Engineering Surface Charge. 2. A Method for Purifying Heterodimers of *Escherichia coli* Glutathione Reductase†

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ABSTRACT: Two *gor* genes encoding different mutants of *Escherichia coli* glutathione reductase have been expressed in the same *E. coli* cell, leading to the creation of a hybrid form of the enzyme dimer. One of the *gor* genes carried, in addition to various directed mutations, a 5′ extension that encodes a benign penta-arginine "arm" added to the N-terminus of the glutathione reductase polypeptide chain [Deonarain, M. P., Scrutton, N. S., & Perham, R. N. (1992) *Biochemistry* (preceding paper in this issue)]. This made possible, by means of ion-exchange chromatography or nondenaturing polyacrylamide gel electrophoresis, the facile separation of the hybrid enzyme from the two parental forms. Moreover, the two subunits in the hybrid enzyme could be made to carry different mutations. In this way, glutathione reductases with only one active site per dimer were generated: the effects of replacing tyrosine-177 with glycine in the NADPH-binding site, which greatly diminishes the K_m for glutathione and switches the kinetic mechanism from ping-pong to ordered sequential, and of replacing His-439 with glutamine in the glutathione-binding site, which greatly diminishes the K_m for NADPH, were both found to be restricted to the one active site carrying the mutations. This system of generating separable enzyme hybrids is generally applicable and should make it possible now to undertake a more systematic study of catalytic mechanism and assembly for the many enzymes with quaternary structure.

The generation of hybrid enzymes has long been recognized as a powerful approach to the study of enzyme structure and function (Markert, 1963; Penhoet et al., 1967). In particular, the study of hybrid enzymes has revealed much about the nature of active sites that are positioned across the subunit interfaces of oligomeric enzymes. For example, the locations of residues intimately involved in the reaction mechanism of aspartate transcarbamoylase have been elucidated by creating hybrid trimers of the enzyme in vitro (Robey & Schachman, 1985; Wente & Schachman, 1987). Similarly, the formation of hybrid enzymes in vivo has been reported by several workers (Larimer et al., 1987; Distefano et al., 1990; Scrutton et al., 1990), thereby avoiding the problems of denaturing and refolding the enzyme in vitro. However, many approaches have been handicapped by the inability to resolve the hybrid species easily from the parental forms of the enzyme.

The flavoprotein glutathione reductase (EC 1.6.4.2) belongs to the small group of enzymes whose active sites reside between subunits of the protein. Such enzymes offer a unique opportunity to study the role of individual subunits in the overall function of the enzyme. We recently demonstrated the creation of heterodimers of *Escherichia coli* glutathione reductase in vivo by coexpressing from the same plasmid two genes that encoded totally inactive or severely crippled enzyme homodimers (Scrutton et al., 1990). The genes carried mutations (H439Q, C47S) at the position either of the active-site base, His-439 (Berry et al., 1989; Deonarain et al., 1989), or of the active-site cysteine residue, Cys-47 (Deonarain et al., 1990). The former functions as an important proton donor/acceptor, the latter as an essential charge-transfer residue in the passage of electrons from the NADPH-binding site to enzyme-bound glutathione (Williams, 1976; Pai & Schulz, 1983; Karplus & Schulz, 1989). However, the assembly in vivo of H439Q and C47S subunits to produce a heterodimer led to complementation of the mutations, one active site dimer being wild-type in nature. Formation of the heterodimer was therefore conveniently monitored by recording the recovery of enzyme activity (Scrutton et al., 1990).

The ability to create heterodimers of glutathione reductase has enabled us to initiate a study of the molecular recognition processes involved in the assembly of the dimeric enzyme. However, we were unable to resolve the heterodimer from the parental homodimers by conventional separation techniques, thereby making study of the heterodimer much more difficult. We present here a strategy for the separation of such heterodimers. In the preceding paper (Deonarain et al., 1992), we describe the addition of a penta-arginine stretch of amino acids to the N-terminus of *E. coli* glutathione reductase. This basic "arm" imparts an extra positive charge that caused the mutant protein to behave differently in chromatographic and electrophoretic separations. However, the kinetic and spectrophotometric properties of the mutant enzyme were unaffected by the presence of the additional positive charge at the N-terminus. It seemed plausible, therefore, to exploit this by creating in vivo hybrid dimers of glutathione reductase in which only one subunit carries the "arm" sequence, thereby enabling them to be separated from "arm" and "arm-less" homodimers.

We describe here the successful application of this method to the isolation and characterization of heterodimers of glutathione reductase in the study of two active-site mutants. These mutants are H439Q, located in the glutathione-binding pocket, which causes a decrease in the K_m for NADPH binding some 1.8 nm for the point of mutation, and Y177G, in the

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NADPH-binding pocket, which causes a decrease in the K_m for glutathione (Berry et al., 1989; Deonarain et al., 1989). The selective inactivation of one active site in the hybrid was achieved by introducing the mutation C47S which removes the essential charge-transfer cysteine residue (Deonarain et al., 1990). The properties of the remaining active site could then be studied in isolation to determine the effects of the H439Q or the Y177G mutations. The potential application of the hybrid dimer technology in a study of subunit–subunit interactions is discussed.

