Switching Kinetic Mechanism and Putative Proton Donor by Directed Mutagenesis of Glutathione Reductase†

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ABSTRACT: By directed mutagenesis of the cloned *Escherichia coli gor* gene encoding the flavoprotein glutathione reductase, Tyr-177 (the residue corresponding to Tyr-197 in the NADPH-binding pocket of the homologous human enzyme) was changed to phenylalanine (Y177F), serine (Y177S), and glycine (Y177G). The catalytic activity of the Y177F mutant was very similar to that of the wild-type enzyme, but that of the Y177S and Y177G mutants was substantially diminished. However, all three mutants retained the ability to protect the reduced flavin from adventitious oxidation, indicating that Tyr-177 does not act as a simple "lid" on the NADPH-binding pocket and that the protection of the reduced enzyme must be due largely to burial of the isoalloxazine ring in the protein. The wild-type enzyme and Y177F mutant displayed ping-pong kinetics, but the Y177S and Y177G mutants appeared to have switched to an ordered sequential mechanism. This could be explained by supposing that the enzyme normally functions by a hybrid kinetic mechanism and that the Y177S and Y177G mutations diverted flux from the ping-pong loop favored by the wild-type enzyme to an ordered sequential loop. The necessary change in the partitioning of the common E–NADPH intermediate could be caused by a slowing of the formation of the EH_2 intermediate on the ping-pong loop, or by the observed concomitant fall in the K_m for glutathione favoring flux through the ordered sequential loop. In another experiment, His-439, thought to act as a proton donor/acceptor in the glutathione-binding pocket, was mutated to a glutamine residue. The H439Q mutant retained ~1% of the catalytic activity of the wild-type enzyme, and the K_m for NADPH bound at a distant site in the enzyme was substantially lowered. Direct protonation of the histidine residue is evidently not essential for the enzymic reaction to occur, although this may be the favored mechanism in the wild-type enzyme. These experiments emphasize the possibility of unforeseen changes in mechanism in mutated enzymes.

Glutathione plays a critical role in the maintenance of reduced thiol groups in the cell and is of particular importance in the biosynthesis of DNA [for a review, see Holmgren (1985)]. Glutathione itself is maintained in a reduced form at the expense of NADPH by the action of the enzyme glutathione reductase (EC 1.6.4.2), one of the growing family of flavoprotein disulfide oxidoreductases. Glutathione reductase is a typical member of this family [others are dihydrolipoamide dehydrogenase (EC 1.8.1.4), an essential component of the 2-oxo acid dehydrogenase multienzyme complexes (Perham et al., 1978, 1987); mercuric reductase, part of a bacterial plasmid-encoded system for the detoxification of mercuric ions (Fox & Walsh, 1983; Brown et al., 1983); and trypanothione reductase, an analogue of glutathione reductase restricted to trypanosomatids (Shames et al., 1986; Krauth-Siegel et al., 1987)], in that it is a dimer with a molecular weight of about 110000 and possesses an intrachain disulfide bridge which is alternately oxidized and reduced as part of the novel catalytic mechanism [reviewed by Willams (1976)]. Considerable homology exists among these enzymes, as shown by a study of the amino acid sequences around the redox-active disulfide bridges (Perham et al., 1978; Williams et al., 1982; Packman & Perham, 1982; Kraugh-Siegel et al., 1982; Fox & Walsh, 1983; Brown et al., 1983; Shames et al., 1988). The best structural information available for any enzyme in this family is that for human glutathione reductase, for which the complete amino acid sequence (Krauth-Siegel et al., 1982) and a crystallographic structure to 1.5-Å resolution have been determined (Thieme et al., 1981; Karplus & Schulz, 1987), contributing to a detailed appreciation of the reaction mechanism (Williams, 1976; Pai & Schulz, 1983).

