Redox Enzyme Engineering: Conversion of Human Glutathione Reductase into a Trypanothione Reductase†

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Received February 4, 1991; Revised Manuscript Received April 4, 1991

ABSTRACT: The substrate specificity of the human enzyme glutathione reductase was changed from its natural substrate glutathione to trypanothione [N^1,N^8-bis(glutathionyl)spermidine] by site-directed mutagenesis of two residues. The glutathione analogue, trypanothione, is the natural substrate for trypanothione reductase, an enzyme found in trypanosomatids and leishmanias, the causative agents of diseases such as African sleeping sickness, Chagas disease, and Oriental sore. The rational bases for our mutational experiments were the availability of a high-resolution X-ray structure for human glutathione reductase with bound substrates, the active site sequence comparisons of human glutathione reductase and the trypanothione reductases from *Trypanosoma congolense* and *Trypanosoma cruzi*, a complementary set of mutants in *T. congolense* trypanothione reductase, and the properties of substrate analogues of trypanothione. Mutation of two residues, A34 → E34 and R37 → W37, in the glutathione-binding site of human glutathione reductase switches human glutathione reductase into a trypanothione reductase with a preference for trypanothione over glutathione by a factor of 700 using k_{cat}/K_m as a criterion.

Few enzymes have been as intensively studied as the almost ubiquitous glutathione reductase (GR), a flavoenzyme that helps to maintain a reducing environment within cells by the reduction of oxidized glutathione in an NADPH-dependent manner [see Figure 1 and Schirmer et al. (1990) and Meister and Anderson (1983)]. The human enzyme has recently been cloned and overexpressed in *Escherichia coli* (Tutic et al., 1990; Bücheler et al., 1990). High-resolution X-ray structures have been determined for human GR and the GR·NADP and GR·NADP·GSSG complexes (Karplus & Schulz, 1987, 1989; Karplus et al., 1989). The enzyme from *E. coli* has been cloned and overexpressed (Greer & Perham, 1986) and has been the subject of numerous elegant mutagenesis studies, which have included a switching of its cosubstrate dependence from NADPH to NADH (Scrutton et al., 1990) and an investigation of the role of the active site histidine residue (Berry et al., 1989). Additionally, thorough kinetic analyses have been carried out for glutathione reductases from various sources (Vanoni et al., 1990; Wong & Blanchard, 1989).

The trypanosomes and leishmania represent a family of parasitic protozoa that have, very unusually, been found to lack glutathione reductase and the high levels of glutathione typically observed in other organisms (Fairlamb et al., 1985; Fairlamb & Cerami, 1985). Instead they have been shown to contain high levels of N^1,N^8-bis(glutathionyl)spermidine, also named trypanothione, glutathionylspermidine, and an enzyme trypanothione reductase (Shames et al., 1986) that maintains these metabolites in the free thiol form (see Figure 1) and that bears many similarities to glutathione reductase (Shames et al., 1986, 1988). In view of the pivotal role of glutathione reductase in oxidative stress management and in the production of deoxyribonucleotides (Schirmer et al., 1990), trypanothione reductase has become an attractive target for the design of trypanocidal agents against human pathogens (Fairlamb, 1989). This concept is supported by the high specificities of host glutathione reductase and parasite trypanothione reductase for their respective substrates (Shames et al., 1988). Given the known propensity of human erythrocyte glutathione reductase to crystallize, the generation of a "new trypanothione reductase" by a site-directed mutagenesis approach in the recently cloned human glutathione reductase cDNA was an attractive proposition. Such a view was enhanced by the recent availability of X-ray grade crystals of trypanothione reductase (Kuriyan et al., 1990; Hunter et al., 1990), which gives rise to the promise of structural comparisons of both mutant and wild-type human glutathione reductases and trypanothione reductase. The amino acid sequence alignment of *Trypanosoma congolense* trypanothione reductase with human glutathione reductase shows 41% amino acid sequence identity, which rises to 70% for the 27 amino acids identified as active site residues and to 85% similarity when taking into account conservative changes (Sullivan et al., 1991; Karplus et al., 1989; Karplus & Schulz, 1989). Three of the four nonconserved residues in the active site (A34, R37, R347) have been identified to be important for substrate binding (Sullivan et al., 1991) (see Table I). These were thus chosen as targets for mutation to the corresponding residues found in trypanothione reductase. Here we report the successful conversion of human glutathione reductase into a trypanothione reductase by the site-directed mutation of two and/or three of these four residues in human glutathione reductase.