Materials and Methods

Materials. Materials were supplied and prepared as described in the previous paper (Deonarain et al., 1992). In addition, the restriction enzymes *Bss*HII, *Bam*HI, *Pst*I, and *Nsi*I were purchased from New England Biolaboratories. Strains of *E. coli* together with their source and genotypes are described in the previous paper (Deonarain et al., 1992).

Site-Directed Mutagenesis, DNA Sequencing, and Bacteriophage Construction. Site-directed mutagenesis was carried out on a derivative of M13 (K19gor3′δEcoRI-Arm) containing the noncoding strand of the *gor* gene (Deonarain et al., 1992). Mutant C47S-Arm was constructed by means of the phosphorothioate method (Taylor et al., 1985) as marketed by Amersham International, using the mutagenic oligonucleotide 5′-TGTTGGCTCTGTGCCGA-3′ (C47S). Putative mutants generated by the phosphorothioate method were screened directly by dideoxy-DNA sequencing (Sanger et al., 1980; Biggin et al., 1983) using the T7 sequencing system (Pharmacia). The whole of the mutated gene was resequenced to ensure that no spurious mutations had been introduced. The isolation of the double-mutant C47S.Y177G-Arm is described below.

Plasmid Construction. Plasmid or bacteriophage RF DNA was prepared by CsCl density gradient centrifugation as described by Maniatis et al. (1982). For screening, plasmids were prepared on a miniscale using the alkaline lysis method described in Maniatis et al. (1982). Restriction endonuclease digestion of DNA was carried out as recommended by the enzyme suppliers. The mutant genes encoding C47S-Arm and C47S.Y177G-Arm were expressed from the vector pKK223-3 and were isolated by restricting bacteriophage RF DNA with *Eco*RI and *Hin*dIII. The *gor* gene fragment was subcloned into the expression vector restricted with the same enzymes, as described by Scrutton et al. (1987) and Deonarain et al. (1989). The resultant constructs were designated pKGR4 C47S-Arm and pKGR4C47S.Y177G-Arm, respectively (Figure 1). The isolation of the pKK223-3-based plasmid expression clone for the mutant H439Q (pKGR3H439Q) is described below. The subsequent isolation of expression clones for the mutant gene H439Q (pGPH439Q) and the wild-type gene (pGPWT) in a system carrying a P15A origin of replication (Figure 1) is also described below.

Growth of Cells and Purification of Glutathione Reductase. Clones expressing the constituent subunits of the hybrid enzymes were propagated in the proteinase-deficient strain of *E. coli* NA33 (Deonarain et al., 1992). The initial stages of enzyme purification were as described by Deonarain et al. (1992). For the isolation of the heterodimers, subsequent modifications to the purification are detailed below.

Measurement of Kinetic Parameters. Specific activities of wild-type and mutant glutathione reductases in the direction of glutathione reduction were measured under saturating conditions of substrates (Scrutton et al., 1987), and the kinetic parameters and kinetic mechanisms of the mutant enzymes were determined as described previously (Berry et al., 1989).

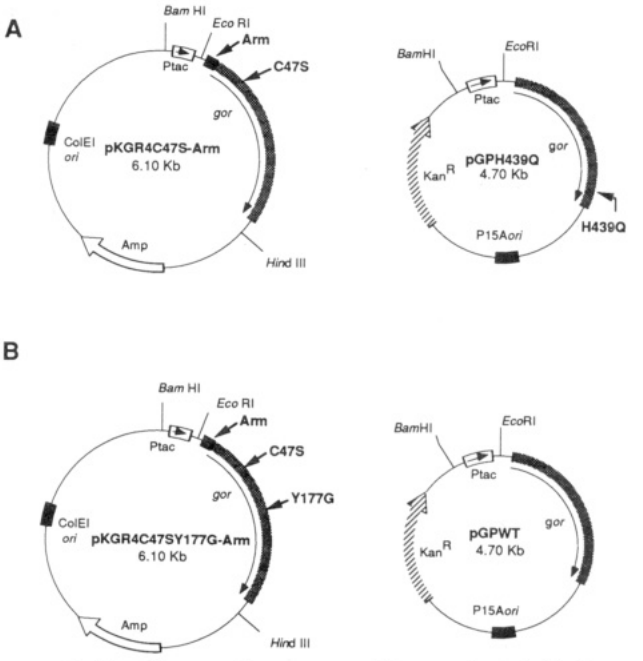

FIGURE 1: Plasmid expression vectors used for creating hybrid dimers of *E. coli* glutathione reductase. (A) Plasmids pKGR4C47S-Arm and pGPH439Q used for the expression of the C47S-Arm/H439Q heterodimer. (B) Plasmids pKGR4C47S.Y177G-Arm and pGPWT used for the expression of the C47S.Y177G-Arm/wild-type heterodimer. See text for details.

Polyacrylamide Gel Electrophoresis. Samples of purified *E. coli* glutathione reductase were submitted to polyacrylamide gel electrophoresis in the presence (Laemmli, 1970) or absence (Davis, 1964; Ornstein, 1964) of SDS in 10% slab gels.

