Folding of the Four Domains and Dimerization Are Impaired by the Gly446→Glu Exchange in Human Glutathione Reductase. Implications for the Design of Antiparasitic Drugs†

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ABSTRACT: Glutathione reductase (NADPH + GSSG + H^+ → $NADP^+$ + 2GSH) is a homodimeric flavoenzyme of known geometry. Each subunit contains four well-defined domains and contributes essential residues to the active sites; consequently, the monomer is expected to be inactive. As part of our program to develop dimerization inhibitors of human glutathione reductase (hGR) as antimalarial agents, we mutagenized the residues 446 and 447 which, together with their counterparts on the other subunit, represent the tightest contact between the subunits [Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 701–729]. Wild-type human glutathione reductase and mutants of this protein were produced in plasmid-transformed *Escherichia coli* SG5 cells. Active enzyme species, namely, wild-type hGR, N-terminally truncated Δ(1–15)hGR, and the point mutant F447P-hGR, were purified by 2′,5′-ADP–Sepharose chromatography and crystallization. Inactive mutants such as G446E-hGR or the double mutants G446E/F447P-hGR and G446P/F447P-hGR were isolated by immunoadsorption chromatography. G446E/F447P-hGR was studied in detail. This mutant behaved like a poorly folded monomeric protein, as indicated by the following properties: absence of the intersubunit disulfide bridge, Cys90–Cys90′; failure to bind FAD; failure to bind NADPH and analogues thereof; a short half-life (<4 min) in *E. coli* cells; and high susceptibility to trypsin in vitro. The results suggest that the sequence around G446 can control dimerization as well as domain folding. This is unexpected since the FAD-binding domain and the NADPH-binding domain occur in many different enzymes and have been regarded as autonomous folding units. The role of dimer interface residues as a folding signal and the prospect to develop inhibitors which render the target protein susceptible to in vivo degradation are discussed.

Many proteins of pathogens—such as trypanothione reductase (Krauth-Siegel et al., 1987) or the enzymes encoded by the HIV[1] virus (Restle et al., 1990)—are obligatory dimers. Dimerization can be regarded as a special case not only of protein folding but also of protein assembly (Jaenicke, 1987; Leistler et al., 1992; Freire et al., 1992). Since compounds which impair protein–protein interactions represent highly specific inhibitors (Rüegg, 1991), dimeric proteins are of general interest as drug targets. A well-studied dimer is human glutathione reductase (hGR). This flavoenzyme helps maintain a reducing milieu within cells by catalyzing the reaction NADPH + GSSG + H^+ → $NADP^+$ + 2GSH (Williams, 1992). Specifically, it has pivotal functions in oxidative stress management and in the production of deoxyribonucleotides. Human GR is also of interest as a target of antitumor and antimalarial drugs (Schirmer et al., 1989).

Studies on human GR mutants have become possible after hGR cDNA was cloned and overexpressed in *Escherichia coli* (Tutic et al., 1990; Bücheler et al., 1990). Specific mutations can be planned in light of the geometric structure of human erythrocyte glutathione reductase, which is known at a resolution of 154 pm (Karplus & Schulz, 1987). The subunits of the homodimeric enzyme are cross-linked by the disulfide bridge, Cys90–Cys90′. Each subunit contains a flexible N-terminal extension (residues 1–18) and four well-defined structural domains: the FAD-binding domain (19–157), the NADPH domain (158–293), the central domain (294–364), and the subunit interface domain (365–478) (Figure 1A). It is inconceivable that monomeric hGR is enzymatically active, since each binding site for the substrate GSSG and each catalytic site are composed of residues from both subunits (Thieme et al., 1981; Krauth-Siegel et al., 1982; Pai & Schulz, 1983).

The center of the parallel-running helices 439–454 and 439′–454′ forms the tightest contact between the subunits (Figure 1B). As described here, a number of single or double mutations at residues 446 and 447 led to the synthesis of inactive monomeric protein species. In contrast, the N-terminal extension of hGR was found not to be essential for catalysis. All mutants were purified and characterized.

