Structure of the NADPH-Binding Motif of Glutathione Reductase: Efficiency Determined by Evolution†

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ABSTRACT: The role of the second glycine residue (Gly-176) of the conserved GXGXXA "fingerprint" motif in the NADPH-binding domain of *Escherichia coli* glutathione reductase has been studied by means of site-directed mutagenesis. This glycine residue occurs at the N-terminus of the α-helix in the $\beta\alpha\beta$ fold that characterizes the dinucleotide-binding domain, in close proximity to the pyrophosphate bridge of the bound coenzyme. Introducing an alanine residue (G176A), the minimum possible change, at this position virtually inactivated the enzyme, as did the introduction of valine, leucine, isoleucine, glutamic acid, histidine, or arginine residues. Only the replacement by serine—a natural substitute for this glycine residue in some forms of mercuric reductase, a related flavoprotein disulfide oxidoreductase—produced a mutant enzyme (G176S) that retained significant catalytic activity. It is conceivable that this is due to a favorable hydrogen bond being formed between the serine hydroxyl and a pyrophosphate oxygen atom. In most of the mutant enzymes, the K_m for NADPH was substantially greater than that found for wild-type glutathione reductase, as expected, but this was accompanied by an unexpected decrease in the K_m for GSSG. The latter can be explained by the observation that the reduction of the enzyme by NADPH, the first half-reaction of the ping-pong mechanism, had become a rate-limiting step of the overall reaction catalyzed, albeit poorly, by the mutant enzymes. The marked preference for a glycine residue at the N-terminus of the α-helix in the $\beta\alpha\beta$ fold revealed by these experiments is in keeping with the view that the lack of a side chain at this position facilitates an electrostatic interaction between the dipole positive charge of the helix and the negative charge of the pyrophosphate bridge of the bound coenzyme. There was no evidence of a significant conformational change in any of the mutant proteins. Replacement of this glycine residue by alanine should act, therefore, as a safe diagnostic test for the involvement of a putative fingerprint motif in the binding of a dinucleotide by an unknown protein.

Glutathione reductase is a member of a family of homologous dimeric enzymes, the flavoprotein disulfide oxidoreductases, that includes, among others, dihydrolipoyl dehydrogenase (Williams, 1976), mercuric reductase (Fox & Walsh, 1983; Brown et al., 1983), and trypanothione reductase (Shames et al., 1986; Krauth-Siegel et al., 1987). For a recent review of their structures and properties, see Williams (1992). Glutathione reductase is particularly important for the biosynthesis of deoxyribonucleotides and for the maintenance of a high ratio of GSH/GSSG via the reaction

$$GSSG + NADPH + H^+ \leftrightarrow 2GSH + NADP^+$$

The three-dimensional structure of human erythrocyte glutathione reductase (Thieme et al., 1981; Karplus & Schulz, 1987) has been used for several years as a reference point for all the related proteins of the flavoprotein disulfide oxidoreductase family. Recently, the crystal structure of *Escherichia coli* glutathione reductase has been determined (Ermler & Schulz, 1991), as have the structures of dihydrolipoyl dehydrogenase (Mattevi et al., 1991, 1992), mercuric reductase (Schiering et al., 1991), and trypanothione reductase (Kuriyan et al., 1991; Hunter et al., 1992; Krauth-Siegel et al., 1993), and it has been possible to confirm the general structural similarity of these proteins implied by the similarity of their amino acid sequences. The cloned (Greer & Perham, 1986) and overexpressed (Scrutton et al., 1987) gene (*gor*) for *E. coli* glutathione reductase has been the subject of numerous studies of protein engineering that have pinpointed amino acid residues involved in the catalytic mechanism (Berry et al., 1989; Deonarain et al., 1990), in the dimerization process (Deonarain et al., 1992a,b; Scrutton et al., 1992), and in conferring coenzyme (Scrutton et al., 1990; Bocanegra et al., 1993) and substrate (Henderson et al., 1991) specificity.

The crystal structures of enzyme–NADPH complexes have also been determined for human (Pai et al., 1988) and *E. coli* (Mittl et al., 1993) glutathione reductase. In both enzymes the NADPH is bound in an extended conformation in a cleft that constitutes part of the active site, the majority of contacts being made with the NADPH-binding domain. This domain contains the characteristic $\beta\alpha\beta$ dinucleotide-binding fold that is a widespread feature of dinucleotide-binding enzymes (Rossmann et al., 1975; Wierenga et al., 1985). Residues from the FAD-binding domain, the central domain, and the interface domain of glutathione reductase contribute to the binding of the reduced nicotinamide moiety, generating the correct orientation needed for the transfer of electrons from it to the isoalloxazine ring of the enzyme-bound FAD.