Results

Creating the C47S-Arm/H439Q Hybrid Enzyme. The construction of an expression plasmid (pKGR4C47S-Arm; Figure 1A) for the *gor* gene carrying the C47S mutation plus the N-terminal arm extension is described above. However, attempts to create a single plasmid harboring the C47S-Arm and H439Q mutant genes (Scrutton et al., 1990) were unsuccessful, since recombination of the plasmid constructs occurred frequently (data not shown). To suppress recombination, therefore, the H439Q gene was maintained on a separate but compatible plasmid (a derivative of pGP1-2) which contains the P15A origin of replication and a kanamycin resistance gene (Tabor & Richardson, 1985). The pGPH439Q expression clone (Figure 1A) was made as follows. The plasmid pKGRH439Q (Berry et al., 1989) was digested with *Eco*RI and *Bss*HII, and the larger fragment was shotgun-ligated with an *Eco*RI/*Bss*HII digest of the plasmid pKGR4WT (Deonarain et al., 1989). The resultant construct, designated plasmid pKGR3H439Q, was verified by plasmid sequencing and restriction analysis. The transcriptional unit from this plasmid was released by digestion with *Bam*HI and *Nsi*I and ligated with *Bam*HI- and *Pst*I-digested pGP1-2. The resultant construct was designated plasmid pGPH439Q. Plasmids pGPH439Q and pKGR4C47S-Arm were subsequently transformed into *E. coli* strain NA33 for hybrid enzyme synthesis (Figure 2a).

Creating the C47S.Y177G-Arm/Wild-Type Hybrid Enzyme. Bacteriophage M13K19gor3′δEcoRIC47S-Arm (Deonarain et al., 1992) was digested with *Eag*I and *Eco*RI, and the smallest fragment was isolated by acrylamide gel electrophoresis (Maniatis et al., 1982). This fragment was ligated with *Eag*I- and *Eco*RI-digested M13K19gorY177G bacteriophage RF DNA (Berry et al. 1989). The resultant

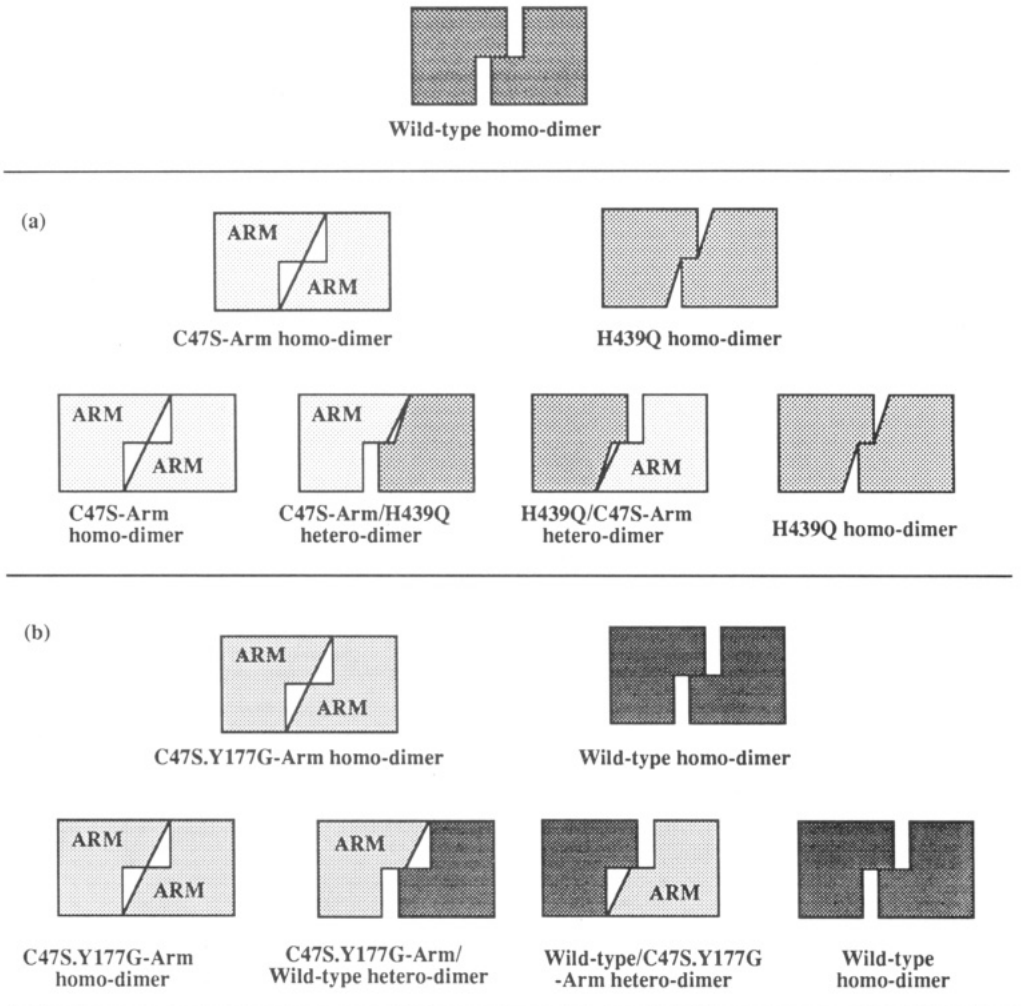

FIGURE 2: Scheme illustrating the formation of heterodimers of *E. coli* glutathione reductase from the parental homodimers C47S-Arm and H439Q, or C47SY177G-Arm and wild type.