EXPERIMENTAL PROCEDURES

Materials and Bacterial Strains. Unless stated otherwise, chemicals and biological reagents were as described previously

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[1] Abbreviations: apo-GR, FAD-free apoglutathione reductase; *E. coli* SG5, an *Escherichia coli* strain not possessing endogenous GR; EDTA, ethylenediaminetetraacetate; FAD, flavin adenine dinucleotide; FPLC, fast protein liquid chromatography; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; hGR, human glutathione reductase; HIV, human immunodeficiency virus; $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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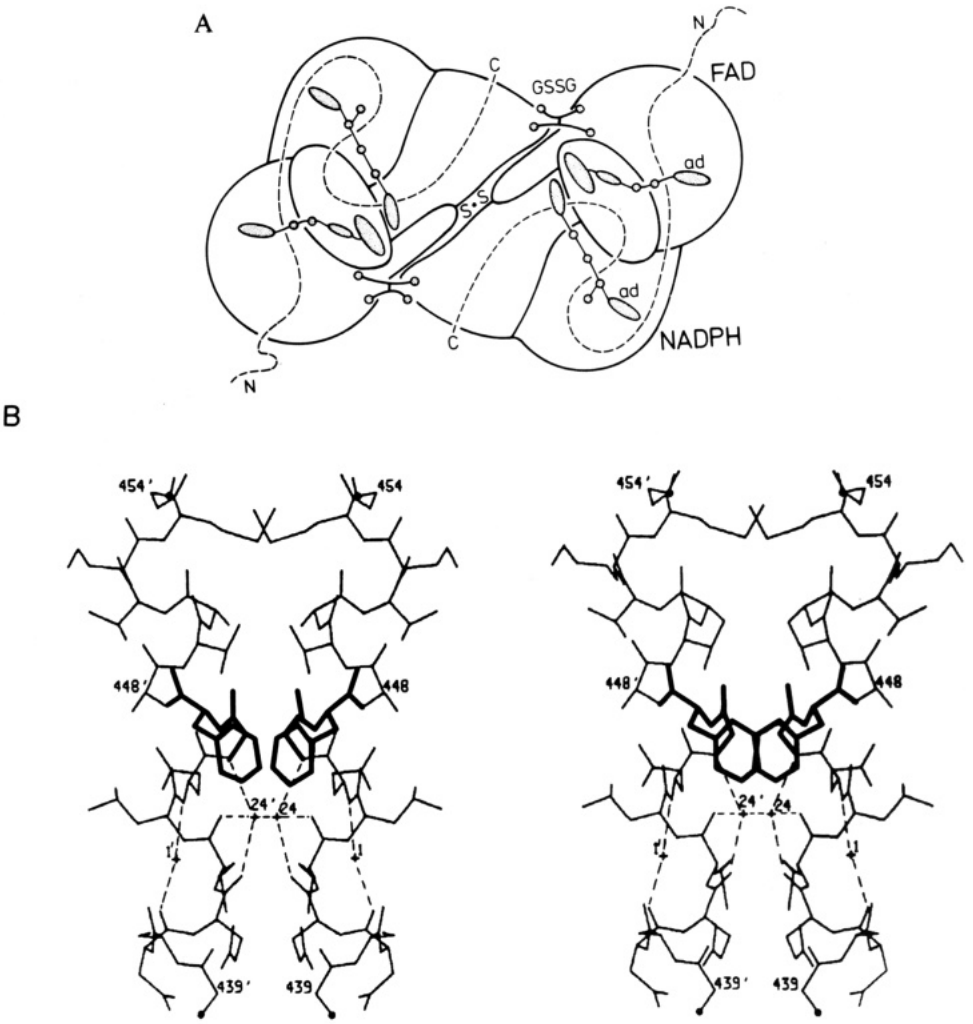

FIGURE 1: (A) Domain sketch of the dimeric enzyme hGR as viewed along the molecular 2-fold axis. At the N-terminus there are 18 flexible residues. The rest of the chain is structurally organized in four domains: the NADPH and FAD domains, the central domain, and the interface domain. The gross positions of the ligands FAD, NADPH, and GSSG are indicated: GSSG binds between the subunits. The intersubunit disulfide bridge, Cys90–Cys90′, is shown at the center of the molecule. (B) Section of the wild-type hGR structure showing the narrow packing of the helical residues 439–454 and 439′–454′. The marked residues G446 and F447 and their counterparts were altered to 446E, 446P, or 447P by site-directed mutagenesis. The numbers 1 and 24 (1′ and 24′) indicate the positions of water molecules whose hydrogen bonds are represented by dashed lines (after Karplus and Schulz (1987)).

(Bücheler et al., 1990, 1992; Tutic et al., 1990).

