSGGVRAARFAAGFG-ARVAVAESR----------LGGTC
TR (*T. congolense*)	IGAGSGGLEAGWNAATLYKKRVAVVDVQTVHGPPFFAALGGTC
Central	
GR	
E. coli	AYIDRIHTSYENVLGKNNVDVIKGFAR
Human	AYVSRLNAIYQNNLTKSHIEIIRGHAA
Ps. aeruginosa	REIQRLNGIYRNLLVNSGVTLLEGHAR
TR (*T. congolense*)	AAVLDINKSYEDMFKDTEGLEFFLGWG
C-terminal	
GR	
E. coli	LTPVAVAAGRRLSERLFNNK
Human	LTPVAIAAGRKLAHRLFEYK
Ps. aeruginosa	LTPVALAEGMAVARRLFKPE
TR (*T. congolense*)	LTPVAINEGASVVDTIFGSK

Alignment of the amino acid sequences of *E. coli* (17), human (16), and *Ps. aeruginosa* (34) GRs and of *T. congolense* TR (14), showing the regions tentatively identified as important in defining substrate specificity.

interact analogously with the positively charged spermidine moiety of $T(S)_2$.

The model of TR (36) was then used for 50-ps molecular dynamics simulations for a 21-Å sphere centered on the active site and included several hundred water molecules (37). These showed important substrate stabilization by a strong hydrogen bond between the N-8 amide of $T(S)_2$ and the carboxylate group of the side chain of Glu-18 (equivalent to Ala-34 in human GR). A comparable GSSG/GR simulation highlighted the importance of Arg-37, Arg-38, and Arg-347 in interactions with the carboxylates of GSSG. As controls, $T(S)_2$/GR and GSSG/TR calculations were also carried out. In both cases, the starting enzyme–substrate geometries were unstable and there was rapid distortion of the starting conformations. Although we had identified residues Asp-112 and Asp-116 in TR as potential contributors to $T(S)_2$ recognition, the dynamics simulations provided no evidence for this.

Substrate Specificity of the Wild-Type Enzymes. Wild-type *E. coli* GR is highly selective for GSSG [(k_{cat}/K_m)GSSG/$(k_{cat}/K_m)T(S)_2 = 240$] but has a higher capacity for reducing $T(S)_2$ than human GR (Table 2). Alignments of the primary structures of the *E. coli*, human, and *Ps. aeruginosa* GRs and *T. congolense* TR reveal that Arg-37 in human GR is conserved in *Ps. aeruginosa* GR but is replaced by an asparagine residue (Asn-21) in *E. coli* GR (Table 1). Given the apparent importance of Arg-37 in the binding of GSSG by human GR, we thought that the replacement of Asn-21 by Arg (N21R) might create an *E. coli* GR with diminished activity toward $T(S)_2$ and a higher relative specificity toward GSSG. The N21R mutant enzyme was overexpressed in, and purified from, the *gor*-deletion *E. coli* NS3. Surprisingly, it still showed activity toward $T(S)_2$. Detailed analysis revealed that the K_m for $T(S)_2$ had risen from ≈2 mM to ≈4.5 mM and that the k_{cat} for the reaction was only slightly affected (Table 3). The fact that human GR discriminates against $T(S)_2$ better than *E. coli* GR, therefore, cannot be explained solely by the possession of a positively charged side chain at position 37 in human GR.

Mutation of Ala-18, Asn-21, and Arg-22. Glu-18, the key substrate-binding residue in TR identified from our modeling studies, is equivalent to Ala-34 in human and to Ala-18 in *E. coli* GR. Similarly, the two arginine residues (Arg-37 and Arg-38) of human GR identified as important for GSSG binding are equivalent to Asn-21 and Arg-22 of *E. coli* GR. The participation of these residues in substrate recognition was tested by the introduction into *E. coli* GR of a glutamate

