Ptitsyn, O. B. (1987) *J. Protein Chem. 6*, 273–293.
Schechter, A. N., Chen, R. F., & Anfinsen, C. B. (1970) *Science 167*, 886–887.
Shortle, D. J. (1989) *J. Biol. Chem. 264*, 5315–5318.
Shortle, D., & Meeker, A. K. (1989) *Biochemistry 28*, 936–944.
Stigter, D., Alonso, D. O. V., & Dill, K. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4176–4180.
Sugawara, T., Kuwajima, K., & Sugai, S. (1991) *Biochemistry 30*, 2698–2706.
Szabo, Z. G. (1969) in *Comprehensive Chemical Kinetics* (Bamford, C. H., & Tipper, C. F. H., Eds.) Vol. 2, Chapter 1, Elsevier, Amsterdam.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.

Engineering Surface Charge. 1. A Method for Detecting Subunit Exchange in *Escherichia coli* Glutathione Reductase†

Mahendra P. Deonarain, Nigel S. Scrutton, and Richard N. Perham*
Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

Received June 14, 1991; Revised Manuscript Received October 30, 1991

ABSTRACT: The gene *gor* encoding *Escherichia coli* glutathione reductase was mutated to create a positively charged N-terminal extension consisting of five arginine residues followed by a factor Xa cleavage site to the enzyme polypeptide chain. The modified protein assembled in vivo to yield a dimeric enzyme with kinetic parameters indistinguishable from those of wild-type glutathione reductase. The N-terminal extension could not be released by treatment with factor Xa but could be removed by exposure to trypsin, again without effect on the enzyme activity. The modified enzyme was readily separated from the wild-type enzyme by means of ion-exchange chromatography or nondenaturing polyacrylamide gel electrophoresis. Incubation of the modified and wild-type enzymes, separately or as a mixture, with NADH led to their partial inactivation, and activity was restored by exposure to 1 mM reduced glutathione. No hybrid dimer was formed in the mixture of modified and wild-type enzymes, as judged by polyacrylamide gel electrophoresis, strongly suggesting that the inactivation induced by NADH was not due to dissociation of the parental dimers. The addition of otherwise benign positively or negatively charged extensions to the N- or C-terminal regions of the constituent polypeptide chains of oligomeric enzymes offers a simple route to detecting hybrid formation and the causative subunit dissociation and exchange.

The flavoprotein glutathione reductase (EC 1.6.4.2) catalyzes the NADPH-dependent reduction of oxidized glutathione according to the equation:

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

Reduced glutathione, GSH, plays an important role in almost all cells. For example, it is required to protect against oxidative damage and for the maintenance of reduced thiol groups, and it is crucially involved in the synthesis of deoxyribonucleotides [for reviews, see Holmgren (1985) and Schirmer et al. (1989)].

Glutathione reductase is a dimer of identical subunits, each of which possesses a small intrachain disulfide bridge that, together with enzyme-bound FAD, forms the redox center of the enzyme. It is a member of the family of flavoprotein disulfide oxidoreductases which share the same general mechanistic and structural features. Other members include dihydrolipoamide dehydrogenase (EC 1.8.1.4), an essential component of the 2-oxo acid dehydrogenase multienzyme complexes (Patel & Roche, 1990; Perham, 1991), mercuric reductase, which forms part of a bacterial system for the detoxification of mercuric ions (Fox & Walsh, 1983; Brown et al., 1983; Walsh et al., 1988), and trypanothione reductase, an analogue of glutathione reductase found in trypanosomatids (Shames et al., 1986, 1988; Krauth-Siegel et al., 1987). These enzymes exhibit a high degree of sequence similarity, especially around the redox-active disulfide bridges, suggesting that the proteins have acquired different substrate specificities by divergent evolution from a common ancestor (Perham et al., 1978; Williams et al., 1982; Packman & Perham, 1982; Krauth-Siegel et al., 1982; Fox & Walsh, 1983; Brown et al., 1983; Shames et al., 1988).

The crystallographic structure of human glutathione reductase in the absence and presence of substrates and substrate analogues is known at high resolution (Thieme et al., 1981; Karplus & Schulz, 1987; Pai et al., 1988; Karplus et al., 1989). The NADPH- and GSSG-binding sites are physically distinct and separated (approximately 1.8 nm apart) by the isoalloxazine ring of the enzyme-bound FAD (Thieme et al., 1981; Karplus & Schulz, 1987). Moreover, the GSSG-binding site is composed of amino acid side chains originating from both enzyme subunits (Pai & Schulz, 1983; Karplus et al., 1989), making it inconceivable that monomers of glutathione reductase are active. The structural detail from X-ray crystallography, in conjunction with protein chemical and kinetic analysis, has enabled a detailed reaction mechanism for the enzyme to be proposed (Williams, 1976; Pai & Schulz, 1983; Wong et al., 1988).