Nomenclature of Plasmids. The vector containing hGR cDNA adapted for overexpression in *E. coli* SG5 cells is pUB302-2 or p(wild-type hGR); it has replaced pUB302-59, the expression plasmid used originally (Bücheler et al., 1992; Bradley et al., 1991). The N-terminally truncated hGR starting at residue 16 of the wild-type enzyme is called Δ-(1–15)hGR; the corresponding expression vector is pUB309-2. All other expression plasmids, derivatives of pUB302-2, are named according to the hGR mutant which they encode, p(G446E-hGR) being a typical example.

Mutagenic Oligodeoxynucleotides. Using an ABI DNA synthesizer, four oligodeoxynucleotides were prepared: 5′-GCAACAGCGAATTCCTGCAGCAT-3′ (G446E); 5′-CTTCACTGCAACAGCCGGCCCCTGCAGCATTTCATC-3′ (F447P); 5′-CTTCACTGCAACAGCCGGCTCCTGCAGCATTTCATC-3′ (G446E/F447P); and 5′-GCAACAGCCGGTGGCTGCAGCATT-3′ (G446P/F447P). Two of these oligodeoxynucleotides (F447P and G446E/F447P) were prepared as a mixture in a single synthetic route, that is, by allowing either a C or a T to be incorporated at the indicated position. For identification of the expected cDNA mutants, an extra *Eco*RI site (G446E) or an *Nae*I site (F447P and G446E/F447P) was generated in the oligodeoxynucleotides. In the case of G446P/F447P, the *Nae*I site was erased again.

Mutagenesis and DNA Sequencing. In all experiments wild-type hGR cDNA served as a template, except for mutant G446P/F447P-hGR where the coding region of p(F447P-hGR) was used. The hGR-encoding *Eco*RI/*Hind*III DNA fragment (noncoding strand) of the expression plasmid pUB302-2 (or of p(F447P-hGR), respectively) was subjected to site-directed mutagenesis in a derivative of bacteriophage M13mp18 on the basis of published procedures using a Biorad kit (Kunkel, 1985; Sambrook et al., 1989). Putative mutants were screened by restriction site analysis, and the selectivity of the mutation(s) was confirmed by dideoxy sequencing (Sanger et al., 1977) of the entire coding region.

The mutant's coding region was excised by restricting the isolated DNA of the bacteriophage with *Eco*RI and *Hind*III and recloned into the original expression plasmid using the same restriction enzymes. The construct was then used to transform *E. coli* strain SG5, which carries a chromosomal deletion of *gor*, the glutathione reductase gene (Greer & Perham, 1986).

In one experiment, two different mutants were generated using a mixture of two oligodeoxynucleotides (F447P and G446E/F447P, see above). Mutants were identified by hybridization of replica filters according to Church and Gilbert (1984) with a ^{32}P-labeled mixture of the oligodeoxynucleotides used for site-directed mutagenesis: By successively decreasing ionic strength and increasing temperature of the washing solutions and by monitoring the amount of radioactivity retained in comparison to control filters, we established conditions which allowed us to distinguish between mutated and wild-type cDNA. Individual mutated clones were then identified by autoradiography of the filters. The final differentiation between clones representing the single mutation (F447P) and the double mutation (G446E/F447P) was established by DNA sequencing.

Protein Determination. For crude fractions, the methods of Ehresman et al. (1973) or Smith et al. (1985) were used. Isolated FAD-containing hGR in oxidized form was determined by absorption measurements at 280 ($A^{1\%}$ = 13.5) and 463 nm ($A^{1\%}$ = 2.16) (Krohne-Ehrich et al., 1977). On the basis of these absorption values, the Ehresman factor of hGR was found to be 0.23. The absorption, $A^{1\%}$, at 280 nm of FAD-free apo-GR is 10.0 (Becker et al., 1991).

Measurement of Kinetic Parameters. Specific activities of wild-type and mutant glutathione reductases in the direction of glutathione reduction were measured under saturating conditions (Worthington & Rosemeyer, 1974; Krohne-Ehrich et al., 1977). The specific activity of pure enzyme preparations tends to increase—for instance from 140 to 220 units/mg—when the protein is stored over months at 4 °C. The kinetic constants of recombinant hGR forms were determined according to Worthington and Rosemeyer (1976).

Equilibrium Dialysis. The method of Tomasselli and Noda (1979) was used for equilibrium dialysis, and the NADPH concentrations in the different compartments were determined according to Roskoski (1987).

SDS–Polyacrylamide Gel Electrophoresis. Cell-free extracts of *E. coli* and samples of purified glutathione reductase were submitted to electrophoresis on 10% polyacrylamide slab gels in the presence of SDS according to Laemmli (1970).