Table 2. Specificity of GR and TR

Enzyme	GSSG			$T(S)_2$		
	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$
T. congolense TR				31	9,600	5.1×10^6
T. cruzi TR				45	14,200	5.3×10^6
C. fasiculata TR		3.1*		53	31,000	9.8×10^6
Human GR	65	12,600	3.2×10^6		9.6†	
E. coli wild-type GR	61 ± 7	44,000 ± 2100	1.2×10^7	2000 ± 400	6,100 ± 740	5.0×10^4
E. coli N21R mutant GR	97 ± 9	32,300 ± 1400	5.5×10^6	4580 ± 440	5,100 ± 300	1.9×10^4

Wild-type and N21R mutant *E. coli* GRs were assayed in 0.1 M potassium phosphate (pH 7.5) at a fixed concentration (100 μM) of NADPH. An approximate K_m for each disulfide substrate was measured and the kinetic parameters were then estimated from initial rates of reaction measured at various substrate concentrations above and below the approximate K_m. The kinetic parameters for the other enzymes are taken from the following references: TRs from *T. congolense* (15), *Trypanosoma cruzi* (11), and *Crithidia fasiculata* (10) and human GR with GSSG as substrate (38) and with $T(S)_2$ as substrate (10).

*Apparent k_{cat} at 50 mM GSSG.

†Apparent k_{cat} with 280 μM $T(S)_2$.

Table 3. Kinetic parameters of mutant forms of *E. coli* GR

Enzyme	GSSG			$T(S)_2$		
	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$
Wild-type	61 ± 7	44,000 ± 2100	1.2×10^7	2000 ± 400	6100 ± 740	5.0×10^4
A18E/N21W/R22N	>>20,000			660 ± 100	9900 ± 900	2.5×10^5
N101D/K105D	120 ± 14	36,000 ± 2300	4.9×10^6	2300 ± 400	3800 ± 400	2.5×10^4
A18E/N21W/R22N/N101D/K105D	>>20,000			330 ± 46	9100 ± 580	4.6×10^5
A18E/N21W/R22N/N101D/K105D/R319A	>>20,000			210 ± 29	4000 ± 210	3.2×10^5

Mutant GRs were prepared from the *gor*-deletion strain NS3 of *E. coli* as described (23) and the kinetic parameters were estimated at a fixed concentration (100 μM) of NADPH as described in Table 1.

residue at position 18, with simultaneous replacement of Asn-21 and Arg-22 by Trp and Asn residues, respectively, as in the parasite enzyme sequence (Table 1) (mutant A18E, N21W, R22N). These changes led to a reversal of substrate specificity that can be divided into two parts: an increase in activity with $T(S)_2$ and a decrease in activity with GSSG. Wild-type *E. coli* GR has a K_m for GSSG of about 60 μM and a k_{cat} of ≈40,000 min^{-1}, giving a specificity constant (k_{cat}/K_m) with GSSG of ≈10^7 $M^{-1}\cdot sec^{-1}$ (Table 3). The mutant enzyme showed very little activity with GSSG in the micromolar range but, on increasing the GSSG concentration to ≈20 mM, some activity with GSSG could be detected. It was impossible to extract kinetic parameters for this enzyme; we can state only that the K_m for GSSG must be >20 mM. The kinetic parameters for $T(S)_2$, however, could be measured. The A18E, N21W, R22N mutant exhibited a decrease in the value of K_m for $T(S)_2$ from ≈2 mM to 660 μM and the k_{cat} for the reaction was little changed. The resulting increase in k_{cat}/K_m was ≈5-fold (Table 3).

Mutation of Asn-101 and Lys-105. The sequence alignment (Table 1) had suggested that the negatively charged aspartic acid residues at positions 112 and 116 in *T. congolense* TR might have a bearing on its substrate specificity. However, our modeling studies suggested that the introduction of two negatively charged residues into the corresponding positions in human GR would have little or no effect. To resolve this question, these mutations were constructed in *E. coli* GR either as the N101D, K105D double mutant or added singly (data not shown) or as a pair to the A18E, N21W, R22N triple mutant. The results of a kinetic analysis of these enzymes (Table 3) confirmed that the changes had very little effect on the enzyme activity. The cumulative effect of the five mutations was to produce an enzyme (A18E, N21W, R22N, N101D, K105D) that possessed a k_{cat} equal to that of the *T. congolense* TR on which the model was based and a value of k_{cat}/K_m ≈10% of that of the trypanosomal enzyme. This difference was entirely due to the difference in K_m (31 μM for the *T. congolense* enzyme and 330 μM for the mutant *E. coli* GR).