The availability of a cloned (Greer & Perham, 1986) and overexpressed (Scrutton et al., 1987; Deonarain et al., 1989)

† This work was supported by the Science and Engineering Research Council and the Cambridge Centre for Molecular Recognition, the Royal Commission for the Exhibition of 1851, and St. John's College, Cambridge. M.P.D. was supported by a research studentship from the SERC and by a benefactors' research scholarship from St. John's College. N.S.S. is a Research Fellow of the Royal Commission for the Exhibition of 1851 and a Research Fellow of St. John's College, Cambridge.
* To whom correspondence should be addressed.

0006-2960/92/0431-1491$03.00/0

gene (*gor*) encoding *Escherichia coli* glutathione reductase has allowed us to study the enzyme by the techniques of protein engineering (Berry et al., 1989; Deonarain et al., 1990; Scrutton et al., 1990a,b). Alignment of the amino acid sequences of the human and *E. coli* glutathione reductases reveals a high degree of similarity (Greer & Perham, 1986). However, two notable differences in the human enzyme are the presence of a pendant N-terminal extension (about 18 amino acid residues) of no defined crystallographic structure and an intersubunit disulfide bridge (Thieme et al., 1981; Karplus & Schulz, 1987). Both of these features are absent from the *E. coli* enzyme (Greer & Perham, 1986). However, the expected similarity in the three-dimensional structures of the two enzymes has been demonstrated by the successful introduction of an intersubunit disulfide bridge in the *E. coli* enzyme at a position equivalent to that found naturally in its human counterpart (Scrutton et al., 1988) and, more recently, by X-ray crystallography of the *E. coli* enzyme (Ermler & Schulz, 1991).

E. coli glutathione reductase can be inactivated by addition of NADPH or NADH in the absence of oxidized glutathione, and it has been suggested (Arscott et al., 1989) that the inactivation is due to the dissociation of the enzyme dimer into monomers. Reactivation of the enzyme with GSH or GSSG was attributed to reconstitution of the dimer from its constituent subunits. However, the proposal of NADPH-induced dissociation rests largely on kinetic arguments, and the reasoning has been complicated by the formation of enzyme–ligand complexes that apparently prevent dissociation of the enzyme dimer (Arscott et al., 1989). Moreover, the inactivation with NADPH can still take place with *E. coli* glutathione reductase into which the intersubunit disulfide bridge has been introduced (Scrutton et al., 1988). This disulfide link prevents complete separation of the enzyme subunits, but it is conceivable that disruption of the dimer interface upon incubation with NAD(P)H may still account for the inactivation observed with this mutant enzyme.

We have therefore sought to observe directly any separation of the enzyme subunits during the NAD(P)H-induced inactivation process. A mixture of two homodimeric enzymes, each differing physically from the other, will produce a heterodimeric species only if there has been a process of complete subunit separation and exchange. We describe here a procedure for selectively tagging subunits of *E. coli* glutathione reductase by engineering benign alterations to the surface charge. Formation of hybrid dimers in vitro can then be detected by separation procedures that exploit differences in the net charge of the proteins, for example ion-exchange chromatography and nondenaturing polyacrylamide gel electrophoresis. We use this technique to reexamine the question of NADPH-induced dissociation of the enzyme. In the following paper (Deonarain et al., 1992), we describe how this technology can be extended to create hybrid enzyme molecules in vivo, which allows a number of interesting questions of subunit and active-site interactions to be tackled.

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. Ethidium bromide, NADPH, NADH, GSH, GSSG, PMSF, benzamidine, and trypsin were from Sigma Chemical Co. Ultrapure agarose and CsCl were from Bethesda Research Labs. Factor Xa, TLCK, and aprotinin were supplied by Boehringer Mannheim GmbH. DE-52 anion-exchange media were obtained from Whatman Ltd. Procion Red He-7B was a gift from Dr. C. R. Lowe (Institute of Biotechnology, University of Cambridge) and was linked to CL-Sepharose 4B as described in Lowe et al. (1980). All other chemicals were of analytical grade wherever possible. Glass-distilled water was used throughout. The restriction enzymes *Hin*dIII and *Eco*RI were purchased from New England Biolaboratories. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. T4 DNA ligase and T4 polynucleotide kinase were from Amersham International. Mono-P and Superose 12 FPLC columns were supplied by Pharmacia.