Purification of Enzymatically Active hGR Species by 2′,5′-ADP–Sepharose Chromatography. Wild-type and mutant hGR species were produced in *E. coli* SG5 transformed with the appropriate plasmid. Plasmid-carrying SG5 cells (5 g) which had been grown for 12–16 h in 2 L of Luria-Bertani medium containing 100 μg/mL ampicillin were harvested by centrifugation (3000*g*, 30 min, 4 °C) and washed in buffer A (20 mM potassium phosphate, 1 mM EDTA, and 1 mM dithioerythritol, pH 7.5). The pellet was then used immediately or stored at −20 °C.

For lysis (Leberman et al., 1980), the cells were suspended in 10 mL of lysing buffer (0.2 mg/mL lysozyme, 10 μM PMSF, 150 nM pepstatin, 40 nM cystatin, and 10 μM FAD in buffer A) and stirred for 5 min. After 200 μL of 4% sodium deoxycholate and 0.1 mg of DNAase I powder were added, the suspension was stirred at room temperature and centrifuged (20 000*g*, 45 min, 4 °C). The supernatant was saved at 4 °C as extract 1, and the pellet was reextracted with another 10 mL of lysing buffer. The combined extracts containing 1050 units of wild-type hGR were brought to a volume of 25 mL by adding buffer A and were then applied at room temperature to a 5-mL column of 2′,5′-ADP–Sepharose (Mannervik et al., 1976) equilibrated with buffer A. The column was washed successively with 3 vol of buffer A, 2 vol of 250 mM KCl in buffer A, 1 vol of 500 mM KCl in buffer A, and finally buffer A until the effluent had the conductivity of buffer A and an absorption difference at 280 nm of less than 0.01. The enzyme was then eluted using 1 vol of 1 mM NADPH in buffer A and stabilized by adding GSSG (2 mM) followed by ammonium sulfate (2 M). After the solution was allowed to stand for 16 h at 4 °C, the precipitate was centrifuged (20 000*g*, 20 min, 4 °C). If necessary, NADP and glutathione were removed from the pellet by washing it three times with 2 M $(NH_4)_2SO_4$ in buffer A; the removal of NADP can be followed by monitoring the absorption at 260 nm.

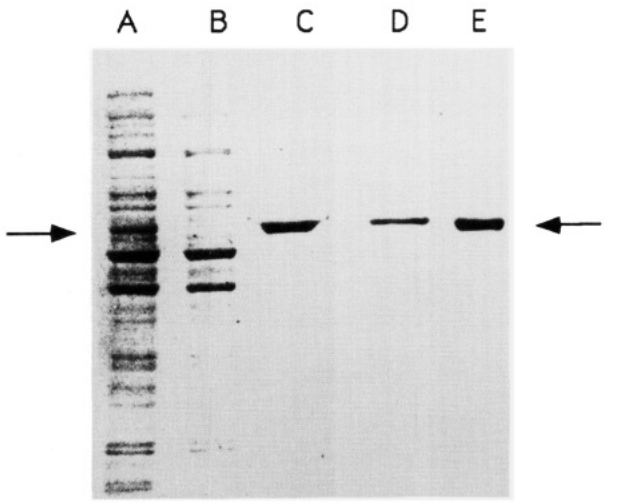

FIGURE 2: Coomassie Blue-stained SDS–polyacrylamide electropherograms of crude and purified hGR fractions. The samples were treated with dithioerythritol prior to electrophoresis in order to break the intersubunit disulfide bridge, Cys90-Cys90′. Lane A: Crude extract of *E. coli* SG5 transformed with p(wild-type hGR). Lane B: Protein fraction washed through the 2′,5′-ADP–Sepharose column. Lane C: Wild-type hGR eluted with 1 mM NADPH (see Table IA). Lane D: Wild-type hGR eluted from the immunoaffinity column (Table IB). Lane E: G446E/F447P-hGR as eluted from this column (Table IC). The arrows indicate the position of hGR isolated from human erythrocytes.

The preparation (940 units of hGR corresponding to 5.1 mg of enzyme protein) was >97% pure as verified by silver- or Coomassie Blue-stained SDS–polyacrylamide electropherograms (Figure 2C). It was crystallized as described for N-terminally truncated glutathione reductase, that is, in the presence of 0.6–1.0 M $(NH_4)_2SO_4$ (Bücheler et al., 1992).