We have recently cloned and sequenced (Greer & Perham, 1986) and overexpressed (Scrutton et al., 1987) the *gor* gene encoding the glutathione reductase from *Escherichia coli*. A comparison of the primary structures of *E. coli* and human glutathione reductases reveals them to be homologous throughout, implying close similarities in the three-dimensional structures of the proteins (Greer & Perham, 1986). In the human enzyme, the side-chain of Tyr-197 lies in the NADPH-binding pocket, but it moves aside when NADPH is bound to allow the nicotinamide ring to come close to the FAD for electron transfer (Pai & Schulz, 1983). Tyr-177 of *E. coli* glutathione reductase is the counterpart of Tyr-197 in the human enzyme, situated in a highly conserved part of the sequence (Greer & Perham, 1986) (the difference in numbering is due to the omission from the *E. coli* enzyme of the N-terminal 17 amino acids of the human protein). It has been postulated (Rice et al., 1984) that this residue might act as a "lid" in the NADPH-binding pocket, shielding the isoalloxazine ring of the FAD and preventing loss of electrons from the reduced flavin. Similarly, a basic amino acid residue was implicated in dihydrolipoamide dehydrogenase as a proton donor/acceptor in the reduction of the substrate (Matthews et al., 1977), and X-ray crystallographic work showed that His-467 was suitably positioned in the glutathione-binding pocket of human glutathione reductase to act in this capacity (Pai & Schulz, 1983). The histidine residue at position 439 in *E. coli* glutathione reductase is equivalent to His-467 in the

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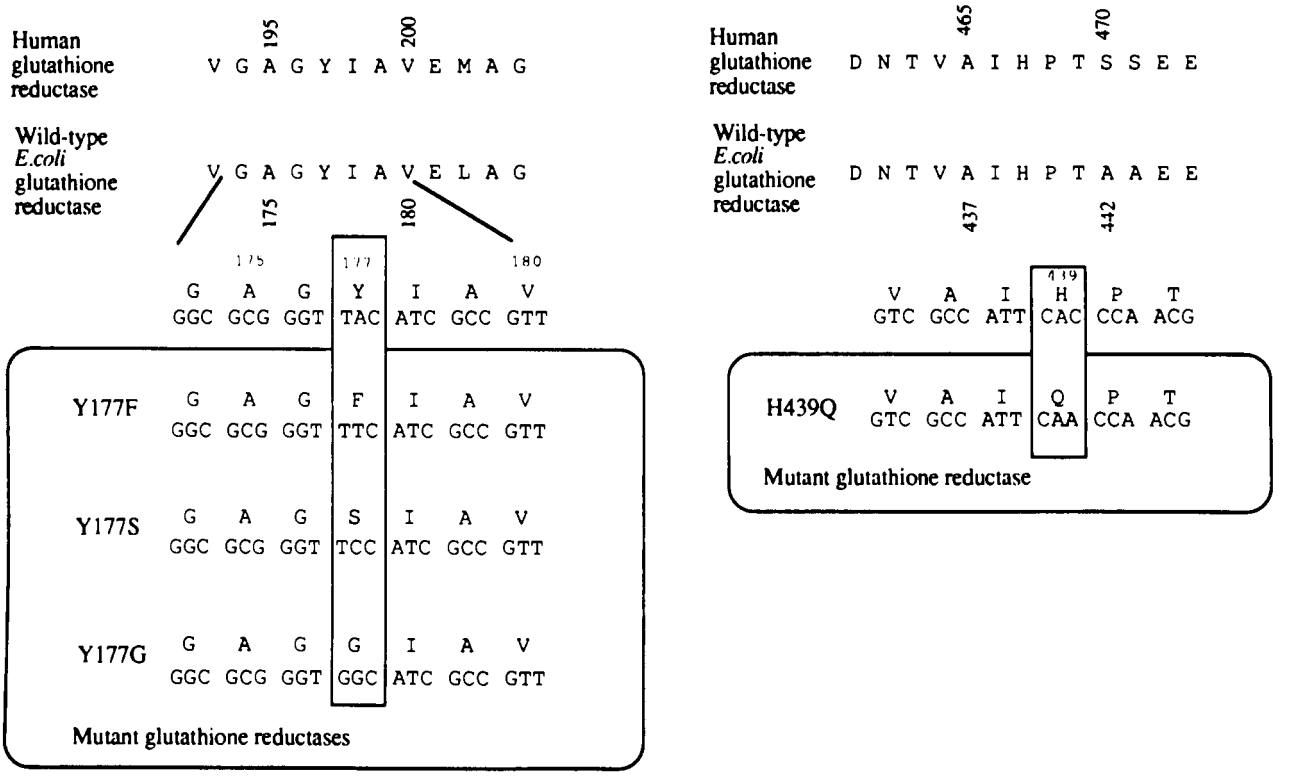