Mutation of Arg-319. The other amino acid residue identified by crystallography (21) and in our dynamics simulations as important in substrate binding in human GR is Arg-347. This residue is conserved as Arg-319 in the *E. coli* enzyme but is replaced by a methionine (Met-317) in *Ps. aeruginosa* GR (Table 1). In *T. congolense* TR, the equivalent residue is Ala-343. To try to decrease further the K_m for $T(S)_2$ in our A18E, N21W, R22N, N101D, K105D mutant, we engineered the relevant change (R319A) into this mutant enzyme. The product enzyme was purified and its kinetic parameters for GSSG and $T(S)_2$ as substrates were measured (Table 3). The addition of the extra mutation did not affect the activity with GSSG and, rather surprisingly, did not significantly alter the parameters with $T(S)_2$ as substrate.

DISCUSSION

TR has become an important target for trypanocidal drug design but a lack of structural information, in the absence of a crystallographic analysis (35), has limited research in this area (13, 39, 40). We have attempted to rectify this lack by molecular modeling and to verify our conclusions by means of protein engineering of a related enzyme, GR, leading to a rationally designed switch of substrate specificity. As the basis for our experiments, we have used the excellent crystallographic analysis of the binding of GSSG to human erythrocyte GR (21). We have then used sequence alignments and molecular dynamics calculations to try to understand the natural discrimination of substrates exhibited by GR and TR. Karplus *et al.* (21) identified six regions of polypeptide chain in human GR involved in binding GSSG. These were residues 30–37, 59–64, 110–117, 339–347, and residues 406 and 467–476 of the second subunit. Studies of the sequence alignments (Table 1) and the results of our molecular dynamics calculations suggested that, as expected, only a small proportion of these residues was important in discriminating between the similar substrates, GSSG and $T(S)_2$. In particular, we would expect any positively charged residues interacting with the glycine carboxylates of GSSG in GR to be replaced in TR, perhaps by negatively charged residues suitably positioned to bind the positively charged spermidine moiety of $T(S)_2$.

Our experiments have confirmed that a major determinant of substrate specificity toward GSSG by GR is the region of polypeptide chain close to the N terminus, around positions 18–22 in the *E. coli* enzyme (Table 3). In human GR, the positively charged side chain of Arg-37 has been shown to interact strongly with the glycine carboxylate of GS-I, whereas the glycine carboxylate of GS-II appears to be bound in a rather nonspecific site formed by residues Ile-113 and Asn-117 (refs. 18 and 21; and see above). Sullivan *et al.* (28) also speculate that the replacement of Arg-37 of human GR [the authors appear to misquote it as Arg-347, which interacts with the α-carboxylate of the γ-glutamyl group of GS-I (18, 21)] by Asn-21 in *E. coli* GR (Table 1) might allow the latter enzyme to function more effectively with $T(S)_2$ as substrate (Table 2 and ref. 28). However, the N21R mutation in *E. coli* GR did not generate an *E. coli* GR showing the kinetic properties of the human enzyme (Table 2). It must be that other residues are playing a more dominant part in substrate discrimination. It should be borne in mind that human GR bears an extra N-terminal segment of ≈17 amino acid residues, compared with the *E. coli* enzyme (16, 17). This segment cannot be seen in the x-ray structure of the enzyme (19) and we cannot assess any part it may play in determining substrate specificity. An interesting possibility is that the more relaxed substrate specificity of the *E. coli* enzyme is in some way related to the fact that, during stationary phase, a large proportion of the spermidine found in *E. coli* exists as glutathionyl spermidine, a compound closely related to $T(S)_2$ (Fig. 1). The function of glutathionyl spermidine in *E. coli* is at present unclear although it may play a part in the control of growth and nucleic acid metabolism (41).