bacteriophage was designated M13K19gorEcoRI.C47S.Y177G-Arm and verified by restriction digest and DNA sequencing. An expression plasmid for this mutant *gor* gene was created by inserting the *Eco*RI/*Hin*dIII *gor* gene-containing fragment from M13K19gorEcoRI.C47S.Y177G-Arm into *Eco*RI/*Hin*dIII-cut pKK223-3 (Scrutton et al., 1987). The plasmid construct was designated pKGR3.C47S.Y177G-Arm. The unwanted 1 kbp stretch of DNA on the 3′ side of the *gor* gene in this plasmid was removed by digestion with *Pst*I and *Nsi*I, followed by religation of the plasmid, to create plasmid pKGR4C47SY177G-Arm (Figure 1B).

To insert the wild-type *gor* gene into plasmid pGP1-2, the following procedure was employed. An *Eco*RI site was engineered on the 5′ side of the gene in the construct M13K19gor as described by Deonarain et al. (1989), to produce M13K19gor EcoRI. The *gor* gene was subcloned as an *Eco*RI/*Hin*dIII fragment into the expression vector pKK223-3 as previously described (Scrutton et al., 1987) to create plasmid pKGR3. The *Bam*HI/*Nsi*I fragment containing the transcriptional unit from plasmid pKGR3 was then inserted into *Bam*HI/*Pst*I-cut pGP1-2 to create pGPWT (Figure 1B). Both pGPWT and pKGR4C47S.Y177G-Arm were then transformed into *E. coli* strain NA33 for hybrid enzyme expression (Figure 2b).

Purification of Hybrid Enzymes from E. coli Strain NA33. *E. coli* strain NA33 was chosen as the expression host for the hybrid enzymes since our previous work (Deonarain et al., 1992) had shown that proteolysis of the N-terminal "arm" is suppressed in this strain. The purification of the C47S-Arm/H439Q and the C47S.Y177G-Arm/wild-type hybrid enzymes followed similar protocols. Thus, a 3-L culture of *E. coli* strain NA33 transformed with plasmids pKGR4C47S-Arm and pGPH439Q was grown for 12 h at 37 °C with shaking in 2 × TY medium (10 g of bactotryptone, 10 g of yeast extract, and 5 g of NaCl per liter). Cells were harvested by centrifugation, and the total glutathione reductase content of the cells was purified as described by Deonarain et al. (1992) up to and including the ion-exchange chromatography step on DE-52. The protein eluted from the DE-52 column was dialyzed against "buffer B" (5 mM potassium phosphate buffer, pH 6.6, and 1 mM EDTA) and applied to a column (5 cm × 10 cm) of Procion Red He-7B linked to CL-Sepharose 4B (Scrutton et al., 1987). The column was washed extensively with buffer B and then with buffer B containing 0.1 M KCl. Glutathione reductase molecules lacking the "arm" sequence were than selectively eluted from the column by the application of buffer B containing 0.4 M KCl.

The homo- and heterodimeric glutathione reductase molecules containing two and one "arm" subunits, respectively, were subsequently eluted from the column by applying buffer B containing 1 M KCl. This latter fraction was concentrated by ultrafiltration and then dialyzed exhaustively against 25 mM *N*-methylpiperazine hydrochloride, pH 5.7, before being applied to a Pharmacia Mono-P column equilibrated with the same buffer. Chromatofocusing was carried out on a Phar-

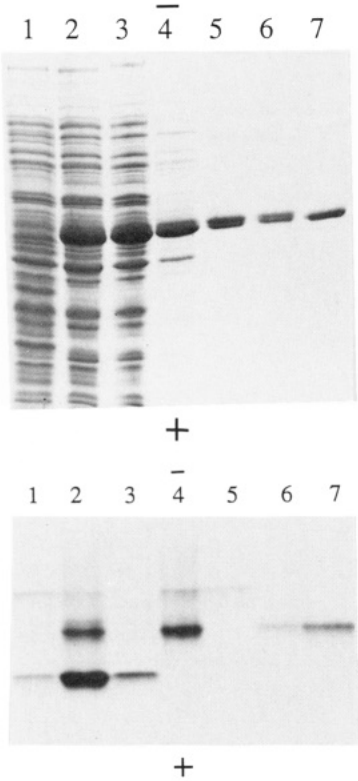

FIGURE 3: Polyacrylamide gel electrophoresis of C47S-Arm/H439Q hybrid glutathione reductase at various stages during its purification. (Top) SDS–polyacrylamide gel electrophoresis. Lane 1, cell-free extract of untransformed *E. coli* strain NA33; lane 2, cell-free extract of *E. coli* strain NA33 transformed with the hybrid expression plasmids; lane 3, 40–80% ammonium sulfate fraction of the cell-free extract; lane 4, proteins eluated from DE-52 with 0.2 M KCL; lane 5, proteins eluted from Procion Red He-7B with 1.0 M KCl; lane 6, second peak (pH 4.5) from the Mono-P chromatofocusing column; lane 7, wild-type glutathione reductase. (Bottom) Nondenaturing polyacrylamide gel electrophoretic analysis of the fractions containing glutathione reductase. Lane 1, mixture of C47S-Arm homodimer and H439Q homodimer; lane 2, proteins eluted from DE-52 with 0.2 M KCl; lane 3, proteins eluted from Procion Red He-7B with 0.4 M KCl; lane 4, proteins eluted from Procion Red He-7B with 1 M KCl; lane 5, first peak (pH 4.7) from the Mono-P chromatofocusing column; lane 6, second peak (pH 4.5) from the Mono-P chromatofocusing column; lane 7, as lane 6, but heavier loading.