E. coli strain TG1 [K12, Δ(*lac-pro*), *sup* E,*thi*, *hsd* D5/F′ *tra* D36, *pro* A$^+$B$^+$, *lac* I^q, *lac* Z ΔM15] was provided by Dr. A. Gibson (MRC Laboratory of Molecular Biology, Cambridge, U.K.). *E. coli* strain NS3 [Δ(*his-gnd*), Δ*gor*, Δ*lac*, *ara* D, Str^R/F′ *pro* A$^+$B$^+$, *lac* I^q, *lac* Z ΔM15] was previously isolated in this laboratory (Deonarain et al., 1989). *E. coli* strain BMH71-18[K12, Δ(*lac-pro*), *sup* E, *thi*/F′ *pro* A$^+$B$^+$, *lac* I^q, *lac* Z ΔM15] was obtained from Dr. G. Winter (MRC Laboratory of Molecular Biology, Cambridge, U.K.). *E. coli* strain AR 68 [F$^-$, *lac* (amb), *Tyr* (amb), *pho* (amb), *sup* C (ts), Str^R, *Htp* R–, λCI$_{857}$, Δ(*int* C3-H1), Δ*Bam*, Tet^R, Tn10, *gal* E, *bio*$^-$, *uvr* B$^-$] was the kind gift of Prof. Shatzman (Smith, Kline & Beecham, King of Prussia, PA).

Site-Directed Mutagenesis and DNA Sequencing. Site-directed mutagenesis was carried out on a derivative of M13 containing the noncoding strand of the *gor* gene [K19gor3′δEcoRI] (Deonarain et al., 1989). Mutants were constructed by means of the phosphorothioate method (Taylor et al., 1985; Scrutton et al., 1990a) as marketed by Amersham International, using the mutagenic oligonucleotides 5′-TAAGGACACTTTGTCATGCGTCGCCGTCGTCGT-CGTATTGAGGGTCGCTAAACACTATGATTACA-3′ (N-terminal extension), 5′-TAAGGACACTTT-GTCATGCGTC-3′ (correction oligonucleotide 1), 5′-CGCCGTCGTCGTCGTATTGAG-3′ (correction oligonucleotide 2), and 5′-TGAGGGTCGTACTAAACA-3′ (correction oligonucleotide 3). Putative mutants generated by the phosphorothioate method were screened directly by dideoxy 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 were introduced during the mutagenesis reactions.

Plasmid Construction. Plasmid or bacteriophage RF DNA was prepared by CsCl density gradient centrifugation as described by Maniatis et al. (1982). For the purposes of 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 (see below) were isolated by restricting 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) and Deonarain et al. (1989).

Growth of Cells and Purification of Glutathione Reductase. Wild-type and mutant glutathione reductases were purified from the *gor* deletion strain of *E. coli* NS3 (Deonarain et al., 1989) or *E. coli* strain NA33 (see below) transformed with the appropriate expression plasmid. Purification of glutathione reductase from *E. coli* strain NS3 was as described by Scrutton et al. (1987) with the modifications of Berry et al. (1989). Purification of glutathione reductase from *E. coli* strain NA33 is described below.

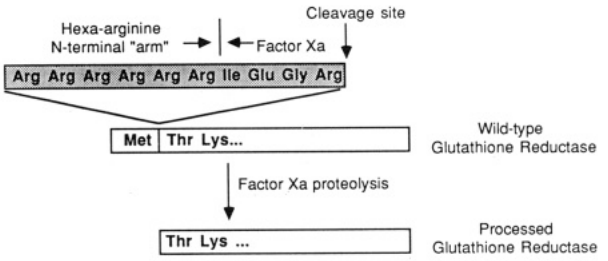

FIGURE 1: Proposed scheme of adding a hexa-arginine "arm" to the N-terminus of *E. coli* glutathione reductase. The mutagenic oligonucleotide encodes the "arm" followed by a factor Xa cleavage site. Insertion is between the first and second codons of the wild-type *gor* gene. Cleavage by factor Xa should yield glutathione reductase as found in vivo.

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 of the mutant enzymes were determined as described previously (Berry et al., 1989).

N-Terminal Sequence Analysis. N-Terminal sequence analysis was kindly performed by Dr. J. McCormick (Department of Biochemistry, University of Cambridge) on an Applied Biosystems Model 477A sequencer.

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.