Modifications for Large-Scale Preparations. When there initially was more than 500 g of cells (from a 50-L culture), the enzyme present in the combined extracts was precipitated by adding $(NH_4)_2SO_4$ to give a final concentration of 3 M. After the suspension stood for >1 h, the precipitate was pelleted by centrifugation (20 000*g*, 20 min, 4 °C) and then dialyzed exhaustively at 4 °C against buffer A. After a clearing spin, the sample was applied to the 2′,5′-ADP–Sepharose column. The additional step (ammonium sulfate precipitation and dialysis) allowed the reduction of the column size—1000 units of hGR was applied per milliliter of matrix—and it prevented the column from plugging.

Purification of Inactive hGR Species by Immunoadsorption Chromatography. The inactive mutant G446E/F447P-hGR, which does not bind to 2′,5′-ADP–Sepharose, was purified by immunoaffinity chromatography. For comparison, wild-type hGR was also isolated using this method (see Table I).

Buffers: 50 mM sodium phosphate, pH 7.2; 200 mM sodium borate, pH 10.0; 100 mM glycine hydrochloride, pH 2.5.

Coupling of Polyclonal Anti-hGR Immunoglobulins to Protein A–Sepharose CL4B According to Gersten and Marchalonis (1978). Protein A–Sepharose CL4B was swollen

Table I: Isolation Procedures for Wild-Type hGR (A and B) and for the Inactive Mutant G446E/F447P-hGR (C)[a]

	volume (mL)	total hGR (units)	total protein (mg)	specif activ (units/mg)	yield of hGR (%) activity	yield of hGR (%) protein
(A) Ligand Affinity Chromatography						
extract of 5 g of SG5 [p(wild-type hGR)]	25	1960	720	2.72	100	100
eluate from 2',5'-ADP–Sepharose	5.0	1850	10.2	181	94	94
(B) Immunoaffinity Chromatography						
extract of 10 mg of SG5 [p(wild-type hGR)]	0.5	3.54	1.60	2.25	100	100
eluate from antibody column	1.0	0.788	0.0145	51.2	22.3	74
(C) Immunoaffinity Chromatography						
extract of 100 mg of SG5 [p(G446E/F447P-hGR)]	5.0	<0.002	14.4	ND	ND	100
eluate from antibody column	1.0	<0.002	0.0156	ND	ND	~70

[a] Specif activ = specific activity = $k_{cat}/52.4$ [μmol/min·mg]; ND = not determinable. For hGR yields (last column), see text.

overnight at 4 °C in phosphate buffer, transferred to a chromatography tube to give a column volume of 1.5 mL, and washed with 5 column volumes of phosphate buffer. Rabbit anti-hGR serum (Hempelmann et al., 1987) diluted 3-fold with phosphate buffer was applied, and the column was left for 2 h at 25 °C. Subsequently, it was rinsed with buffer until the absorption of the effluent at 280 nm was less than 0.05. For cross-linking the immunoglobulins with the matrix, the column was equilibrated with borate buffer, pH 10.0, and then treated over a period of 30 min with 3 mL of dimethylsuberimidate (3 mg/mL in borate buffer) and washed with 7.5 mL of this buffer. It was left then for 18 h at 4 °C in borate buffer containing 1 M ethanolamine and subsequently washed with 7.5 mL of borate buffer, 30 mL of phosphate buffer, 4.5 mL of glycine hydrochloride buffer, and 7.5 mL of phosphate buffer.

Immunoadsorption Chromatography. Approximately 20 μg of wild-type or mutant hGR was chromatographed. This quantity is present in ca. 10 mg of SG5 transformed with p(wild-type hGR) and 100 mg of SG5 transformed with p(G446E/F447P-hGR), respectively. The SG5 pellet was taken up in 50 mM sodium phosphate, pH 7.2, to give a 4% cell suspension, sonicated, and cleared by centrifugation; this extract was applied immediately to the antibody column. After the column was washed with phosphate buffer until the ΔA_{280} value of the effluent fell below 0.05, the antigen was eluted with 2 column volumes of 100 mM glycine hydrochloride, pH 2.5; 100-μL fractions were collected in tubes containing 5 μL of 1 M Tris base. The fractions were monitored for GR activity, protein content, and FAD binding. In addition, they were subjected to immunoblotting.