Materials and Methods

Materials and bacterial strains were as previously reported (Sullivan et al., 1989, 1991). pUB302(59), a vector containing the human glutathione reductase cDNA adapted for overexpression in *E. coli* has been described (Bücheler et al., 1990). Mutant genes for the double mutant and the single mutant

† This work was supported in part by grants from the National Institutes of Health (GM21643), the Deutsche Forschungsgemeinschaft, the McArthur Foundation, and a SERC/NATO postdoctoral fellowship (M.B).

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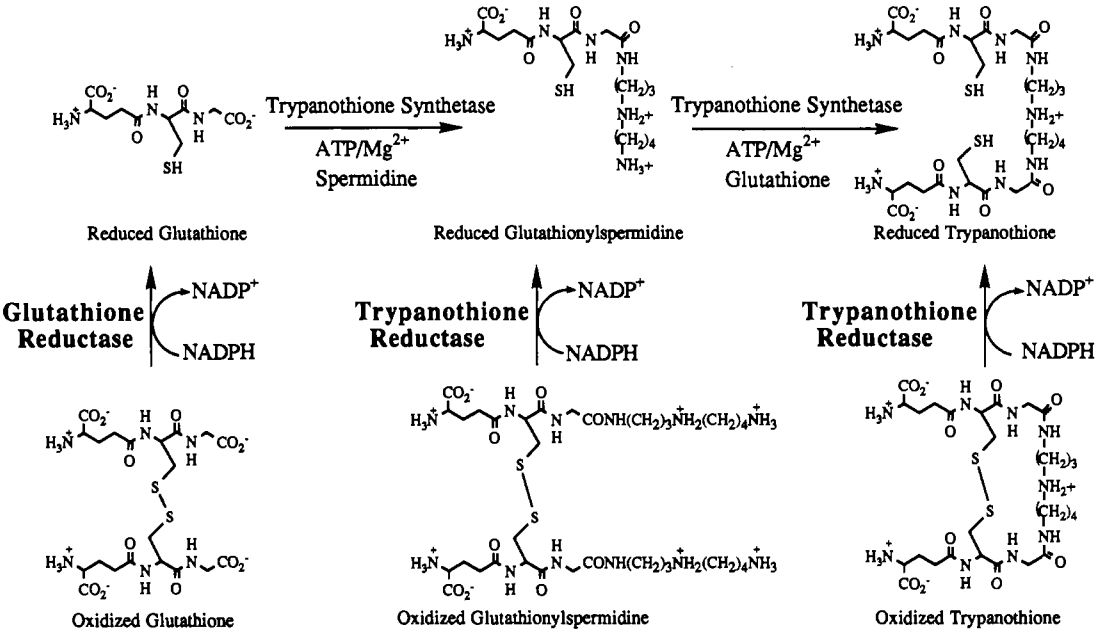

FIGURE 1: The structures of oxidized and reduced glutathione, glutathionylspermidine, and trypanothione and the reactions catalyzed by the specific reductases.

Table I: The Mutational Strategy for the Conversion of Human Glutathione Reductase into a Trypanothione Reductase

role in GR[a]	residue in GR: wild type	residue in GR: mutant[b]	proposed role in TR
main chain interaction	A34	E34	H bonds to secondary ammonium ion of spermidine
H bond to the glycine carboxylate of GSSG	R37	W37	hydrophobic interaction with the spermidine bridge. (There is no free glycine carboxylate in trypanothione.)
H bond to the γ-glutamate carboxylate of GSSG	R347	A347	unknown

[a] Karplus et al. (1989). [b] These residues indicate the amino acids found at the corresponding positions in the sequences of the two trypanothione reductases from *T. cruzi* and *T. congolense*.

were generated by the phosphothioate procedure (Taylor et al., 1985a,b) using a single-stranded DNA template of the human glutathione reductase cDNA. The single-stranded DNA template was generated by restriction of pUB302(59) with *Hin*dIII and *Xho*I, and the appropriate fragment obtained after electroelution was ligated with T4 DNA ligase into double-stranded M13mp18, which had been cut with *Hin*dIII and *Sal*I. Single-stranded DNA was then prepared according to standard procedures (Ausubel et al., 1987). The oligonucleotides used for mutagenesis were (R347A) 5′-AAAAGTCGATGGGCAAGTTTTGCGCCAGCAGCTAT (A34E and R37W) 5′-GGCCGCCCTCCACGCGCTCT-CCAGCCCGCCCGA.