Proteolytic Cleavage by Proteinases. Digestions with factor Xa were carried out using a substrate:proteinase ratio of 200:1 or 20:1 (w/w) in 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 mM DTT, and 100 mM NaCl at 37 °C. Limited digestions with trypsin were carried out in 50 mM Tris-HCl (pH 7.8) at a substrate:proteinase ratio of 5000:1 (w/w) at 0 °C, and complete digestion was achieved by using a 100:1 (w/w) ratio at 30 °C for 6 h (see below). All proteinase digests were stopped by adding PMSF (final concentration 1 mM) and freezing at −20 °C. Samples were subsequently analyzed by electrophoresis in nondenaturing 10% polyacrylamide gels.

Results

A Positively Charged N-Terminal Extension on Glutathione Reductase. To generate a subunit of *E. coli* glutathione reductase bearing additional positive charge relative to the wild-type subunit, an extension of the polypeptide chain including several arginine residues was engineered onto the N-terminus of the protein. This extension is referred to as a positive "arm" (Figure 1). The N-terminus was favored for this purpose because the closely related human enzyme already carries an additional flexible N-terminal sequence of about 18 amino acid residues that does not obviously contribute to catalytic function (Greer & Perham, 1986), whereas the C-terminal region forms an important part of the GSSG-binding site (Karplus & Schulz, 1987; Karplus et al., 1989). Similarly, the C-terminal region of another related enzyme, mercuric reductase, appears to be involved in providing additional ligands for Hg^{2+} binding (Distefano et al., 1990), and that of dihydrolipoamide dehydrogenase may have a part to play in stabilizing the dimer and binding the substrate (Schulze et al., 1990; N. S. Scrutton, M. P. Deonarain, and R. N. Perham, unpublished experiments).

A 67-mer oligonucleotide was designed to attach a hexa-arginine extension and a unique proteinase (factor Xa) cleavage site onto the N-terminus of *E. coli* glutathione reductase. The factor Xa cleavage site created the potential for the "arm" to be removed proteolytically after purification of the enzyme (Figure 2). However, after the directed mutagenesis, the number of putative mutant plaques was found to be consistently low (about 10 per reaction), which no doubt reflects the difficulty of inserting large stretches of DNA sequence into a target gene by the phosphorothioate method. Moreover, a large number of spurious mutations was found to accompany a successful insertion of DNA sequence into the N-terminal region of the gene. This may be due to difficulty in hybridizing the highly repetitive sequence of the insertion oligonucleotide to the single-stranded DNA template owing to the undesirable formation of secondary structure. Of all

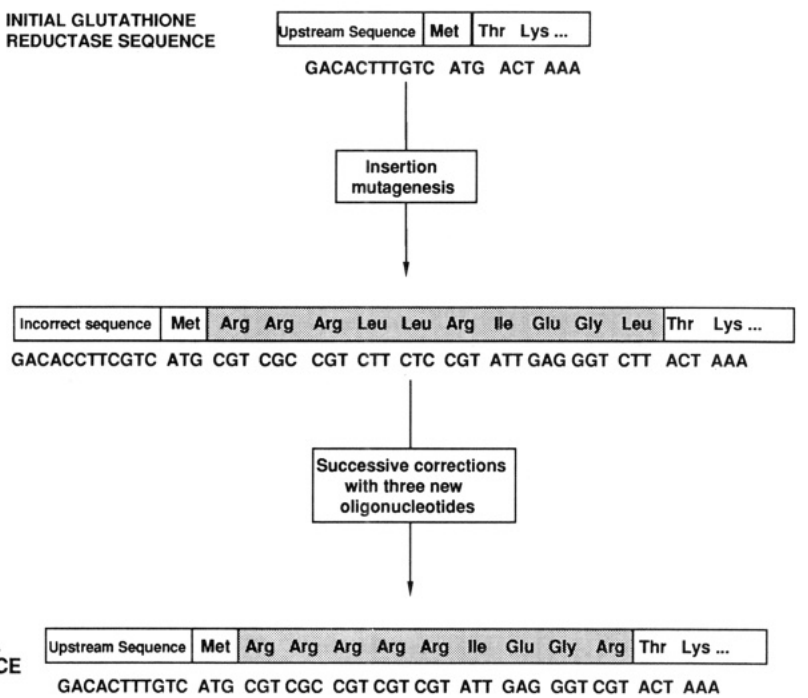

FIGURE 2: Scheme illustrating the mutagenic route to obtain the gene encoding the "arm" protein. The oligonucleotides used to direct the changes are described in the text. The final product after performing the three correction–mutagenesis reactions lacked one of the arginine codons encoded by the original insertion–mutagenesis oligonucleotide.