RESULTS

Purification of Enzymatically Active Glutathione Reductase Species by 2',5'-ADP–Sepharose Chromatography. Wild-type hGR was isolated using a column which carries the NADP analogue adenosine 2',5'-bisphosphate as ligand (Table IA). The purification procedure exploits the facts that *E. coli* SG5 does not possess endogenous glutathione reductase (Greer & Perham, 1986) and that the hGR-encoding cDNA has been adapted to high-level expression (Bücheler et al., 1990, 1992). In short, after the cell extract was applied and the column was washed with concentrated salt solutions (up to 0.5 M KCl in buffer A), hGR was eluted with substrate (1 mM NADPH) at low ionic strength. Scale-up of the isolation procedure to 500 g of *E. coli* SG5 as starting material was achieved by including an ammonium sulfate precipitation step.

The protocols for expression and isolation of the mutants F447P-hGR and Δ(1–15)hGR are identical with those for wild-type hGR. This is consistent with the finding that the kinetic properties do not vary appreciably among these enzyme species (Table II). The high steady-state level of Δ(1–15)-hGR indicates that the absence of 15 N-terminal residues does not affect the in vivo stability of the enzyme.

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

hGR species	k_{cat} (min^{-1})	K_M(GSSG) (μM)	K_M(NADPH) (μM)
wild-type hGR (from erythrocytes)	8900	66	6.6
wild-type hGR (recombinant)	9500	65	6.6
Δ(1–15)hGR	10000	66	6.6
F447P-hGR	10000	77	6.4
G446E/F447P-hGR, G446P/F447P-hGR, or G446E-hGR	<1	ND	ND

[a] The k_{cat} values of hGR preparations vary between 8000 and 12 000 min^{-1} and increase by up to 40% on storage. ND = not determinable.

Isolation of Wild-Type hGR and G446E/F447P-hGR by Immunoadsorption Chromatography. Wild-type hGR was subjected to the procedure shown in Table IB in order to define the conditions for the isolation of those inactive hGR species which do not bind any specific ligand of the enzyme; a point in case is G446E/F447-hGR (Table IC). An extract containing 3.54 units of wild-type hGR was applied (Table IB). This corresponds to 19.6 μg of hGR, with the specific activity of the enzyme being 181 units/mg (Table IA). Pure protein (14.5 μg) was recovered after immunoaffinity chromatography (Table IB and Figure 2D). In contrast to this protein yield of 74%, only 22.3% of the enzyme activity was recovered. The low activity yield is due to the instability of hGR in the buffer used for eluting hGR from the antibody column (100 mM glycine hydrochloride, pH 2.5). Apart from collecting the column eluate in Tris base, which raises the pH to 7.3, no measures were taken to improve the yield in activity at this point.

With respect to the inactive mutant, G446E/F447P-hGR, immunoblots had shown that cell extracts contain ca. 10 times less reactive material when compared to extracts from cells producing active hGR species (Figure 3). Consequently, 10-fold starting material was used for the purification of G446E/F447P-hGR and processed as fast as possible (Table IC). The yield of 15.6 μg of pure protein compares well with the value of 14.5 μg obtained for wild-type hGR (Table IB), which means that ca. 70% of the inactive mutant was recovered after immunoaffinity chromatography; as shown in Figure 2E, the resulting protein of 52 kDa was >97% pure. The isolation procedure for inactive mutants can be scaled up by using (and reusing) 10 times larger columns.

Kinetic Parameters. As shown in Table II, human glutathione reductase samples isolated from red blood cells, recombinant wild-type hGR, N-terminally truncated hGR (Δ(1–15)hGR), and F447P-hGR have similar enzymic parameters. This proves that the N-terminal extension of wild-

Table III: Comparison of G446E/F447P-hGR with Recombinant Wild-Type hGR[a]

property	G446E/F447P-hGR		wild-type hGR
enzyme activity in *E. coli*	<0.2 mU/A_{600}		250 mU/A_{600}
binding to 2′,5′-ADP–Sepharose	<10 μg/mL gel		5000 μg/mL gel
specific activity of the enzyme isolated by affinity chromatography	not detectable		181000 mU/mg
reaction with anti-hGR serum		similar	
purification by immunoadsorption chromatography		similar	
specific activity of the enzyme isolated by immunoadsorption chromatography	<10 mU/mg		41200 mU/mg
flavin spectrum	absent		present
apparent M_r after SDS treatment	<52000		105000
apparent M_r after treatment with SDS and dithioerythritol	52000		52000
half-life in *E. coli* cells (Figure 2)	<4 min		>200 min
half-life in vitro in the presence of trypsin (hGR/trypsin, 20:1 w/w)	<10 min		>200 min

[a] The mutants G446E-hGR and G446P/F447P-hGR resemble G446E/F447P-hGR, whereas G446P-hGR appears to be indistinguishable from wild-type hGR.