The region around residues Asn-116 and Lys-120 of human GR, identified by sequence alignment studies as potentially important in substrate discrimination, showed no evidence of

this in our molecular dynamics simulations. This was confirmed by a study of the appropriate mutant enzymes (Table 3). Similarly, Arg-347 has been shown to interact with the α-carboxyl group of the γ-glutamyl residue of the GS-I moiety of bound GSSG in human GR (20). This implies that mutation of Arg-347 might not affect substrate discrimination, since the γ-glutamyl group is common to GSSG and $T(S)_2$ (Fig. 1), but the distance from Arg-347 to the glycine α-carboxylate (~5Å) is small enough that a slight rotation of the arginine side chain might allow it to interact with the glycine residue of GSSG. Arg-347 is conserved in the *E. coli* enzyme (as Arg-319) but is replaced by a methionine residue (Met-317) in *Ps. aeruginosa* GR and by an alanine residue (Ala-343) in TR (Table 1). Not unexpectedly, the R319A mutation in *E. coli* GR brought about no significant increase in specificity for $T(S)_2$ (Table 3).

The *E. coli* mutant enzyme (A18E, N21W, R22N, N101D, K105D), which most resembles the trypanosomal enzyme, has a K_m for $T(S)_2$ only 10 times higher than that of the *T. congolense* TR on which it was modeled and shows a k_{cat} almost identical to that of the trypanosomal enzyme (Table 3). Attempts to lower the K_m for $T(S)_2$ still further must await the determination of more sequences for other TRs and the solution of the crystallographic structure of our mutant *E. coli* GR. They will be greatly facilitated by knowledge of the structure of TR (35). Even so, with the results from other laboratories (21, 28), we have delineated many of the structural features that govern substrate recognition by these enzymes, which should prove useful in the design of potential trypanocidal drugs. Given the earlier success in redesigning the coenzyme specificity of *E. coli* GR (7), the present results make this enzyme unique in that both its substrate and coenzyme specificities have now been rationally engineered.

This paper is dedicated to the memory of G.B.H. We thank Ronald M. Levy and Donna A. Bassolino for assistance in the construction of the homology model, Professor G. E. Schulz for providing the coordinates of GSSG bound to GR before publication, and Mahendra P. Deonarain for collaboration on plasmid DNA sequencing. This work was supported by the Cambridge Centre for Molecular Recognition, the Science and Engineering Research Council, The Royal Society, The Royal Commission for the Exhibition of 1851, St John's College, Cambridge, National Institutes of Health grants (AI26784) to G.B.H. and (AI19428) to A.C., and a National Science Foundation grant of computer time to J.K. K.O. is on leave from EGIS Pharmaceuticals, H-1475 Budapest, P.O. Box 100, Hungary.