The entire mutagenized genes were sequenced in M13mp18 with use of eight short synthetic primers. Each mutant gene was then reinserted into the original expression system (Bücheler et al., 1990). The triple mutant was made by swapping appropriate restriction fragments of the double and single mutants, using the unique sites of the enzymes *Bsm*I and *Hin*dIII, which were ligated by use of T4 DNA ligase and sequenced in the regions of the two mutations and the two restriction sites. All the mutated human glutathione reductase genes were transformed into the glutathione reductase deletion *E. coli* strain SG5 (Greer & Perham, 1986) for protein expression. Purification of the human glutathione reductases mutants was identical with the procedures recently described for trypanothione reductase (Sullivan et al., 1991), with use of a 2′,5′-ADP affinity column and a Mono Q (HR5/5) column, except that 20 mM potassium phosphate/1 mM EDTA/5 mM β-mercaptoethanol, pH 7.2, was used throughout as the purification buffer. Assays for glutathione and trypanothione reductase activities were identical with those previously described (Meister & Anderson, 1983; Sullivan et al., 1989, 1991), with errors in k_{cat} and K_m typically being less than ±5%.

Results and Discussion

To evaluate the role of specific residues in trypanothione reductase with respect to catalysis and binding and, in particular, to obtain structural information concerning the mode of binding of trypanothione to the enzyme, we have undertaken the construction of mutants of human glutathione reductase, an enzyme of known crystal structure. Indeed it was this high-resolution X-ray structure (Karplus et al., 1989; Karplus & Schulz, 1989) together with the sequence comparison of human glutathione reductase and parasite trypanothione reductases (Karplus et al., 1989; Sullivan et al., 1991; Sullivan & Walsh, 1991) that provided the basis of our mutational strategy (see Table I).

The mutant and wild-type glutathione reductases were assayed with oxidized trypanothione and glutathione as substrates; the results are as shown in Table II. The activity of each enzyme was optimized with respect to pH with use of 100 mM phosphate/50 mM citrate titrated with NaOH, as buffer. The mutant R347A shows the importance of the hydrogen-bonding contact between the γ-glutamyl residue of oxidized glutathione (GS_1) and the arginine residue 347 in the glutathione reductase structure. [The importance of this residue has also been shown by crystallographic and kinetic studies on human glutathione reductase using synthetic analogues of glutathione (Janes & Schulz, 1990).] The fall in k_{cat}/K_m, i.e., in substrate specificity, for this mutant is es-

Table II: Kinetic Constants for Wild-Type and Mutant Human Glutathione Reductases[a]

enzyme	wild type	mutants		
		347	34, 37	34, 37, 347
K_m(GSSG) (μM)	66	1800	ND	ND
k_{cat}(GSSG) (min^{-1})	8200	7500	ND	ND
k_{cat}/K_m(GSSG) (M^{-1} min^{-1})	1.2×10^8	4.2×10^6	9×10^3	4×10^3
pH optimum (GSSG)	7.3	6.2	5.6	6.6
$K_m(TS_2)$ (μM)	ND	ND	120	76
$k_{cat}(TS_2)$ (min^{-1})	ND	ND	730	500
$k_{cat}/K_m(TS_2)$ (M^{-1} min^{-1})	1.4×10^4	2×10^5	6×10^6	6×10^6
pH optimum (TS_2)	7.4	6.2	6.5	7.0

[a] GSSG = oxidized glutathione; TS_2 = oxidized trypanothione; ND = not determined. The K_m values for these substrates and the corresponding mutants were at least 50 mM for glutathione and at least 5 mM for trypanothione. The ratio of k_{cat}/K_m was determined as previously described in these cases (Sullivan et al., 1991). 347 = R347A; 34, 37 = A34E, R37W; 34, 37, 347 = A34E, R37W, R347A.

sentially dominated by the 27-fold increase in K_m when compared with the wild-type enzyme (wild type, 66 μM, R347A, 1.8 mM for glutathione); interestingly, this single amino acid substitution also influences the specificity for trypanothione, which is increased by a factor of 14 from that of wild-type glutathione reductase. The ratio of specificity constants for this single mutant for glutathione/trypanothione is now 21, compared to the value of 9000 in the case of unchanged glutathione reductase.