FIGURE 1: Homology between human and *E. coli* glutathione reductases and the mutations at positions 177 and 439 of *E. coli* glutathione reductase. Partial nucleotide sequences of the wild-type *gor* gene (Greer & Perham, 1986) and of its mutated forms are shown. Mutagenesis was carried out as described under Materials and Methods.

human enzyme and also lies within a highly conserved region of the protein sequence (Greer & Perham, 1986).

To explore the role of Tyr-177, we have generated three site-directed mutants of *E. coli* glutathione reductase at position 177: one (Y177F) of tyrosine to phenylalanine, one (Y177S) of tyrosine to serine, and the third (Y177G) of tyrosine to glycine. These mutations were designed to change systematically the hydrophobicity and size of the amino acid side chain at this site (Figure 1). We have also investigated the role of His-439 by mutating it to a glutamine residue (Figure 1). Both sets of mutations have unexpected effects on the mechanism, in the one case inducing a change of apparent kinetic mechanism from ping-pong to ordered sequential and in the other failing to bring about a complete inhibition of activity.

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) and [γ-^{32}P]ATP triethylammonium salt (>5000 Ci/mmol; for end labeling of oligonucleotides) were from Amersham International. Ethidium bromide, isopropyl β-thiogalactoside (IPTG), 5-bromo-4-chloroindol-3-yl β-galactoside (X-gal), NADPH, NADP$^+$, and oxidized and reduced glutathione (GSSG and GSH) were from Sigma Chemical Co. Deoxy- and dideoxynucleoside 5′-triphosphates used in DNA sequencing were from P-L Biochemicals. Ultrapure agarose, dithiothreitol, and cesium chloride were from Bethesda Research Laboratories. Sodium borohydride was obtained from BDH Chemicals Ltd. Glass-distilled water was used throughout.

The restriction enzymes *Hin*dIII, *Eco*RI, and *Bam*HI were purchased from New England Biolaboratories. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. T4 DNA ligase and the Klenow fragment of *E. coli* DNA polymerase I were generously made available by Dr. R. T. Hunt (Department of Biochemistry, University of Cambridge, U.K.). T4 polynucleotide kinase was from Amersham International.

Mutagenesis and DNA Sequencing. A 3.3-kbp DNA fragment containing the *gor* gene was excised from an M13mp8/*gor* clone (Scrutton et al., 1987) by digestion with *Bam*HI and *Eco*RI and directionally ligated into M13K19 (Carter et al., 1985) restricted with the same enzymes. The clone, designated K19gor, carrying the noncoding strand of the *gor* gene, was used to carry out the mutagenesis. The mutagenic oligonucleotides 5′-CGCGGGTTTCATCGCCG-3′ (Y177F), 5′-CGCGGGTTCCATCGCCG-3′ (Y177S), 5′-GGCGCGGGTGGCATCGCCGTT-3′ (Y177G), and 5′-GCCATTCAACCAACGGC-3′ (H439Q) were annealed to single-stranded template, and the *Eco* K/*Eco* B double primer with selection procedure (Carter et al., 1985) was used to produce mutants. Putative mutants were screened by colony hybridization with a mutagenic oligonucleotide, and the mutations were confirmed by dideoxy sequencing (Sanger et al., 1980; Biggin et al., 1983). 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 isolated 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 strain (SG5) of *E. coli* carrying a chromosomal deletion of the *gor* gene (Greer & Perham, 1986).