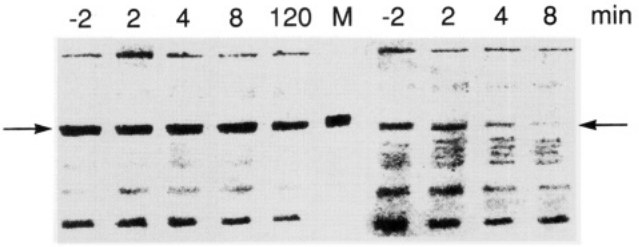

FIGURE 3: Immunoblots demonstrating the contrasting stability of wild-type hGR (left panel) and G446E/F447P-hGR (right panel) in vivo. To transformed *E. coli* SG5 growing in the logarithmic phase was added chloramphenicol (300 μg/mL) at zero time. Two minutes before (–2) and up to 120 min after the translation inhibitor was added, samples were withdrawn, mixed with trichloroacetic acid, and prepared for immunoblot analyses. The arrows point to the position of hGR; purified hGR was loaded on the marker lane (M). The nonspecific bands are due to antibodies against *E. coli* proteins of the serum. These bands become prominent only after prolonged exposure.

type hGR does not influence substrate binding nor catalysis. The hGR species with mutagenized residues in the contact helix were expected to show changes in k_{cat} and K_M for GSSG, since the orientation of the two interface domains relative to each other directly affects the catalytic sites as well as the binding sites for GSSG (Karplus et al., 1989). The slightly higher K_M value for GSSG of F477P-hGR is consistent with this prediction. More conspicuous effects of a mutation in this region were observed for *E. coli* glutathione reductase. When G418, the structural equivalent of G446 in hGR, was mutagenized to a tryptophan residue, the enzyme became cooperative (Hill coefficient 1.76) for glutathione binding (Scrutton et al., 1992; Berry et al., 1990).

Properties of the Inactive Double Mutant G446E/F447P-hGR. This protein was chosen for detailed studies because it could be isolated in higher yields than the other inactive hGR species, with mutations in the contact helices (G446E-hGR, G446P/F447P-hGR).

According to the data of Table III, there is no indication that any of the four structural domains along each polypeptide of the mutant is properly folded or that it interacts with its cognate partner (FAD, NADPH, GSSG, and the other subunit, respectively). Above all, the susceptibility to degradation points to the absence of folded domains. Whereas wild-type hGR can be incubated with proteases such as trypsin or subtilisin (at a w/w ratio of 20:1) for hours with no loss of activity, the mutant is degraded in less than 10 min without any evidence of a protease-resistant core. This means that the mutant protein behaves like SDS-denatured wild-type protein (Untucht-Grau, 1982).

In another set of experiments it was shown that the mutant hGR is also rapidly degraded in vivo (Figure 3). SG5[p(wild-type hGR)] and SG5[p(G446E/F447P-hGR)] were cultured for 5 h. Then, at $t = 0$ min, chloramphenicol was added to parallel cultures as an inhibitor of protein synthesis. Subsequently, samples were withdrawn at distinct time intervals and subjected to immunoblot analyses (Figure 3). In the case of the mutant, the immunoreactive material was shown to disappear in the cells with a half-life of less than 4 min, whereas wild-type hGR was stable for at least 120 min. This instability explains the apparently low yield of G446E/F447P-hGR (Table IC) when compared to wild-type hGR (Table IB).

Lack of Ligand Binding. After G446E/F447P-hGR was incubated (1–2 nmol/mL assay buffer) with 50 μM FAD and the excess FAD was removed by gel filtration, less than 0.01 μmol of FAD per micromole of protein subunit was present as shown by absorption measurements at 460 nm (Krohne-Ehrich et al., 1977) and by apoglutathione reductase complementation assays (Krauth-Siegel et al., 1987). The failure to bind cofactor cannot be ascribed solely to the transient exposure of G446E/F447P-hGR to pH 2.5 during the purification procedure. Wild-type hGR isolated by immunoaffinity chromatography was shown to incorporate more than 0.15 μmol of FAD per 1 μmol of subunit and regain activity correspondingly. The proposition that the FAD-binding domain does not adopt its native conformation in G446E/F447P-hGR is supported by the absence of the intersubunit disulfide bridge, Cys90–Cys90′ (Figure 1A). In SDS–polyacrylamide electropherograms (Figures 2E and 3), the mutant protein showed an apparent M_r of ca. 50 000, irrespective of preincubation with dithioerythritol. In contrast, wild-type apo-GR has an M_r of 104 000 in the absence of added thiols (Worthington & Rosemeyer, 1974) due to the covalent link between the subunits (Thieme et al., 1981; Untucht-Grau, 1982).