The pyrophosphate bridge of the NADPH is bound at the C-terminal end of the first β-strand and the N-terminus of the succeeding α-helix in the $\beta\alpha\beta$ fold (Figure 1). As in many

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Table 1: Alignment of Some Sequences of Dinucleotide-Binding Domains Deviating from the Conserved Fingerprint

protein (coenzyme)	source	first residue	sequence	reference
glutathione reductase (NADPH)	*E. coli*	174	G A G Y I A V E L	Greer et al. (1986)
mercuric reductase (NADPH)	*Pseudomonas aeruginosa* Tn501	277	G S S V V A L E L	Brown et al. (1983)
mercuric reductase (NADPH)	*Shigella flexneri* R100	276	G S S V V A L E L	Misra et al. (1985)
mercuric reductase (NADPH)	*Thiobacillus ferrooxidans*	263	G S S V V A L E L	Inoue et al. (1989)
trypanothione reductase (NADPH)	*Trypanosoma congolense*	195	G G G F I S V E F	Shames et al. (1988)
6-phosphogluconate dehydrogenase (NADPH)	sheep	9	G L A V M G	Adams et al. (1991)
alcohol dehydrogenase (NAD)	yeast	177	G A A G G L G S L A	Jörnvall (1977)
malate dehydrogenase (NAD)	pig heart	10	G A A G Q I A Y S L	Joh et al. (1987)

FIGURE 1: Superposition based on the conserved glycine residues of the $\beta\alpha\beta$ folds from the coenzyme-binding domains of *E. coli* glutathione reductase (shown in yellow), horse liver alcohol dehydrogenase (blue), sheep 6-phosphogluconate dehydrogenase (red), and pig heart malate dehydrogenase (purple). The residue numbering and the position of the bound NADPH are taken from *E. coli* glutathione reductase. Although in 6-phosphogluconate dehydrogenase and malate dehydrogenase the glycine-rich sequence deviates from the conserved fingerprint (see Table 1), the region involved in the binding of the pyrophosphate bridge of the coenzyme is essentially superimposable. The figure was generated using the program MOLSCRIPT (Kraulis, 1991).

other dinucleotide-binding proteins (Rossmann et al., 1975; Wierenga et al., 1985), an electrostatic interaction between the negative charge of the pyrophosphate moiety and the dipole positive charge at the N-terminus of the α-helix can be inferred. The fingerprint motif GXGXXA(G), where X = any amino acid, is highly conserved among dinucleotide-binding proteins (Wierenga et al., 1985; Hanukoglu & Gutfinger, 1989; Scrutton et al., 1990). The first glycine residue of the fingerprint is responsible for the tight turn of the main chain at the end of the first β-strand and is retained in all known structures. The second glycine is at the N-terminus of the following α-helix and is highly conserved except in some mercuric reductases, where it is replaced by a serine residue, and in sheep 6-phosphogluconate dehydrogenase, where there is an inversion in the order of the second and third conserved residues (GXAXXG) of the fingerprint (Table 1). The absence of a side chain is thought to facilitate the electrostatic interaction with the coenzymes (Wierenga et al., 1985). The third conserved residue is either an alanine (mostly NADPH-binding proteins) or a glycine (mostly NADH-binding proteins), and in glutathione reductase the presence or absence of the side-chain methyl group has been demonstrated to dictate different sets of hydrogen bonds with the bound coenzyme that contribute toward coenzyme specificity (Scrutton et al., 1990; Mittl et al., 1993). This is in accord with a detailed analysis of the structural relationship between coenzyme specificity and sequence patterns generally in the nucleotide-binding fold (Baker et al., 1992).

Although mutagenesis studies have been carried out on the first glycine residue of the conserved motif (Chen et al., 1990; Ma et al., 1992), a systematic study of the role of the second glycine residue has not hitherto been attempted. In order to investigate the effects of introducing other amino acid side chains at this position, we have generated eight point mutations at this site, Gly-176, in the gene (*gor*) encoding *E. coli* glutathione reductase and studied the effects of the changes on the binding of NADPH and the catalytic activity of the enzyme. The residues substituted (alanine, serine, valine, leucine, isoleucine, glutamic acid, histidine, and arginine) were deliberately chosen to change systematically the size and charge of the side chain at this position. With one exception, the mutant enzymes were found to retain little catalytic activity. Some mutations inhibited the enzyme without substantially decreasing its affinity (as judged by the values of K_m) for the dinucleotide, and there were changes in the rate-determining step in the catalytic mechanism that manifested themselves as major decreases in the value of K_m for the other substrate, GSSG.

MATERIALS AND METHODS

Materials. Complex bacteriological media were from Difco Laboratories, and all media were prepared as described in Maniatis et al. (1982). [^{35}S]dATP-αS triethylammonium salt (400 Ci/mmol) for DNA sequencing was supplied by Amersham International. [1-^{2}H]-D-Glucose (>98 atm % excess ^{2}H) was purchased from Aldrich. Ethidium bromide, NADPH, $NADP^+$, NAD^+, and GSSG were from Sigma. Ultrapure agarose, dithiothreitol, and CsCl were from Bethesda Research Labs. All other chemicals were of analytical grade wherever possible. Glass-distilled water was used throughout. The restriction enzymes *Eco*RI and *Hin*dIII, calf intestinal alkaline phosphate, glucose 6-phosphate de-

Table 2: Apparent Kinetic Parameters for Wild-Type and Mutant Glutathione Reductases[a]

enzyme	K_m for NADPH[b] (μM)	K_m(mut)/K_m(WT)	k_{cat} (min^{-1})	% of WT	k_{cat}/K_m (min$^{-1}\cdot\mu$M)	% of WT	K_m for GSSG[c] (μM)	K_m(WT)/K_m(mut)
wild type	19 ± 2.7	1	32000	100	684	100	97 ± 9.1	1
G176A	334 ± 51	17.5	18	0.06	0.05	0	<0.2[d]	>485
G176S	39 ± 5.5	2.1	4986	15.6	131	19	2.86 ± 0.34	33
G176V	>2500[e]	>130	ND[f]	ND[f]	ND[f]	ND[f]	0.2 ● 0.05	485
G176L	627 ● 140	33	603	1.9	0.96	0.1	3 ± 0.6	32
G176I	31 ± 2.8	1.63	49	0.15	0.63	0.09	16.4 ± 2.2	5.9
G176E	192 ± 27	10	74	0.23	0.39	0.06	0.49 ± 0.09	198
G176H	921 ± 158	48.5	229	0.71	0.25	0.04	0.2 ± 0.04	485
G176R	>2500[e]	>130	ND[f]	ND[f]	ND[f]	ND[f]	11 ± 0.47	8.8