Growth of Cells and Purification of Glutathione Reductase. Wild-type and mutant glutathione reductases were purified from the *gor* deletion strain of *E. coli* (SG5) transformed with the appropriate expression plasmid according to the protocol described in Scrutton et al. (1987), except that a column of DEAE-cellulose (DE-52), run in 20 mM potassium phosphate

Table I: Specific Catalytic Activities of the Wild-Type and Mutant Enzymes[a]

	wild type	Y177F	Y177S	Y177G	H439Q
Specific Activities (under Saturating Assay Conditions)					
units/mg	252	246	72	7	3
Oxidase Activity					
half-life of $NaBH_4$-reduced enzyme (min)	60	22	25	26	not determined

[a] Enzyme specific activities were measured in the direction of glutathione reduction at saturating concentrations of all substrates. For the oxidase activity, half-lives (mean of two determinations) were measured for the pseudo-first-order reoxidation of sodium borohydride reduced wild-type and mutant enzymes by monitoring the absorbance at 540 nm.

buffer, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol and eluted with 0.2 M KCl in the same buffer, was included after the ammonium sulfate fractionation.

Measurement of Kinetic Parameters. Specific activities of wild-type and mutant glutathione reductases in the direction of glutathione reduction were measured under saturating conditions (Scrutton et al., 1987). The reverse reaction was estimated by the method of Icēn (1971). The kinetic parameters were measured with a 5 × 5 matrix of substrate concentrations at 30 °C. The results for the forward reaction were analyzed by a least-squares regression analysis (Cleland, 1979) and for the reverse reaction by application of the statistical software package BMDP (BMDP Statistical Software Inc., 1440 Sepulveda Blvd., Los Angeles, CA 90025) modified to fit to the overall rate equation for a ter-bi reaction in which substrates B and C are the same. The apparent oxidase rates, under aerobic or anaerobic conditions, were estimated at 30 °C at 540 nm after sodium borohydride reduction of all the enzymes in 0.1 M potassium phosphate buffer, pH 7.5. Half-lives for the pseudo-first-order reoxidation of the flavin (mean of two determinations) were determined by regression analysis. The regression coefficient R had a value of 0.99–1.00 in all cases.

Fluorescence Measurements. Fluorescence measurements were carried out with a Perkin-Elmer MPS-44E fluorescence spectrophotometer. Enzymes were dissolved in 0.1 M potassium phosphate buffer, pH 7.5.

Results

The wild-type and mutant enzymes were purified from extracts of *E. coli* SG5 cells overexpressing (about 200-fold) the wild-type or mutated *gor* gene from the expression vector pKK223-3, as previously described (Scrutton et al., 1987). The mutant Y177F was found to be almost as active as wild type, whereas Y177S showed only about 25% and Y177G only about 3% of the wild-type activity (Table I). However, despite the substantial fall in catalytic activity, all three mutants were clearly active.

During the catalytic cycle of glutathione reductase, the enzyme is reduced by NADPH to generate a two-electron-reduced form of the enzyme, thought to be a charge-transfer complex. It is essential for efficient functioning that adventitious reoxidation of this reduced form of the enzyme by oxygen be prevented. The rate of reoxidation of the reduced enzyme (conveniently generated with sodium borohydride) can be monitored by the disappearance of the charge-transfer band at 540 nm (Williams, 1976). This oxidase rate is an indication of the amount of protection afforded to the charge-transfer complex by the protein, including the ability of the residue at position 177 to shield the NADPH-binding site and prevent loss of electrons from flavin in the charge-transfer complex to oxygen. Under anaerobic conditions, the borohydride-reduced wild-type and mutant (Y177F, Y177S, Y177G) enzymes showed no "apparent oxidase" activity. However, under aerobic conditions in air-saturated buffer, we were able to measure rates of reoxidation of the flavin (Table I). The half-life of the reduced flavin in the wild-type enzyme was about 60 min whereas the half-lives in the mutant forms of the enzyme were all 20–25 min. The only comparison we have is with $FADH_2$, which free in aqueous solution has a half-life of less than 1 s, suggesting a significant protection of the reduced flavin in the enzyme. Given the differences in size of the amino acid side chains engineered at position 177 in the mutants, it is perhaps surprising that the oxidase rates of all the mutant enzymes were similar. It therefore appears that protection of the reduced flavin in glutathione reductase is largely due to burial of the isoalloxazine ring within the protein (Thieme et al., 1981) and not to the particular properties of Tyr-177.