1. Graf, L., Craik, C. S., Petthy, A., Rocznick, S., Fletterick, R. J. & Rutter, W. J. (1987) *Biochemistry* **26,** 2616–2623.
2. Cronin, C. N., Malcolm, B. A. & Kirsch, J. F. (1987) *J. Am. Chem. Soc.* **109,** 2222–2223.
3. Lindberg, R. L. P. & Negishi, M. (1989) *Nature (London)* **339,** 632–634.
4. Bone, R., Silen, J. L. & Agard, D. A. (1989) *Nature (London)* **339,** 191–195.
5. Kim, J., Luzicka, F. & Frey, P. A. (1990) *Biochemistry* **29,** 10590–10593.
6. Clarke, A. R., Atkinson, T. & Holbrook, J. J. (1989) *Trends Biochem. Sci.* **14,** 145–148.
7. Scrutton, N. S., Berry, A. & Perham, R. N. (1990) *Nature (London)* **343,** 38–43.
8. Holmgren, A. (1985) *Annu. Rev. Biochem.* **54,** 237–271.
9. Fairlamb, A. H., Blackburn, P., Ulrich, P., Chart, B. T. & Cerami, A. (1985) *Science* **227,** 1486–1487.
10. Shames, S. L., Fairlamb, A. H., Cerami, A. & Walsh, C. T. (1986) *Biochemistry* **25,** 3519–3526.
11. Krauth-Siegel, R. L., Enders, B., Henderson, G. B., Fairlamb, A. H. & Schirmer, R. H. (1987) *Eur. J. Biochem.* **164,** 123–128.
12. Henderson, G. B., Fairlamb, A. H., Ulrich, P. & Cerami, A. (1987) *Biochemistry* **26,** 3023–3027.
13. Jockers-Scherübl, M. C., Schirmer, R. H. & Krauth-Siegel, R. L. (1989) *Eur. J. Biochem.* **180,** 267–272.
14. Shames, S. L., Kimmel, B. E., Peoples, O. P., Agabian, N. & Walsh, C. T. (1988) *Biochemistry* **27,** 5014–5019.
15. Sullivan, F. X., Shames, S. L. & Walsh, C. T. (1989) *Biochemistry* **28,** 4986–4992.
16. Krauth-Siegel, R. L., Blatterspiel, R., Saleh, M., Schulz, G. E., Schirmer, R. H. & Untucht-Grau, R. (1982) *Eur. J. Biochem.* **121,** 259–267.
17. Greer, S. & Perham, R. N. (1986) *Biochemistry* **25,** 4082–4088.
18. Pai, E. F. & Schulz, G. E. (1983) *J. Biol. Chem.* **258,** 1751–1757.
19. Karplus, P. A. & Schulz, G. E. (1987) *J. Mol. Biol.* **195,** 701–729.
20. Pai, E. F., Karplus, P. A. & Schulz, G. E. (1988) *Biochemistry* **27,** 4465–4474.
21. Karplus, P. A., Pai, E. F. & Schulz, G. E. (1989) *Eur. J. Biochem.* **178,** 693–703.
22. Scrutton, N. S., Berry, A. & Perham, R. N. (1987) *Biochem. J.* **245,** 875–880.
23. Deonarain, M. P., Berry, A., Scrutton, N. S. & Perham, R. N. (1989) *Biochemistry* **28,** 9602–9607.
24. Scrutton, N. S., Berry, A. & Perham, R. N. (1988) *FEBS Lett.* **241,** 46–50.
25. Berry, A., Scrutton, N. S. & Perham, R. N. (1989) *Biochemistry* **28,** 1264–1269.
26. Ermler, U. & Schulz, G. E. (1991) *Proteins: Struct. Funct. Genet.* **9,** 174–179.
27. Perham, R. N., Scrutton, N. S., Berry, A. & Deonarain, M. P. (1991) in *Flavins and Flavoproteins*, eds. Curti, B. & Ronchi, S. (de Gruyter, Berlin), in press.
28. Sullivan, F. X., Sobolov, S. B., Bradley, M. & Walsh, C. T. (1991) *Biochemistry* **30,** 2761–2767.
29. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
30. Taylor, J. W., Schmidt, W., Cosstick, R., Okruszek, A. & Eckstein, F. (1985) *Nucleic Acids Res.* **13,** 8749–8764.
31. Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. M. & Roe, B. A. (1980) *J. Mol. Biol.* **143,** 161–178.
32. Biggin, M. D., Gibson, T. J. & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3963–3965.
33. Cleland, W. W. (1979) *Methods Enzymol.* **63,** 103–138.
34. Perry, A. C., NiBhriain, N., Brown, N. L. & Rouch, D. A. (1991) *Mol. Microbiol.* **5,** 163–171.
35. Kuriyan, J., Kong, X.-P., Krishna, T. S. R., Sweet, R. M., Murgolo, N. J., Fields, H., Cerami, A. & Henderson, G. B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8764–8768.
36. Murgolo, N. J., Cerami, A. & Henderson, G. B. (1989) *Ann. N.Y. Acad. Sci.* **569,** 193–200.
37. Chen, L. X. Q., Engh, R. A., Brunger, A. T., Nguyen, D. T., Karplus, M. & Fleming, G. R. (1988) *Biochemistry* **27,** 6908–6921.
38. Worthington, D. J. & Rosemeyer, M. A. (1976) *Eur. J. Biochem.* **67,** 231–238.
39. Henderson, G. B., Ulrich, P., Fairlamb, A. H., Rosenberg, I., Pereira, M., Sela, M. & Cerami, A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5374–5378.
40. Fairlamb, A. H., Henderson, G. B. & Cerami, A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2607–2611.
41. Tabor, H. & Tabor, C. W. (1975) *J. Biol. Chem.* **250,** 2648–2654.