[a] Glutathione reductase activity was measured at 30 °C in 100 mM potassium phosphate buffer, pH 7.5. ND, not determined. [b] The concentration of GSSG was 0.012 or 1.2 mM, and the concentration of NADPH was varied between 10 and 750 μM. [c] The concentration of NADPH was 200 μM, and the concentration of GSSG was varied between 0.02 μM and 1.2 mM. [d] The apparent value of K_m for GSSG could not be measured since discrimination in rate could not be achieved even at GSSG concentrations as low as 0.02 μM. [e] The apparent value of K_m for NADPH could not be measured because saturating levels of NADPH could not be reached. [f] These kinetic parameters could not be determined because saturating levels of NADPH could not be reached.

hydrogenase from *Leuconostoc mesenteroides*, and yeast hexokinase were purchased from Boehringer Mannheim. T4 polynucleotide kinase was from Pharmacia. T4 DNA ligase was supplied by Promega.

Site-Directed Mutagenesis and DNA Sequencing. A derivative of M13 containing the noncoding strand of the *gor* gene (K19*gor3′δEco*RI) was used to carry out the mutagenesis (Deonarain et al., 1989). The mutagenic oligonucleotides used were 5′-TTGGCGCGGCTACATCG-3′ (G176A), 5′-TTGGCGCGGTTACATCG-3′ (G176V), 5′-TTGGCGCGGATACATCG-3′ (G176E), 5′-TTGGCGCGCATACATCG-3′ (G176L), 5′-TTGGCGCGCTTACATCG-3′ (G176R), 5′-TTGGCGCGCCTACATCG-3′ (G176H), 5′-TTGGCGCGAGTACATCG-3′ (G176S), and 5′-TTGGCGCGATTACATCG-3′ (G176I). The mismatched bases are underlined. The mutants were constructed by using the phosphorothioate method (Taylor et al., 1988), as supplied by Amersham International. Putative mutants were screened by dideoxy sequence analysis using the T7 sequencing system purchased from Pharmacia. The whole of the mutated gene was then resequenced to ensure that no mutations had been introduced elsewhere during the mutagenesis procedure. Mutant genes were excised by restricting the bacteriophage RF DNA with *Eco*RI and *Hin*dIII, and the *gor* gene fragment was subcloned into the expression vector pKK223-3 restricted with the same enzymes, as described by Scrutton et al. (1987). Constructs were transformed into a *lac* I^q strain (NS3) of *E. coli* carrying a chromosomal deletion of the *gor* gene (Deonarain et al., 1989).

Growth of Cells and Purification of Glutathione Reductase. Wild-type and mutant glutathione reductases were purified from the *gor*-deletion *lac* I^q strain of *E. coli* (NS3) transformed with the appropriate expression plasmid according to the method described by Berry et al. (1989).

Preparation of (4S)-[4-^{2}H]NADPH. NADP$^+$ was reduced in the presence of [1-^{2}H]-D-glucose 6-phosphate by the action of *L. mesenteroides* glucose 6-phosphate dehydrogenase in 10 mM TEA·HCl buffer, pH 7.8 (Vanoni et al., 1990). The reaction was monitored spectrophotometrically at 380 nm (ϵ_{380} for NADPH = 1230 M$^{-1}\cdot$cm^{-1}) and the pH maintained at 7.8 by the addition of 1 M NaOH. At the end of the reaction, the solution was filtered through a 10K exclusion-size Filtron macrosep centrifugal concentrator and diluted to 10 mL with water. (4*S*)-[4-^{2}H]NADPH was then purified from residual oxidized nucleotide on a FPLC Mono Q anion-exchange column equilibrated in 10 mM TEA·HCl buffer, pH 7.8, and eluted isocratically as described by Orr and Blanchard (1984). The column effluent was monitored at 380 nm, and peak fractions were diluted 1:20 and their spectra recorded on a Hewlett-Packard HP 8452A diode array spectrophotometer. Only fractions with an $A_{260}/A_{340} \leq 2.4$ were pooled, and they were used within 24 h. [1-^{2}H]-D-Glucose 6-phosphate was prepared from [1-^{2}H]-D-glucose by phosphorylation with yeast hexokinase and MgATP (Vanoni et al., 1990). It was purified by adsorption to Dowex 1-X8 (Cl$^-$ form, 200–400 mesh) and elution with 10 mM HCl (Bartlett, 1959).