The kinetic mechanism envisaged for human glutathione reductase is ping-pong, addition of NADPH leading to formation of an EH_2 intermediate, which is subsequently reoxidized by GSSG with formation of GSH (Williams, 1976; Pai & Schulz, 1983). The results of a detailed study of the kinetics of the wild-type *E. coli* enzyme and of the mutant enzymes are shown in Table II. The wild-type enzyme displayed ping-pong kinetics, consistent with the mechanism postulated for the human enzyme (Williams, 1976; Pai & Schulz, 1983), and the kinetic mechanism for mutant Y177F was also found to be predominantly ping-pong. However, the mutants Y177S and Y177G differed signficantly in that kinetic plots indicated an ordered sequential mechanism (Figure 2). In addition to this novel result, the mutations Y177S and Y177G were found to have resulted in a substantially lowered K_m for GSSG (Table II). This was also unexpected since GSSG binds at a separate site on the enzyme, at some distance from the point of mutation in the NADPH-binding pocket (Thieme et al., 1981; Pai & Schulz, 1983). No change was detected in the kinetic mechanism of the reverse reaction (Table II). Since all the mutants were active to some extent and were all purified

Table II: Kinetic Parameters of Wild-Type and Mutant Forms of *E. coli* Glutathione Reductase

	wild type	Y177F	Y177S	Y177G	H439Q
forward reaction					
mechanism	ping-pong	ping-pong	sequential	sequential	ND[a]
K_m(NADPH) (μM)	38 ± 4	24 ± 5	30 ± 8	18 ± 9	ND[b]
K_m(GSSG) (μM)	97 ± 12	53 ± 9	2 ± 1.5	5 ± 2.5	310 ± 30[c]
k_{cat} (min^{-1})	3600 ± 2600	31000 ± 3900	8200 ± 1400	280 ± 70	140 ± 10
reverse reaction					
mechanism	ter-bi sequential	ter-bi sequential	ter-bi sequential	ND	ND
K_m($NADP^+$) (μM)	120 ± 20	70 ± 13	80 ± 9	ND	ND
K_m(GSH) (μM)	1300 ± 1000	1200 ± 300	700 ± 100	ND	ND
k_{cat} (min^{-1})	3000 ± 250	1400 ± 70	100 ± 3	ND	ND

[a] ND, not determined. [b] The true value of K_m for NADPH in this mutant could not be measured since discrimination in rate could not be achieved even at a concentration of NADPH as low as 2 μM. [c] Apparent K_m for GSSG at 50 μM NADPH.