macia FPLC apparatus with polybuffer 74, pH 4.0. The homodimeric "arm" species of glutathione reductase was found to elute before the heterodimeric "arm" species, at pH values of approximately 4.7 and 4.5, respectively. The pH of the fractions was raised to about pH 8.0 immediately after elution by the addition of a small quantity of 2.5 M K_2HPO_4. A final purification step was achieved by gel filtration through a Superose-12 column attached to a Pharmacia FPLC apparatus equilibrated with 100 mM potassium phosphate buffer, pH 7.5. A typical analysis by means of SDS– and nondenaturing polyacryamide gel electrophoresis at various stages of the purification process is shown in Figure 3.

Spectral Properties of the Heterodimers of Glutathione Reductase. The absorption spectrum of the C47S homodimer is different from the spectra of the wild-type, H439Q, and Y177G homodimers. The absorption peak of the enzyme-bound flavin in the C47S homodimer is blue-shifted by about 10 nm, suggesting that the environment of the isoalloxazine ring of FAD is perturbed in this mutant (Deonarain et al., 1990). Also, unlike the wild-type enzyme and the H439Q and Y177G mutants, the C47S mutant cannot be reduced by NADPH or by sodium borohydride to generate the reduced (EH_2) enzyme since Cys-47, which as its thiolate forms an

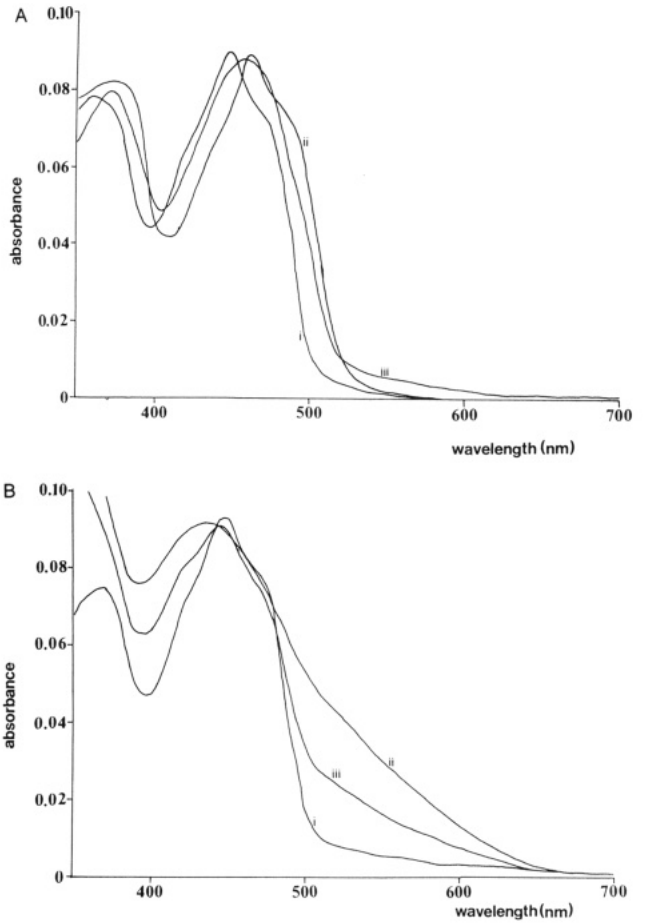

FIGURE 4: Absorption spectra of enzyme-bound FAD in the C47S-Arm/H439Q hybrid enzyme. The enzyme was dissolved in 50 mM potassium phosphate buffer, pH 7.5. Panel A: (i) oxidized C47S-Arm homodimer, (ii) oxidized H439Q homodimer, (iii) oxidized C47S-Arm/H439Q heterodimer. Panel B: (i) reduced C47S-Arm homodimer; (ii) reduced H439Q homodimer; (iii) reduced C47S-Arm/H439Q heterodimer.

essential charge-transfer complex with the FAD, has been removed [see Deonarain et al. (1990) and references cited therein]. Because of these differences in spectral properties, a hybrid enzyme that carries the C47S mutation in only one subunit should exhibit an absorption spectrum characteristic of both the C47S mutant and the wild-type enzyme.