Measurements of Kinetic Parameters and Data Analysis. Glutathione reductase activity was measured at 30 °C in 0.1 M potassium phosphate buffer, pH 7.5. The concentration of GSSG, when kept constant, was 10- and 100-fold the apparent K_m values for GSSG of each mutant, whereas the concentration of NADPH, when kept constant, was 200 μM. Initial velocities were measured by monitoring the decrease in absorbance at 340 nm of the reduced pyridine nucleotide (ϵ_{340} = 6220 M$^{-1}\cdot$cm^{-1}) in a Hewlett-Packard HP 8452A diode array spectrophotometer and were plotted against the concentration of the varied substrate (GSSG, NADPH, and (4*S*)-[4-^{2}H]NADPH). The kinetic parameters (V and V/K) were calculated by fitting the data, using the least-squares method, to the equation $v = VA/(K + A)$. Isotope effects were calculated by dividing the kinetic parameters determined in the presence of protium-containing substrate by those determined in the presence of deuterium-containing substrate (Northrop, 1975).

Thermal Stability. Wild-type and mutant *E. coli* glutathione reductases were incubated for 10 min at several temperatures in the range of 0–100 °C. Glutathione reductase activity was then measured at 30 °C in potassium phosphate buffer, pH 7.5. The concentrations of NADPH and GSSG for the assays were 200 μM and 1.2 mM, respectively.

RESULTS

Measurements of Apparent Kinetic Parameters. The first mutation carried out was the replacement of glycine-176 by alanine (G176A). Although this substitution is the minimal possible change, the introduction of a methyl group as a side chain at this position was sufficient essentially to inactivate the enzyme: the apparent K_m for NADPH was more than 15-fold higher and the k_{cat} 1700-fold lower than the respective values for wild-type glutathione reductase (Table 2). The catalytic efficiency ($k_{cat}/K_{m\ NADPH}$) was thus 13 000 times lower than that of the wild-type enzyme. Of the other mutations (Table 2), the replacement of the glycine residue

by serine, G176S, was found to be the least damaging. The G176S mutant retained 19% of the wild-type catalytic efficiency, the apparent K_m for NADPH (39 μM) being similar to that of the wild-type enzyme (19 μM). This is an interesting result, since in some mercuric reductases serine is a natural substitute for this second glycine residue of the fingerprint (Table 1).

All the other substitutions effectively inactivated the enzyme (Table 2). Surprisingly, however, replacing the glycine residue by amino acids with different branched side chains conferred differing characteristics on the resultant proteins. The substitution of Gly-176 by valine (G176V) effected a marked increase in K_m for NADPH, so marked that it was impossible to evaluate it because saturating concentrations of the coenzyme could not be reached. The G176I mutant retained an apparent K_m for NADPH (31 μM) similar to that of wild-type glutathione reductase (19 μM), whereas that of the G176L mutant (627 μM) was 33 times higher. However, the catalytic efficiencies of the G176L and G176I mutants were both <2% that of the wild-type enzyme. Thus, assuming that the low K_m for NADPH in the G176I mutant means that the binding of NADPH has been little affected, the orientation of the bound dinucleotide in its changed environment is presumably not compatible with efficient electron transfer from the reduced nicotinamide moiety to the enzyme-bound flavin.

In spite of the inherent electrostatic repulsion between the negative charge of the glutamate side chain and the pyrophosphate moiety of the incoming coenzyme, the apparent K_m for NADPH for the G176E mutant was only 10-fold higher than that of the wild-type enzyme, but the k_{cat} was very low (0.23% that of the wild type). The replacement of Gly-176 with histidine (G176H), a bulkier residue with a protonatable side chain, caused a further increase of the apparent K_m for NADPH and likewise a decrease in the catalytic activity. Finally, for the G176R mutant, the presence of the larger side chain that might hinder the binding of the coenzyme was not offset by any effect of its positive charge interacting with the negative charge of the pyrophosphate moiety of the dinucleotide. Indeed, the K_m for NADPH was too high for it to be determined.

Thermal Stability. In order to test if the replacements of Gly-176 had induced any significant conformational change, all the mutant enzymes were incubated for 10 min at several different temperatures in the range of 0–100 °C. Glutathione reductase activity was then measured at 30 °C except for the G176V mutant, for which the very low enzymatic activity and high K_m for NADPH precluded this. The mutant proteins otherwise were all found to undergo cooperative thermal denaturation at the same temperature (approximately 65 °C) as wild-type *E. coli* glutathione reductase (Figure 2). The lack of change in thermal stability strongly suggests that none of the mutations has led to a major conformational change in the enzymes. This is in keeping with the fact that all the enzymes could be purified by means of the same experimental procedure, which also suggests that the proteins were not significantly different in three-dimensional structure.

Values of the Apparent K_m for GSSG. All the mutant enzymes were found to exhibit lower values of the apparent K_m for GSSG than for wild-type glutathione reductase (Table 2). Indeed, the apparent K_m for GSSG for the G176A mutant could not be determined as discrimination in rate could not be achieved at concentrations of GSSG as low as 0.02 μM. The apparent K_m values for GSSG for the G176V and G176H mutants were more than 400 times lower than that of the wild

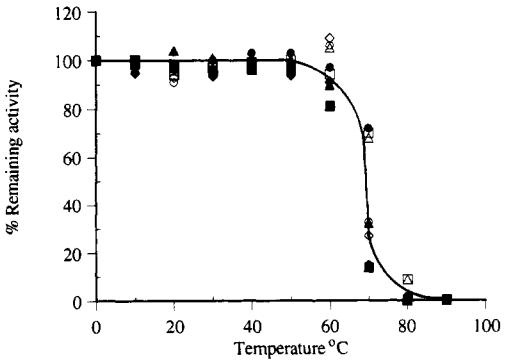

FIGURE 2: Thermal stability of wild-type and mutant *E. coli* glutathione reductases. The enzymes were incubated for 10 min at various temperatures. Glutathione reductase activity was measured at 30 °C in 100 mM phosphate buffer, pH 7.5, in 200 μM NADPH and 1.2 mM GSSG. Symbols: (○) wild type; (●) G176A; (△) G176E; (▲) G176H; (□) G176I; (■) G176L; (◇) G176R; (◆) G176S.

type, whereas the apparent K_m for the G176E mutant was 200-fold lower. For the G176L, G176S, G176I, and G176R mutants, the apparent K_m values for GSSG were somewhat bigger, but still lower than that of wild-type glutathione reductase by factors of 32, 33, 6, and 9, respectively.