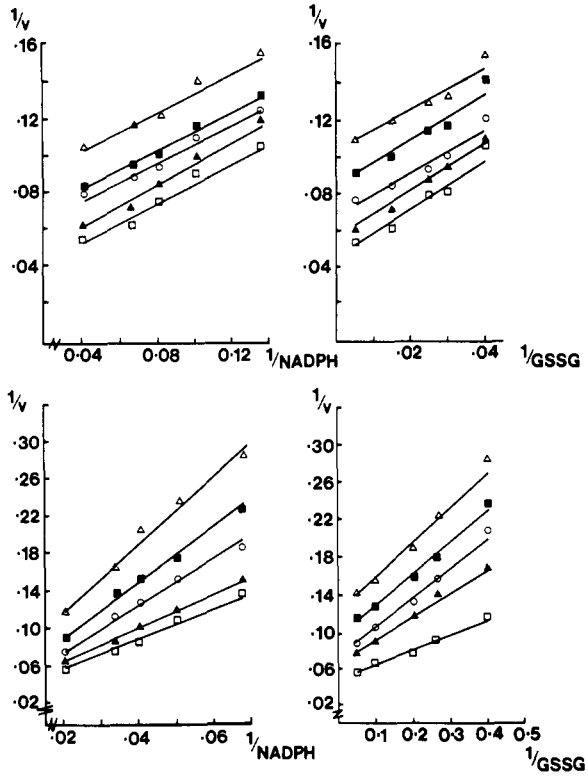

FIGURE 2: Kinetic plots for mutant forms of *E. coli* glutathione reductase. Enzyme activities were measured with a 5 × 5 matrix of substrate concentrations at 30 °C. Double-reciprocal plots of $1/v$ against $1/[GSSG]$ and $1/v$ against $1/[NADPH]$ were constructed. The results were analyzed by least-squares regression analysis (Cleland, 1979). (A) Mutant Y177F: left-hand panel, GSSG concentrations are (top to bottom) 25, 33.3, 40, 70, and 250 μM; right-hand panel, NADPH concentrations are (top to bottom) 7.5, 10, 12.5, 15, and 25 μM. (B) Mutant Y177S: left-hand panel, GSSG concentrations are (top to bottom) 2.5, 3.75, 5, 10, and 18.75 μM; right-hand panel, NADPH concentrations are (top to bottom) 7.5, 10, 12.5, 15, and 25 μM. The wild-type enzyme behaved similarly to mutant Y177F and mutant Y177G behaved similarly to mutant Y177S, although the inferred kinetic parameters were different (see Table II).

by the same method, including an affinity chromatography step on Procion Red HE-7B, we do not expect the mutations to have led to major structural changes in the enzyme but rather that the effects of the mutations are due to subtle changes in and around the active sites of the enzyme.

Mutant H439Q was also not totally inactive despite the loss of the putative proton donor in the mechanism, and it showed about 1% of the activity of the wild-type enzyme (Table I). That this level of activity was due to contaminating wild-type enzyme can be ruled out since the expression of the mutant *gor* gene was carried out in *E. coli* strain SG5 which carries a deletion of the chromosomal *gor* gene. We were unable to measure the true values of K_m for NADPH and GSSG in this mutant, since no discrimination in the enzymic rate of reaction could be obtained even at NADPH concentrations as low as 2 μM. The apparent K_m for GSSG was determined at 50 μM NADPH and was found to be about 300 μM. It therefore appears that in this case a mutation in the GSSG-binding pocket has led to a fall in the apparent K_m for the other substrate, NADPH, which binds at a different and physically distant site. This further supports the idea that the 1% level of activity is really due to the mutant H439Q enzyme and not to a small contamination with wild-type enzyme, since in such a case the measured K_m would be equal to that of the wild-type enzyme.

We also tried to probe the flavin environment in the various proteins by fluorescence studies. Excitation of the flavin in human glutathione reductase at 458 nm results in almost no emission at 531 nm, the quenching being attributed mainly to a dynamic mechanism and flexibility of the bound flavin within the protein (de Kok & Visser, 1987). The wild-type *E. coli* enzyme was found to exhibit very little fluorescence under similar conditions (λ^{abs} 461 nm, λ^{emis} 525 nm) when examined in 0.1 M potassium phosphate buffer, pH 7.5. However, the mutant enzymes Y177F, Y177S, and Y177G were fluorescent and exhibited similar relative quantum efficiencies of ~40% compared with FAD in free solution. These values are 25-fold higher than the quantum efficiency of the wild-type *E. coli* enzyme. This acquisition by enzyme-bound FAD of the ability to fluoresce may be reflecting any conformational differences in the enzymes that are responsible for the lowered K_m values of the mutants for GSSG (Table II). In contrast, mutant H439Q glutathione reductase showed only 0.1% of the relative quantum efficiency of FAD in free solution, similar to (or even less than) the fluorescence of the wild-type enzyme.

Discussion

The techniques of directed mutagenesis allow us to probe the role of any residue of *E. coli* glutathione reductase in the structure and catalytic mechanism of the enzyme. The experiments reported here were concentrated on Tyr-177, the putative lid in the NADPH-binding site, and His-439, the postulated proton acceptor/donor in the reduction of GSSG.

We converted the tyrosine residue naturally present at position 177 of the *E. coli* glutathione reductase to phenylalanine, serine, and glycine in a series of mutants. All the mutant enzymes showed some glutathione reductase activity (Table I), the mutant Y177F being almost as active as the wild-type enzyme. Despite the substantial loss of activity in mutants Y177S and Y177G, our results show that neither a phenolic hydroxyl group nor an aromatic side chain is essential at position 177 for the catalytic activity of *E. coli* glutathione reductase. Furthermore, the half-lives for adventitious reoxidation of the reduced enzymes were of the order of 20 min for all the mutants (Table I). This demonstrates that the nature of the residue at position 177 plays only a minor role in the protection of the reduced flavin in the charge-transfer complex in the enzyme and that any shielding must be largely due to burial of the FAD within the protein.