For the purified C47S-Arm/H439Q hybrid dimer, the spectrum of the oxidized enzyme shows a broadening of its peak around 458 nm, which is consistent with the presence of both the C47S-Arm and H439Q mutant subunits (Figure 4). Reduction of the enzyme with sodium borohydride caused an increase in the absorbance at 540 nm, which was half that seen for an equivalent concentration of the H439Q homodimer (Figure 4). These observations are consistent with the supposed composition of the C47S-Arm/H439Q hybrid dimer. Similar results were obtained with the absorption spectrum of the C47S.Y177G-Arm/wild-type hybrid enzyme, confirming the identity of that species too (data not shown).

Kinetic Characterization of the C47S-Arm/H439Q Hybrid. The C47S-Arm and H439Q parental homodimers possess little (H439Q) or no (C47S-Arm) ability to catalyze the NADPH-dependent reduction of glutathione (Table I). However, the hybrid enzyme was found to possess a specific catalytic activity approximately half that of the wild-type enzyme (Table I), indicating that active-site complementation had occurred and that one of the two active sites in the dimer was fully active (Figure 2a).

Table I: Kinetic Properties of Wild-Type and Hybrid Glutathione Reductases of *E. coli*

enzyme	sp act. (units/mg)[a]	K_m(GSSG) (μM)	K_m(NADPH) (μM)	k_{cat} (min^{-1})	kinetic mechanism[b]
wild type	334	97 ± 12	38 ± 4	36000 ± 2600	ping-pong
H439Q	3	310 ± 30	<2[c]	140 ± 10	
Y177G	7	5 ± 2.5	18 ± 9	280 ± 70	ordered sequential
C47S	0				
heterodimer H439Q/C47S.Arm	158	86 ± 7	54 ± 6	17600 ± 1400	ping-pong
C47S.Y177GArm/wild-type	160	79 ± 8	52 ± 7	18700 ± 1660	ping-pong

[a] Enzyme specific activities were measured at saturating concentrations of all substrates. [b] Kinetic mechanism was determined as described by Berry et al. (1989). [c] The true value of the 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.

Replacement of His-439 with glutamine (or alanine) in the glutathione-binding site of *E. coli* glutathione reductase causes a substantial decrease in the K_m for NADPH, which is bound in a separate and physically distinct site (Berry et al., 1989; Deonarain et al., 1989). We showed previously (Scrutton et al., 1990) that for a mixed population of C47S homodimers (catalytically inactive), H439Q homodimers (almost inactive), and C47S/H439Q heterodimers (active), the K_m for NADPH was similar to that found for the wild-type enzyme. We inferred that the effect of the H439Q mutation is confined to the active site of which the histidine residue is a member and that the effect of mutation is not transmitted to the other active site across the subunit interface (Figure 5A). The C47S-Arm/H439Q heterodimer was also found to possess a K_m for NADPH that was comparable in value to that of the wild-type enzyme (Table I). In addition, the k_{cat} for glutathione reduction was, as expected, approximately half that of the wild-type enzyme, and the kinetic mechanism was found to be ping-pong. Thus, our experiments on the isolated C47S-Arm/H439Q hybrid serve to confirm our previous observation (Scrutton et al., 1990) on the mixed population of homo- and heterodimers.

Kinetic Characterization of the C47S.Y177G-Arm/Wild-Type Hybrid. Replacement of Tyr-177 with a serine or glycine residue in the NADPH-binding site of *E. coli* glutathione reductase leads to a dramatic fall in the K_m for glutathione, which is bound in a separate and physically distinct site. Interestingly, this fall in K_m for glutathione was also accompanied by a switch in kinetic mechanism (Berry et al., 1989) from ping-pong (seen for the wild-type enzyme) to ordered sequential (for the Y177S and Y177G mutants). As with the fall in K_m for NADPH in the H439Q mutant, the fall in K_m for glutathione might be localized within the active site that carries the mutation, but it could conceivably be due to communication across the subunit interface to the second active site. To address this question, we created an enzyme in which the Y177G mutation was united with the C47S mutation in one subunit and the double mutant was assembled with a wild-type subunit.

In this hybrid, the active site carrying the Y177G mutation will be inactivated owing to the simultaneous presence of the C47S mutation. If the effect of the mutation is transmitted to the second active site, which is wild type in nature (Figure 2b), the observed K_m for glutathione should be decreased. However, if the effect is restricted to the active site that carries the mutation, the second active site ought to possess wild-type kinetic parameters. A full kinetic characterization of the hybrid enzyme revealed that indeed the second active site exhibited a K_m for glutathione that was similar in value to that measured for the wild-type enzyme (Table I). As expected for a hybrid enzyme possessing only one functional active site, the measured k_{cat} was half that previously determined for the wild-type enzyme. In addition, the kinetic mechanism was found to be ping-pong, like that of the wild-type enzyme but unlike that (ordered sequential) seen for the Y177G homodimer. All these results indicate that the effect of mutating Tyr-177 is restricted to the mutated active site (Figure 5B).