The double mutation A34E, R37W dramatically switches the substrate specificity of human glutathione reductase from glutathione to trypanothione. This double mutant has a specificity constant k_{cat}/K_m for trypanothione only 20-fold lower than wild-type glutathione reductase has for glutathione. This factor of 20 can be subdivided into approximately a 10-fold reduction in k_{cat} (8200 min^{-1} vs 730 min^{-1}) and, quite remarkably, only a 2-fold increase in K_m (120 μM for trypanothione in A34E, R37W vs 66 μM for glutathione in wild type). There is also a dramatic influence on the catalytic efficiency of this double mutant for glutathione, with k_{cat}/K_m being a mere 9000 min^{-1} M^{-1}. This value is approximately 14 000-fold lower than for wild-type human glutathione reductase. The triple mutant (A34E, R37W, R347A) has approximately the same catalytic efficiency as the double mutant for trypanothione. However, whereas its K_m value for typanothione at 76 μM is improved (within experimental error, the same as that of glutathione with wild-type human glutathione reductase), the turnover number has fallen from 730 min^{-1} to 500 min^{-1} (Table II). With trypanothione as substrate, the specificity constant (k_{cat}/K_m) for both the double and triple mutants of glutathione reductase is 6×10^6 M^{-1} min^{-1} vs 1.4×10^4 M^{-1} min^{-1} for wild-type GR, which corresponds to a 430-fold increase. The recognition of trypanothione has been improved to a point, where in the case of the triple mutant the K_m is equal to that of wild-type glutathione reductase for glutathione and in the same range as the K_m values measured for trypanothione with the trypanothione reductase from *Trypanosoma cruzi* (Jockers-Scherübl et al., 1989). We thus observe a specificity change from 1×10^4 for GSSG/TS_2 in wild-type human glutathione reductase to 700 in favor of TS_2/GSSG, a dramatic switch from host-type molecular recognition to parasite-type molecular recognition by only two (or three) mutations. In view of these results, it is informative to look at the kinetic data for *E. coli* glutathione reductase, which has an asparagine residue in the position corresponding to arginine 37. The two residues corresponding to positions A34 and R347 are identical with those in human glutathione reductase. The *E. coli* enzyme has a k_{cat}/K_m for trypanothione of (8900 min^{-1}/2.7 mM) = 3.6×10^6 M^{-1} min^{-1} (Sullivan et al., 1991), suggesting that the mutation A34E (and to some degree R347A), although enhancing binding, does somewhat lower the turnover number.

This double mutant of human glutathione reductase, a functional trypanothione reductase, has been crystallized, under identical conditions as wild-type glutathione reductase (E. F. Pai, M. Bradley, and C. T. Walsh, unpublished results). This provides the hope that it will be possible to compare the structure of an isomorphous double-mutant enzyme with bound trypanothione with wild-type glutathione reductase containing bound glutathione (Karplus et al., 1989). This comparison should yield insight into the mode of binding of trypanothione to the trypanocidal target enzyme trypanothione reductase and, inter alia, facilitate the design of parasite enzyme-specific inhibitors.

ACKNOWLEDGMENTS

We acknowledge the generous gift by Heiner Schirmer and D. Werner of a vector containing the human glutathione reductase cDNA overexpression system and the thorough reading and very helpful comments on the manuscript by Heiner Schirmer.