A detailed study of the wild-type enzyme (Table II) revealed that it displayed ping-pong kinetics, as previously described for the human enzyme (Williams, 1976; Pai & Schulz, 1983). The same was true of the mutant Y177F enzyme. On the other hand, the mutation of residue 177 from an aromatic to a smaller hydrophilic side chain substantially lowered k_{cat} and simultaneously lowered the K_m for the other substrate, glutathione (Table II). Moreover, these mutants (Y177S and Y177G) now displayed ordered sequential kinetic mechanisms, as judged by the converging nature of the kinetic plots (Figure 2). We can rule out the possibility that these plots are made to converge by the decomposition of an intermediate (e.g., EH_2) in the ping-pong pathway, since all the mutants (Y177F, Y177S, and Y177G) have the same half-life for reoxidation of this intermediate (Table I) and mutant Y177F still shows predominantly ping-pong kinetics. Thus, there appears to have been a switch in kinetic mechanism in mutants Y177S and Y177G.

These unusual results are most readily explained by supposing that *E. coli* glutathione reductase actually follows a hybrid kinetic mechanism (Figure 3), such as that already postulated for the yeast enzyme (Mannervik, 1973). In the wild-type enzyme the ping-pong loop dominates. It would then

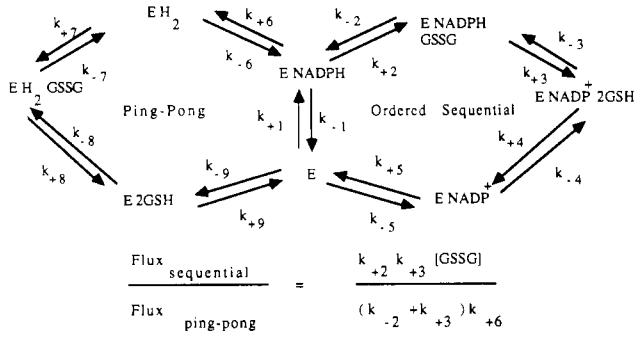

FIGURE 3: Kinetic mechanism for glutathione reductase. The hybrid ping-pong/bi-bi ordered sequential mechanism is that proposed for the yeast enzyme (Mannervik, 1973).

appear that the mutations Y177S and Y177G are diverting flux from the ping-pong loop to the ordered sequential loop of this hybrid mechanism. There are two possible ways to account for the proposed diversion, both involving a change in the partitioning of the intermediate E–NADPH. First, if the mutation slowed or blocked the E–NADPH to EH_2 step (Figure 3), it would tend to force the intermediate E–NADPH through the ordered sequential pathway. This pathway might coincidentally have a lower K_m for GSSG, thereby accounting for the change observed in this kinetic parameter (Table II). An alternative explanation arises from the fact that high concentrations of GSSG favor the ordered sequential pathway in a hybrid ping-pong/ordered sequential mechanism (Mannervik, 1973). Thus, if the mutation caused the observed lowering of the K_m for GSSG in mutants Y177S and Y177G, it would be expected to have the effect of favoring the ordered sequential pathway (Figure 3). This latter explanation might require some action at a distance in the protein, given that the binding site for glutathione is about 1.8 nm from the NADPH-binding pocket (Thieme et al., 1981; Pai & Schulz, 1983). The same is true for the lowering of the K_m for NADPH by the H439Q mutation in the GSSG-binding pocket (Table II). In this case, a simple diversion of flux through the alternative pathway of the hybrid mechanism does not appear able to account for the change in K_m, since NADPH is the first substrate to bind and this step is common to both pathways (Figure 3). However, a proper understanding of the molecular basis of the apparent change in kinetic mechanism must await a crystallographic analysis of the structures of the mutant enzymes.