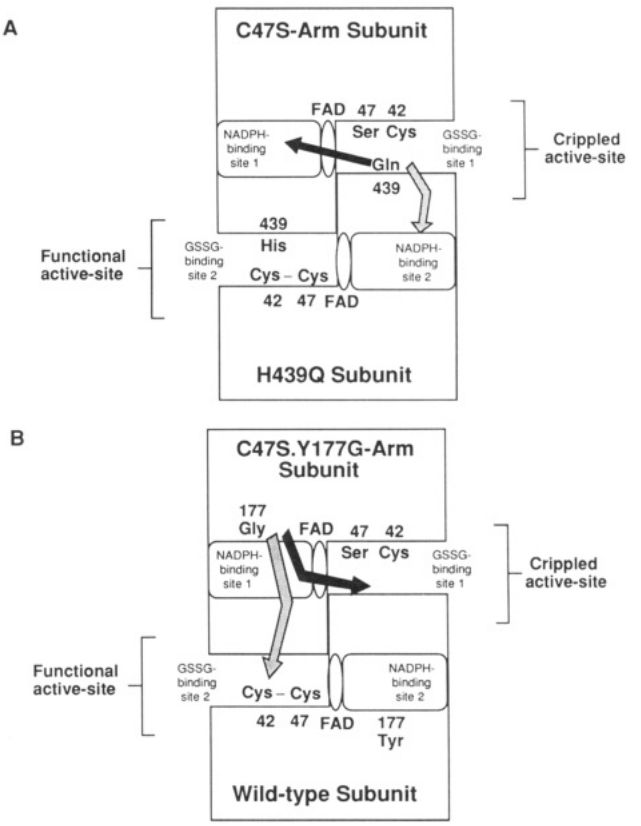

FIGURE 5: Schematic diagram illustrating the active sites of *E. coli* glutathione reductase and possible effects of mutation of either His-439 or Tyr-177. The brackets represent the active-site boundaries. (A) Scheme for the C47S-Arm/H439Q heterodimer. The darkened arrow represents communication within the active site carrying the H439Q mutation. The lightened arrow indicates the additional/alternative route of communication across the subunit interface to the second active site. (B) Scheme for the C47SY177G-Arm/wild-type heterodimer. The darkened arrow represents communication within the active site carrying the Y177G mutation. The lightened arrow indicates the additional/alternative route of communication across the subunit interface to the second active site.

Discussion

To create separable hybrid species, the parental forms of enzymes that hybridize must be well resolved by chromatographic or electrophoretic techniques. Traditionally, this was achieved by using naturally occurring isoenzymes (Markert, 1963; Penhoet et al., 1967). To make this easier, and especially to extend the study to enzymes for which isoenzyme forms either did not exist or were unsuitable, a technique of reversible chemical modification was devised, by means of which suf-

ficient difference in chromatographic or electrophoretic mobility was imparted to one of the parental forms. This approach was used successfully with rabbit muscle aldolase modified by citraconylation of surface lysine residues (Gibbons & Perham, 1974) and with aspartate transcarbamoylase similarly modified reversibly by treatment with 3,4,5,6-tetrahydrophthalic anhydride (Gibbons et al., 1974; Gibbons & Schachman, 1976), in studies of subunit interactions and cooperative interactions between subunits, respectively. However, the heterogeneity of the chemical modification can complicate the experimental analysis, and the possibility of inactivating the enzyme in the process is real.

The formation of hybrid enzymes through the reconstitution in vitro, or the assembly in vivo, of genetically modified enzyme subunits is now a more practicable proposition. Schachman and co-workers, for example, have elegantly demonstrated that the active site of aspartate transcarbamoylase is located across the subunit interface by the formation in vitro of hybrid enzymes created by genetic means (Robey & Schachman, 1985; Wente & Schachman, 1987). The presence of shared active sites had previously been inferred from the crystallographic structure of the enzyme (Monaco et al., 1978; Honzatko et al., 1982). Similarly, the intersubunit location of the active site in ribulosebisphosphate carboxylase has been demonstrated by the hybridization in vivo of site-directed mutants (Larimer et al., 1987). The ability to create hybrids of ribulosebisphosphate carboxylase has recently been exploited in a study of subunit interactions in this enzyme (Soper et al., 1989).

The high-resolution crystallographic structure of human erythrocyte glutathione reductase shows how glutathione is bound across the subunit interface of the dimeric enzyme (Karplus & Schulz, 1987; Pai et al., 1988). Glutathione reductase is thus an ideal candidate for studies of subunit interactions and the effects they may have on biological function. An important step forward in a study of this kind is the ability to create and isolate hybrid forms of the enzyme. We have described here a method by which molecules of glutathione reductase can be created and readily purified to homogeneity. The method is based on the addition of a short arginine-rich extension to the N-terminus of one subunit. This is benign, yet sufficient to impart to the hybrid chromatographic and electrophoretic properties from those of the two parental homodimers (Deonarain et al., 1992). Expression of the different subunits is achieved in vivo, thereby circumventing the need for disruptive denaturation and reconstitution in vitro. Hybrid enzyme molecules are obtained in high yield and with relative ease. The new method is thus a substantial advance on previous experiments, in which a mixture of inseparable homo- and heterodimers was created (Scrutton et al., 1990).