Certain replacements of the neighboring amino acid (Tyr-177) in the NADPH-binding site of *E. coli* glutathione reductase also produce mutant enzymes (Y177G, Y177S) that exhibit a lower K_m for GSSG (Berry et al., 1989). Moreover, the Y177G and Y177S mutant enzymes display a switch of kinetic mechanism, from ping-pong to ordered sequential. However, the double-reciprocal kinetic plots for the G176S mutant showed clearly parallel lines, as expected for a ping-pong mechanism (Figure 3). The high K_m for NADPH and the low k_{cat} and K_m for GSSG prevented the determination of the kinetic mechanism for the other mutant proteins. The decrease in the K_m values for GSSG accompanying these mutations in the NADPH-binding site is at first sight unexpected, because NADPH and GSSG bind in physically separate sites on the enzyme with the isoalloxazine ring of the FAD in between (Karplus & Schulz, 1987) and there was no evidence (see above) for the occurrence of major conformational changes in the mutant proteins. Thus it was unlikely that any of the mutations increased the affinity for GSSG by causing a physically remote change in the local structure of the substrate-binding site. On the other hand, in a ping-pong kinetic mechanism, as displayed by *E. coli* glutathione reductase, the lower K_m for GSSG displayed by the mutant proteins can be explained on the basis of the theoretical model proposed by Matthews (1990). Thus, if a mutation in the binding site of one substrate causes a decrease in the catalytic velocity of one of the two half-reactions that constitute the mechanism, there is a consequent reduction in the value of K_m for the substrate of the half-reaction that becomes less rate-limiting.

Primary Deuterium Kinetic Isotope Effects for Wild-Type and Mutant Glutathione Reductases. In order to determine whether the mutations in *E. coli* glutathione reductase had led to falls in the rate of the reduction of the enzyme by NADPH, primary deuterium kinetic isotope effects for NADPH were determined at saturating levels of GSSG. When (4*S*)-[4-^{2}H]NADPH was used as the variable substrate, only a small primary deuterium isotope effect on V (^DV = 1.45) was observed with wild-type glutathione reductase (Table 3). This value is in good agreement with that previously obtained for the same enzyme (Vanoni et al., 1990). However, most

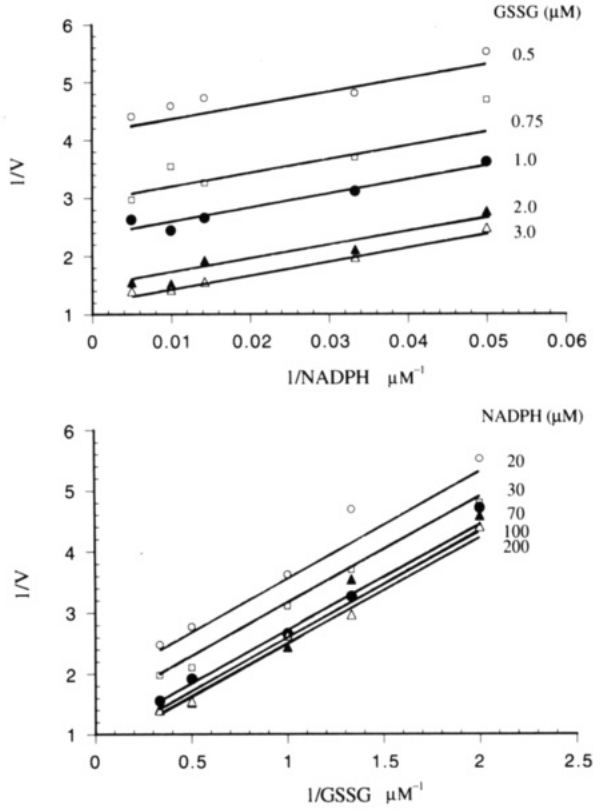

FIGURE 3: Double-reciprocal plots of $1/v$ against $1/[NADPH]$ and $1/v$ against $1/[GSSG]$ for the G176S mutant of glutathione reductase. Initial velocities (arbitrary units) were measured with a 5×5 matrix of substrate concentrations at 30 °C in 100 mM potassium phosphate buffer, pH 7.5. The results were analyzed by least-squares regression analysis (Cleland, 1979). The concentrations of the fixed substrate are indicated.