His-439 in the *E. coli* enzyme (the equivalent of His-467 in the human enzyme) is thought to be the proton donor/acceptor in the glutathione-binding site required by the catalytic mechanism (Matthews et al., 1977; Pai & Schulz, 1983), and it was therefore expected that mutation of this residue to a glutamine residue would render the mutant (H439Q) catalytically inactive. However, contrary to expectation, mutant H439Q showed about 1% of the activity of wild-type glutathione reductase (Tables I and II). A glutamine side chain can enter into hydrogen bonding like the imidazole side chain of histidine, but cannot be protonated with anything like the same facility. Thus, it may be significant that a recent refinement of the crystallographic structure suggests that the geometry of the contact between His-467 and Cys-58 of the redox-active disulfide bridge in the human glutathione re-

Enzyme	Source	Sequence	Ref.
		467	
Glutathione reductase	Human	F D N T V A I H P T S	Krauth-Siegel et al., 1982
		439	
Glutathione reductase	*Escherichia coli*	F D N T V A I H P T A	Greer & Perham, 1986
		461	
Trypanothione reductase	*Trypanosoma congolense*	F Y N T I G V H P T S	Shames et al., 1988
		452	
Dihydrolipoamide dehydrogenase	Human	I A R V C H A H P T L	Otulakowski & Robinson, 1987
		444	
Dihydrolipoamide dehydrogenase	*Escherichia coli*	I A L T I H A H P T L	Stephens et al., 1983
		450	
Dihydrolipoamide dehydrogenase	*Azotobacter vinelandii*	L G M M V F A H P A L	Westphal & de Kok, 1988
		535	
Mercuric reductase	*Pseudomonas aeruginosa* Tn501	L A D Q L F P Y L T M	Brown et al., 1983
		538	
Mercuric reductase	*Shigella flexnerii* Tn21	L A D Q L F P Y L T M	Misra et al., 1985
		521	
Mercuric reductase	*Stapylococcus aureus* pI258	L T D S F A P Y L T M	Laddaga et al., 1987

FIGURE 4: Protein sequence homologies in flavoprotein disulfide oxidoreductases around the putative proton donor, His-439, of *E. coli* glutathione reductase.

ductase is not ideal for a direct proton transfer and that a solvent water molecule, which is in an ideal position to protonate Cys-58 on reduction, is hydrogen bonded to His-467 (Karplus & Schulz, 1987). On reduction, however, Cys-58 moves by about 1 Å, generating a "charge-relay" system between Glu-472, His-467, and Cys-58, in which the direct H-bonding geometry is improved (Karplus & Schulz, 1987). It may also be significant that whereas the enzymes glutathione reductase, dihydrolipoamide dehydrogenase, and trypanothione reductase all have a histidine residue in this position, in the homologous mercuric reductase the histidine is replaced by a tyrosine residue (Figure 4). Other amino acid changes accompany the loss of the histidine residue, for example, the replacement of the succeeding proline residue by a leucine residue (Figure 4), but the importance of this has yet to be explored. Mercuric reductase is of course exceptional in that the substrate is not an organic disulfide, but our experiments indicate that a protonatable side chain in this position is not essential to the mechanism of glutathione reductase either. Whether the small amount of residual activity in the H439Q mutant is due to reliance on another (perhaps normally superfluous) protonatable side chain in the enzyme, to the hydrogen-bonding capacity of the glutamine side chain only partly mimicking that of the replaced histidine residue, or to another subtle change in mechanism remains to be determined by further experiments. This result is similar to that recently reported for the enzyme triosephosphate isomerase (Nickbarg et al., 1988) where mutation of residue His-95, the electrophilic component in catalysis, to glutamine resulted in an enzyme that retained 0.25% of wild-type activity. In the mutant triosephosphate isomerase there was also a subtle change in catalytic mechanism, with Glu-165 having to generate and reprotonate the enediolate intermediate without the catalytic assistance of the histidine residue (Nickbarg et al., 1988).

None of these experiments is interpretable simply in terms of an all-or-nothing contribution by a particular side chain to the molecular mechanisms of the enzymes concerned. The results emphasize the possibility of unforeseen consequences and underline the need for detailed analysis of the effects of mutations in the active sites of enzymes, a pursuit regrettably much more time consuming than the generation of the mutations themselves.

Acknowledgments

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Registry No. GSSG, 27025-41-8; GSH, 70-18-8; NADPH, 53-57-6; $NADP^+$, 53-59-8; L-Tyr, 60-18-4; L-His, 71-00-1; L-Glu, 56-85-9; L-phenylalanine, 63-91-2; L-serine, 56-45-1; L-glycine, 56-40-6.

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