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Determination of Kinetic Constants for Peptidyl Prolyl Cis–Trans Isomerases by an Improved Spectrophotometric Assay†

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Received March 5, 1991; Revised Manuscript Received April 26, 1991

ABSTRACT: The kinetic properties and substrate specificity of two well-characterized peptidyl prolyl cis–trans isomerases (PPIases), cyclophilin and the FK-506 binding protein (FKBP), have been previously examined [Fischer, G., Bang, H., Berger, E., & Schellenberger, A. (1984) *Biochim. Biophys. Acta 791*, 87–97; Harrison, R. K., & Stein, R. L. (1990) *Biochemistry 29*, 1684–1689; Albers, M. W., Walsh, C. T., & Schreiber, S. L. (1990) *J. Org. Chem. 55*, 4984–4986]. The chymotrypsin-coupled enzymatic assay employed in these studies suffers from two serious shortcomings. Due to the low equilibrium population of the X-*cis*-Pro-Phe-pNA isomer (the PPIase substrate), in conjunction with the low solubility of *p*-nitroaniline generated by chymotrypsin hydrolysis, substrate concentrations in the saturating region are not experimentally attainable. Secondly, the uncatalyzed cis–trans isomerization obscures the interpretation of the initial velocity. As a result of these limitations, the steady-state kinetic parameters (K_m, k_{cat}) have not been determined. Here we introduce an improved version of the spectrophotometric assay and report for the first time the Michaelis constants and turnover numbers for both PPIases with established substrates. The improvements in the experimental conditions originate in a medium-induced increase in the equilibrium population of the cis X-Pro conformer and in conducting the assay at 0 °C to suppress the uncatalyzed thermal isomerization. In addition, we present a rigorous mathematical model of the spectrophotometric progress curves that accounts for the contributions of the residual background rate. For Suc-Ala-Ala-*cis*-Pro-Phe-pNA with bovine cyclophilin, K_m = 0.98 ± 0.14 mM and k_{cat} = 13200 ± 880 s^{-1}; for recombinant human cyclophilin, K_m = 0.87 ± 0.084 mM and k_{cat} = 12700 ± 550 s^{-1}. The kinetic parameters for Suc-Ala-Leu-*cis*-Pro-Phe-pNA with FKBP are K_m = 0.520 ± 0.08 mM and k_{cat} = 344 ± 26 s^{-1}. We also demonstrate that [(Boc)Dab]^8-CsA, a cyclosporin A analogue, is a tight-binding, slow-binding inhibitor of cyclophilin and that another cyclosporin A analogue, [Me^{5}Bth]^1-CsA, is a competitive inhibitor of the same enzyme.

Peptidyl prolyl cis–trans isomerase[1] (PPIase,[2] EC 5.2.1.8), discovered by Fischer and co-workers in 1984 (Fischer et al., 1984a), catalyzes the cis–trans isomerization of X-Pro peptide bonds. This class of enzymes facilitates the refolding of some denatured proteins in vitro (Lang et al., 1987; Lang & Schmid, 1988; Lin et al., 1988; Schönbrunner et al., 1991) and is believed to have an important role in the folding of newly synthesized proteins in vivo, where proline isomerization has been proposed as a potential rate-limiting step (Brandts et al., 1975). The discovery of PPIase coincided with the discovery of cyclophilin, a protein receptor for the immunosuppressive drug cyclosporin A (Handschumacher et al., 1984). The two proteins were subsequently shown to be identical (Takahashi et al., 1989; Fischer et al., 1989a). Intriguingly, the FK-506 binding protein, a receptor for the powerful immunosuppressant FK-506, also has PPIase activity (Siekierka et al., 1989; Harding et al., 1989). The fact that both PPIases bind

† This research was supported by Grants AR32007 and AI24650 from the National Institutes of Health.
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[1] Enzymes that catalyze an intramolecular cis–trans isomerization are categorized under the EC 5.2 class, cis–trans isomerases. This enzyme has also been referred to as a "rotamase".

[2] Abbreviations: PPIase, peptidyl prolyl cis–trans isomerase, (CyP, cyclophilin); TFE, trifluoroethanol; Suc, succinyl; pNA, *p*-nitroanilide; AU, absorbance units; PMSF, phenylmethanesulfonyl fluoride; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; CsA, cyclosporin A; [(Boc)Dab]^8-CsA, [N^{ϵ}-*t*-butoxycarbonyl diaminobutyryl]^8-CsA; [Me^{5}Bth]^1-CsA, [5-methyl-3-hydroxy-2-(methylamino)-6-octenoic acid]^1-CsA.

0006-2960/91/0430-6127$02.50/0