The inability of G446E/F447P-hGR to attach to adenosine 2′,5′-bisphosphate–Sepharose—behavior that was observed for both the crude extract and the purified protein (Table III)—strongly suggests that the NADPH-binding domain is not properly folded. This result was corroborated by preliminary equilibrium dialysis studies which showed that the dissociation constant K_d for NADPH was not measurable (that is, above 200 μM) in the case of G446E/F447P-hGR, whereas it was found to be 10 ± 2 μM with recombinant wild-type apoglutathione reductase (R. H. Schirmer, unpublished results). The FAD-free apoenzyme was used as a control because G446E/F447P-hGR does not bind FAD.

DISCUSSION

In our attempts to identify regions of human GR which are sensitive to dimerization inhibition, we chose the N-terminal and C-terminal sections of the polypeptide chain (Figure 1A).

The hypothesis that the N-terminal extension of 18 residues might play a role for the formation of the active dimer was falsified by constructing a deletion mutant which lacks the first 15 residues. This mutant, Δ(1–15)hGR, does not differ in yield, kinetic constants, or stability from wild-type hGR (Table II). In contrast, a number of mutations in the C-terminal region did affect dimerization. Here, in the interface domain, an area of 21 nm^2 around Gly446 forms a close contact with its counterpart in the other subunit (Karplus & Schulz, 1987).

Whereas the mutation F447P (plus F447′P) had little effect (Table II), G446E/F447P-hGR and G446E-hGR were found to be enzymically inactive. The point mutation G446E in hGR is reminiscent of the clinically important mutation F412V in a neuraminidase-protecting protein: this mutation impairs the dimerization of the protecting protein and renders it unstable (Zhou et al., 1991). A deficit of native structure is also apparent in the inactive hGR mutants. The data presented in Table III suggest for G446E/F447P-hGR that the FAD domain, the NADPH domain, and the interface domain do not recognize their cognate partners. By inference, it is unlikely that the small central domain (residues 294–364), whose shape is maintained by the other three domains in wild-type hGR (Thieme et al., 1981), adopts its native conformation in the mutant. Whether the individual domains contain residual structural motifs as is the case for the λ-repressor mutants described by Lim et al. (1992) remains to be studied. Physical studies on G446E/F447P-hGR appear to be feasible since even at a concentration of 50 μM (2.5 mg/mL) the mutant protein does not form precipitating aggregates in vitro or in vivo.

The nonnative structures of the dinucleotide-binding domains in the hGR mutant present an unexpected finding. These domains occur—in different orientations relative to each other—as modules in many different enzymes, with hGR, lipoamide dehydrogenase, mercuric ion reductase, and NADH oxidase being typical examples (Lohrer & Krauth-Siegel, 1990; Williams, 1992). Consequently, such modules are generally regarded as autonomous folding units (Schulz & Schirmer, 1979; Nilsson & Anderson, 1991). For a number of protein domains it has been shown that minor mutations in the domain core can prevent the polypeptide from folding (Reidhaar-Olson & Sauer, 1988; Dill, 1990; Nilsson & Anderson, 1991).

Our results suggest that one or two residues of a dimeric multidomain protein can affect the folding processes of *all* domains. Such pivotal sequences of a large protein may play a role in biological processes which require an unfolded form of the polypeptide(s) (Rothman, 1989). For instance, passing on a heptapeptide (Flynn et al., 1992) like the segment 443–449 of hGR in a relay team of chaperones (Langer et al., 1992) might be instrumental in distributing glutathione reductase species among intracellular compartments (Schirmer et al., 1989).

From a pharmacological viewpoint, our results demonstrate that there is a sensitive point of the hGR structure which can be considered as a target for dimerization inhibitors. Such compounds would lead not only to inactive monomers but also to degradation of the protein (Figure 3), as they disturb the order in an intersubunit core region (Dill, 1990; Lim et al., 1992). Consequently, dimerization inhibitors are likely to act catalytically in vivo so that the dosage can be smaller than anticipated for a stoichiometric inhibitor.

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