Table 3: Primary Deuterium Kinetic Isotope Effects for Wild-Type and Mutant Glutathione Reductase[a]

enzyme	$^{D}V/K$[b]	^{D}V[c]	enzyme	$^{D}V/K$[b]	^{D}V[c]
wild type	1.15	1.45	G176I	0.82	1.08
G176A	1.04	2.31	G176E	0.98	1.3
G176S	1.66	1.95	G176H	1.51	8.5
G176V	ND[d]	ND[d]	G176R	ND[d]	ND[d]
G176L	2.01	4.77			

[a] Glutathione reductase activity was measured at 30 °C in 100 mM potassium phosphate buffer, pH 7.5. The concentrations of substrates were varied in Table 2. [b] Isotope effect on $V/K = (V/K)_H/(V/K)_D$. [c] Isotope effect on $V_{max} = (V_{max})_H(V_{max})_D$. [d] These values could not be determined (see Table 2).

of the mutant enzymes exhibited much higher deuterium isotope effects on V (up to a maximum ^{D}V value of 8.5 measured for the G176H mutant). This suggests that in most of the mutant proteins the reduction of the enzyme had become more rate-limiting in the overall reaction, thereby allowing the isotope effect, previously silent, to become partly expressed.

The increase in K_m for NADPH was correlated with an increase in the isotope effect on V_{max} (Figure 4). Since the K_m for NADPH, the first substrate to bind, is dependent on both binding and catalytic steps, whereas ^{D}V depends on all the steps after the binding of the labeled substrate, the increase in K_m accompanying the increase in ^{D}V is mainly due to a slower rate of enzyme reduction rather than to an increase in the dissociation constant for NADPH. Assuming that the intrinsic isotope effect on the C–H (^{2}H) bond-breaking step for NADPH is not changed by the above mutations, its value for wild-type *E. coli* glutathione reductase is ≥8.5. The majority of the mutants showed a deuterium isotope effect on

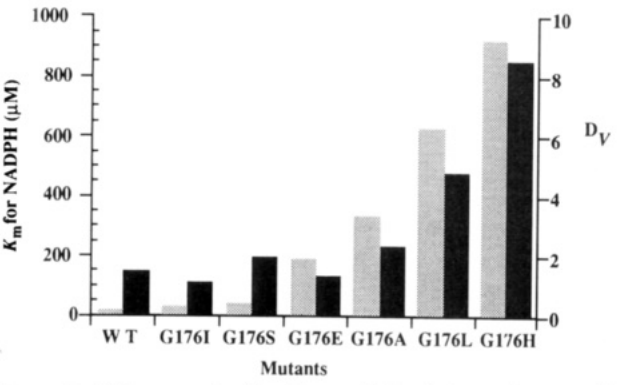

FIGURE 4: Histogram showing the correlation between increases in the apparent K_m values for NADPH and increases in the primary deuterium kinetic isotope effects on the maximal velocity for wild-type and mutant glutathione reductases. Bars: (shaded) K_m for NADPH; (solid) ^{D}V.

the slope of the reciprocal plots ($^{D}V/K$) very close to 1.0, as found for the wild-type protein. Only for the G176S, G176L, and G176H mutants were the $^{D}V/K$ values (1.6, 2.0, and 1.5, respectively) significantly greater than 1.0.

DISCUSSION

In spite of the great structural similarity exhibited by the $\beta\alpha\beta$ dinucleotide-binding folds (Rossmann et al., 1975; Wierenga et al., 1985), only a short amino acid sequence is highly conserved, the GXGXXG(A) motif (Table 1). The first glycine residue is retained in all known primary structures [in the NAD-linked yeast alcohol dehydrogenase and pig heart malate dehydrogenase, the second intervening alanine residue (Table 1) can be viewed as an insertion that has subtle but important effects on the binding of the coenzyme adenine ribose (Baker et al., 1992)]. The second glycine residue has been replaced by serine in some mercuric reductases and by alanine in sheep 6-phosphogluconate dehydrogenase, although in the latter, unusual for NADPH-linked enzymes, the consensus sequence differs also by having a glycine residue instead of an alanine in the third conserved position (Table 1).

In several dinucleotide-binding proteins of unknown three-dimensional structure, it has been proposed that sequences rich in glycine residues are involved in the binding of the coenzyme because of their homology with the consensus fingerprint. Mutagenesis studies on the first of the conserved glycine residues, which showed that the mutant enzymes displayed either a fall in or an absence of catalytic activity, were used to justify the involvement of these sequences in binding the dinucleotide (Chen et al., 1990; Ma et al., 1992). Although it may be acceptable to replace the first glycine residue to assess the involvement of a putative sequence motif in dinucleotide binding, it has to be borne in mind that in the Rossmann fold this residue displays positive ϕ and φ angles and has an important structural role, being responsible for a tight turn between the first β-strand and the following α-helix of the $\beta\alpha\beta$ fold (Wierenga et al., 1985). Replacement of this residue by site-directed mutagenesis could thus cause a damaging distortion of the three-dimensional structure of the mutant enzyme. On the other hand, the second glycine residue is at the N-terminus of the α-helix and in close proximity to the pyrophosphate bridge of the bound coenzyme. The absence of a side chain at this position has more of a functional than a structural role (Wierenga et al., 1985). This is confirmed by its negative ϕ and φ angles. Thus, replacement of this glycine residue might be expected to interfere with the binding

of NAD(P), without significantly changing the structure of the folding unit.