The hybrid forms of glutathione reductase we have generated have enabled us to shed more light on the possibility of communication between the two active sites. We demonstrated earlier (Berry et al., 1989; Deonarain et al., 1989) that conversion of His-439 in the glutathione-binding site to an alanine residue (H439A) or a glutamine residue (H439Q) results in a much diminished K_m for NADPH, which binds in a physically separate site (Pai & Schulz, 1983; Karplus & Schulz, 1987). The results of our present experiments with the C47S-Arm/H439Q hybrid (Table I) indicate that the effect of the H439Q mutation is restricted to the active site of which it is a part and is not transmitted across the subunit interface to the second active site (Figure 5). This is the conclusion we reached from a study of a mixture of parental forms and the C47S/H439Q hybrid (Scrutton et al., 1990). A kinetic explanation of the effect has been advanced (Matthews, 1990). In addition, we have demonstrated for the first time in this work that the reciprocal effect of mutating residue Tyr-177 (located in the NADPH-binding site), which results in a substantial decrease in the K_m for glutathione and a switch in kinetic mechanism from ping-pong to ordered sequential (Berry et al., 1989), is also retained in the active site that harbors the Y177G mutation. The kinetic parameters of the single active site in the hybrid C47SY177G-Arm/wild-type enzyme are similar to those of the wild-type enzyme itself, and it operates by a ping-pong kinetic mechanism (Table I), conclusive proof that the effects of mutating Tyr-177 are contained within the active site carrying the mutation (Figure 5).

Conventional homodimeric mutants only permit a study of the effects of engineering identical residues at the same position in each subunit, owing to the inability to introduce dissimilar (opposing or complementary) mutations at equivalent positions in the enzyme subunits. The system of separable hybrid enzyme formation overcomes this. The formation of hybrid glutathione reductases altered in the region of the active site has already allowed us to answer some of the questions left unanswered in previous studies of mutations in this enzyme. Armed with an extensive knowledge of the three-dimensional structures of human glutathione reductase (Thieme et al., 1981; Pai & Schulz, 1983; Karplus & Schulz, 1987; Pai et al., 1988; Karplus et al., 1989) and of the *E. coli* enzyme itself (Ermler & Schulz, 1991), our ability to create hybrid dimers of the *E. coli* enzyme will now make it possible to undertake a systematic study of further aspects of the enzyme mechanism and of the molecular recognition processes involved in subunit–subunit interactions. The approach described in this and the previous paper (Deonarain et al., 1992) should find a wide and general applicability in the study of enzymes that have the complication, and added interest, of quaternary structure.

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Registry No. NADPH, 53-57-6; GSSG, 27025-41-8; His, 71-00-1; Tyr, 60-18-4; Gly, 56-40-6; Gln, 56-85-9; glutathione reductase, 9001-48-3.

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Apparent Oxygen-Dependent Inhibition by Superoxide Dismutase of the Quinoprotein Methanol Dehydrogenase†

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ABSTRACT: Methanol dehydrogenase activity, when assayed with phenazine ethosulfate (PES) as an electron acceptor, was inhibited by superoxide dismutase (SOD) and by Mn^{2+} only under aerobic conditions. Catalase, formate, and other divalent cations did not inhibit the enzyme. The enzyme also exhibited significantly higher levels of activity when assayed with PES under anaerobic conditions relative to aerobic conditions. The oxygen- and superoxide-dependent effects on methanol dehydrogenase were not observed when either Wurster's Blue or cytochrome *c*-551i was used as an electron acceptor. Another quinoprotein, methylamine dehydrogenase, which possesses tryptophan tryptophylquinone (TTQ) rather than pyrroloquinoline quinone (PQQ) as a prosthetic group, was not inhibited by SOD or Mn^{2+} when assayed with PES as an electron acceptor. Spectroscopic analysis of methanol dehydrogenase provided no evidence for any oxygen- or superoxide-dependent changes in the redox state of the enzyme-bound PQQ cofactor of methanol dehydrogenase. To explain these data, a model is presented in which this cofactor reacts reversibly with oxygen and superoxide, and in which oxygen is able to compete with PES as an electron acceptor for the reduced species.

Inhibition of a process by superoxide dismutase (SOD)[1] is usually assumed to be due to blockage of a reaction involving superoxide as an intermediate (Fridovich, 1975). We report here that under certain conditions the activity of methanol dehydrogenase from *Paracoccus denitrificans* is inhibited by SOD in an unusual oxygen-dependent manner. Methanol dehydrogenase has long been considered to be an important enzyme due to its central role in bacterial methanol metabolism and the potential importance of methylotrophic bacteria to biotechnology (Anthony, 1982, 1986). Methanol dehydrogenase is also of great interest to enzymologists (Duine et al., 1987) who wish to elucidate its reaction mechanism and the role of its redox cofactor, pyrroloquinoline quinone (PQQ)[1] (Salisbury et al., 1979) (Figure 1), in catalysis and electron transfer. Although methanol dehydrogenase was first characterized in 1964 (Anthony & Zatman, 1964) and has subsequently been purified from several sources, the details of its reaction mechanism and its interaction with artificial and

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[1] Abbreviations: PQQ, pyrroloquinoline quinone; PES, phenazine ethosulfate; DCIP, 2,6-dichlorophenolindophenol; CuZnSOD, copper-zinc-containing superoxide dismutase; MnSOD, manganese-containing superoxide dismutase; O_2^-, superoxide; TTQ, tryptophan tryptophylquinone.

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