For these reasons, we have focused on the second glycine residue, Gly-176, of the conserved sequence motif in *E. coli* glutathione reductase. This residue has been replaced by eight different amino acids in order to investigate the effect on the binding of the coenzyme of systematically changing the size and charge of the side chain at this site (Table 2). Introducing even a methyl group at position 176 (G176A) was sufficient to produce an inactive enzyme, presumably by disruption of the potential electrostatic interaction between the α-helix dipole and the pyrophosphate bridge of the incoming NADPH. This is consistent with predictions based on inspection of the crystal structure of the *E. coli* glutathione reductase–NADPH complex (Mittl et al., 1993) and the observations of Pai et al. (1988) on human glutathione reductase: the approach of Gly-176 (Gly-196 in human glutathione reductase) to the pyrophosphate bridge of the dinucleotide is so close that a larger side chain at this position could not easily be accommodated (see Figure 1). Thus, it would appear that the electrostatic interaction is of major importance in the correct binding of NADPH. Further evidence of this can be inferred from the low number and weakness of the hydrogen bonds formed between the pyrophosphate of the coenzyme and the main-chain amide protons.

It is interesting that the replacement of Gly-176 by serine—a natural substitute for this glycine residue in some forms of the related flavoprotein disulfide oxidoreductase, mercuric reductase (Table 1)—produced the only mutant enzyme (G176S) that retained significant catalytic efficiency (19% of the wild type). The proposed electrostatic interaction between the protein α-helix and the pyrophosphate of NADPH should be disfavored by the presence of the larger amino acid side chain, but the binding of the dinucleotide may be stabilized by other interactions. Although no crystal structure is available of a mercuric reductase with a serine residue at this position, it is conceivable, for example, that a hydrogen bond forms between the serine hydroxyl and a pyrophosphate oxygen atom. Close scrutiny of the crystal structure of *E. coli* glutathione reductase indicates that a serine residue at position 176 could form a hydrogen bond with one or the other or both of two phosphate oxygen atoms in the pyrophosphate bridge of the bound coenzyme, but further consideration of this must await a structure determination of the G176S mutant. Other changes to the size and nature of the side chain generated enzymes that were virtually inactive, and in the valine (G176V) and arginine (G176R) mutants the affinity for the coenzyme was so low that the K_m for NADPH could not be determined (Table 2). Surprisingly, the replacement of Gly-176 with isoleucine caused only a small increase in the K_m for NADPH, but the very low value of k_{cat} exhibited by this mutant is indicative of a large increase in the energy of the transition state, suggesting a distorted binding of the coenzyme and an incorrect orientation for electron transfer to the enzyme-bound flavin.

With (4*S*)-[4-^{2}H]NADPH as substrate, most of the mutant enzymes exhibited primary deuterium isotope effects on V_{max} higher than those of wild-type glutathione reductase (Table 3), and the increase in K_m for NADPH displayed by the mutants was correlated with the increase in the isotope effect (Figure 4). The increase in the isotope effect on V_{max} for the mutants indicates that the reduction of the enzymes by NADPH, the first half-reaction of the ping-pong mechanism (Figure 3), has become a rate-limiting step of the overall reaction. As demonstrated by Matthews (1990), for an enzyme with a ping-pong mechanism, a decrease in the catalytic velocity of one of the two half-reactions will manifest itself in a decrease in the K_m for the substrate of the other half-reaction. Given that none of the mutations appears to have caused a significant change in the conformation of the protein (Figure 2), this offers a plausible explanation of the decreases in the K_m for GSSG exhibited by the mutant enzymes (Table 2).

From our experiments, it is clear that there is a marked preference for a glycine residue at the N-terminus of the α-helix in the $\beta\alpha\beta$ fold of the NADPH-binding domain of *E. coli* glutathione reductase. The lack of a side chain at this position allows the desired electrostatic interaction between the α-helix dipole and the pyrophosphate moiety of the coenzyme, enabling the NADPH to adopt the correct orientation for the subsequent electron transfer to the enzyme-bound FAD. The glycine-rich sequence, -GXGXXG(A)-, has evidently evolved as the most effective structure to optimize the catalytic efficiency of perhaps otherwise unrelated dinucleotide-binding proteins. In order to assess the involvement of a glycine-rich sequence in the binding of the dinucleotide in a putative dinucleotide-binding protein, it is suggested that the second glycine residue of the motif be replaced with an alanine. This minimal substitution should not significantly alter the local enzyme structure, and any loss of catalytic activity would add weight to other results that implicate the protein in the binding of coenzyme.

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REFERENCES

Adams, M. J., Gover, S., Leaback, R., Phillips, C., & Somers, D. O'N. (1991) *Acta Crystallogr. B47*, 817–820.

Baker, P. J., Britton, K. L., Rice, D. W., Rob, A., & Stillman, T. J. (1992) *J. Mol. Biol. 228*, 662–671.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 459–465.

Berry, A., Scrutton, N. S., & Perham, R. N. (1989) *Biochemistry 28*, 1264–1269.

Bocanegra, J. A., Scrutton, N. S., & Perham, R. N. (1993) *Biochemistry 32*, 2737–2740.

Brown, N. L., Ford, S. J., Pridmore, R. D., & Fritzinger, D. C. (1983) *Biochemistry 22*, 4089–4095.

Chen, Z., Lu, L., Lee, W. R., & Chang, S. (1990) *Biochemistry 29*, 1112–1118.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Deonarain, M. P., Berry, A., Scrutton, N. S., & Perham, R. N. (1989) *Biochemistry 28*, 9602–9607.

Deonarain, M. P., Scrutton, N. S., & Perham, R. N. (1992a) *Biochemistry 31*, 1491–1497.

Deonarain, M. P., Scrutton, N. S., & Perham, R. N. (1992b) *Biochemistry 31*, 1498–1504.

Ermler, U., & Schulz, G. E. (1991) *Proteins: Struct., Funct., Genet. 9*, 174–179.

Fox, B. S., & Walsh, C. T. (1983) *Biochemistry 22*, 4082–4088.

Greer, S., & Perham, R. N. (1986) *Biochemistry 25*, 2736–2742.

Hanukoglu, I., & Gutfinger, T. (1989) *Eur. J. Biochem. 180*, 479–484.

Henderson, G. B., Murgolo, N. J., Kuriyan, J., Ospray, K., Kominos, D., Berry, A., Scrutton, N. S., Hinchcliffe, N. V., Perham, R. N., & Cerami, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8764–8768.

Hunter, W. N., Bailey, S., Habash, J., Harrop, S. J., Helliwell, J. R., Aboagye-Kwarteng, T., Smith, K., & Fairlamb, A. H. (1992) *J. Mol. Biol. 227*, 322–333.
Inoue, C., Sugawara, K., Shiratori, T., Kusano, T., & Kitagawa, Y. (1989) *Gene 84*, 47–54.
Joh, T., Takeshima, H., Tsuzuki, T., Setoyama, C., Shimada, K., Tanase, S., Kuramitsu, S., Kagamiyama, H., & Morino, Y. (1987) *J. Biol. Chem. 262*, 15127–15131.
Jörnvall, H. (1977) *Eur. J. Biochem. 16*, 25–40.
Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 701–729.
Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946–950.
Krauth-Siegel, R. L., Enders, B., Henderson, G. B., Fairlamb, A. H., & Schirmer, R. H. (1987) *Eur. J. Biochem. 164*, 123–128.
Krauth-Siegel, R. L., Sticherling, C., Jost, I., Walsh, C. T., Pai, E. F., Kabsch, W., & Lantwin, C. B. (1993) *FEBS Lett. 317*, 105–108.
Kuriyan, J., Kong, X.-P., Krishna, T. S. R., Sweet, R. M., Murgolo, N. J., Field, H., Cerami, A., & Henderson, G. B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8764–8768.
Ma, Q., Cui, K., Xiao, F., Lu, A. Y. H., & Yang, C. S. (1992) *J. Biol. Chem. 267*, 22298-22304.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mattevi, A., Schierbeek, A. J., & Hol, W. G. J. (1991) *J. Mol. Biol. 220*, 975–994.
Mattevi, A., Obmolova, G., Kalk, K. H., Sokatch, J., Betzel, C. H., & Hol, W. G. J. (1992) *Proteins: Struct., Funct., Genet. 13*, 336–351.
Matthews, R. (1990) in *Flavins and Flavoproteins* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 593–597, Walter de Gruyter, Berlin and New York.
Misra, T. K., Brown, N. L., Haberstroh, L., Schmidt, A., Goddette, D., & Silver, S. (1985) *Gene 34*, 253–262.
Mittl, P. R. E., Berry, A., Scrutton, N. S., Perham, R. N., & Schulz, G. E. (1993) *J. Mol. Biol. 231*, 1921–1925.
Northrop, D. B. (1975) *Biochemistry 14*, 2644–2651.
Orr, G. A., & Blanchard, J. S. (1984) *Anal. Biochem. 142*, 232–234.
Pai, E. F., Karplus, P. A., & Schulz, G. E. (1988) *Biochemistry 27*, 4465–4474.
Rossman, M. G., Liljas, A., Branden, C. I., & Banaszak, L. J. (1975) *Enzymes, 2nd Ed. 11*, 61–102.
Schiering, N., Kabsch, W., Moore, M. J., Distefano, M. D., Walsh, C. T., & Pai, E. F. (1991) *Nature 352*, 168–172.
Scrutton, N. S., Berry, A., & Perham, R. N. (1987) *Biochem. J. 245*, 875–880.
Scrutton, N. S., Berry, A., & Perham, R. N. (1990) *Nature 343*, 38–43.
Scrutton, N. S. Deonarain, M. P., Berry, A., & Perham, R. N. (1992) *Science 258*, 1140–1143.
Shames, S. L., Fairlamb, A. H., Cerami, A., & Walsh, C. T. (1986) *Biochemistry 25*, 3519–3526.
Shames, S. L., Kimmel, B. E., Peoples, O. P., Agabian, N., & Walsh, C. T. (1988) *Biochemistry 27*, 5014–5019.
Taylor, J. W., Schmidt, W., Cosstick, R., Okruszek, A., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8749–8764.
Thieme, R., Pai, E. F., Schirmer, R. H., & Schulz, G. E. (1981) *J. Mol. Biol. 151*, 763–782.
Vanoni, M. A., Wong, K. W., Ballou, D. P., & Blanchard, J. S. (1990) *Biochemistry 29*, 5790–5796.
Wierenga, R. K., De Maeyer, M. C. H., & Hol, W. G. J. (1985) *Biochemistry 24*, 1346–1357.
Williams, C. H., Jr. (1976) *Enzymes, 3rd Ed. 13*, 89–173.
Williams, C. H., Jr. (1992) in *Chemistry and Biochemistry of Flavoproteins* (Müller, F., Ed.) Vol. III, pp 121–211, CRC Press, Boca Raton, FL.