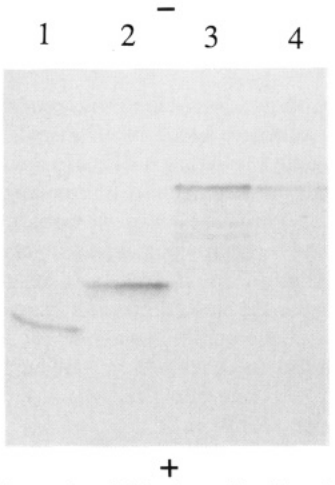

FIGURE 3: Nondenaturing 10% polyacrylamide gel electrophoresis of "arm" glutathione reductase purified from *E. coli* strains NS3 and NA33. Lane 1, wild-type glutathione reductase; lane 2; "arm" protein purified from strain NS3; lane 3, "arm" protein purified from strain NA33 in the absence of proteinase inhibitors; lane 4, "arm" protein purified from strain NA33 in the presence of proteinase inhibitors. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

the plaques screened, none was free of corrupted DNA sequence. Three further rounds of mutagenesis (described under Materials and Methods) were therefore performed to correct the least corrupted sequence. These further mutagenesis reactions were also, but to a lesser extent, hampered by spurious changes, and the final product, M13K19gor3′δ*Eco*RI-Arm, still lacked one of the arginine residues originally encoded by the insertion oligonucleotide (Figure 2). The mutant glutathione reductase gene, encoding the protein with the penta-arginine "arm", was excised from M13K19gor3′δ*Eco*RI-Arm by restriction with *Eco*RI and *Hin*dIII and ligated into the expression vector pKK223-3 previously cut with *Eco*RI and *Hin*dIII (Scrutton et al., 1987; Deonarain et al., 1989). This yielded the expression vector pKGR4-Arm.

Purification of Glutathione Reductase with a Positive N-Terminal "Arm". The expression vector pKGR4-Arm was transformed into the *E. coli gor* deletion strain NS3 (Deonarain et al., 1989). The level of *gor* gene expression, as judged by estimation of catalytic activity in cell-free extracts, was found to be significantly lower (approximately 10 times) from this plasmid construct than from the wild-type expression plasmid pKGR4 (Deonarain et al., 1989).

The modified enzyme was purified to homogeneity as described previously for wild-type glutathione reductase (Deonarain et al., 1989). Surprisingly, no difference in chromatographic properties was noticed between the modified and wild-type enzymes. Nondenaturing polyacrylamide gel electrophoresis, however, indicated that the modified enzyme differed in charge since it migrated more slowly (Figure 3, lanes 1 and 2).

N-Terminal sequence analysis revealed that only one arginine residue, rather than the five expected before the factor Xa cleavage site, was present in the "arm"-bearing protein. Since direct DNA sequencing of the expression plasmid confirmed that it contained the full insert, the loss of the four arginine residues was thought to have occurred by proteolysis. To circumvent this problem, a host strain deficient in proteinases was sought. The host strain must be *lac* I^q, in order to maintain the pKGR4 plasmid and to impart control of the *tac* promoter (Deonarain et al., 1989). First, *E. coli* strain AR68 (Str^R), which is deficient in the heat-shock regulatory protein *htp*R and is therefore unable to induce the heat-shock proteinase of the *lon* gene, was utilized as host. This strain was made *lac* I^q by conjugation (Lederberg & Zinder, 1948; Deonarain et al., 1989) with strain BMH71-18 (Str^S), which carries the *lac* I^q gene on its episome. Following conjugation, streptomycin-resistant colonies were screened for susceptibility to infection with the filamentous bacteriophage fd, thereby confirming that episome transfer from BMH71-18 to AR68 had occurred. The resulting strain was designated *E. coli* strain NA33.

Purification of the "arm" protein from this strain was identical to that of the wild-type enzyme, except that a higher concentration (1 M) of KCl was required to elute the enzyme from the matrix in the final affinity chromatography step on Procion Red He-7B (see below for details). Nondenaturing polyacrylamide gel electrophoresis of the purified enzyme revealed a new protein species which migrated much more slowly than the wild type, but a succession of fainter bands of higher mobility approaching that of the wild type was also present (Figure 3, lane 3). Hence, it appeared that in *E. coli* strain NA33, a complete "arm" protein was being synthesized and that it was also being proteolyzed but to a much smaller extent than in *E. coli* strain NS3. Other strains deficient in intracellular proteinases were also tested as host, including a *htp*R^- *lon*$^-$ strain and a Δ*omp*T strain, but no improvement in preventing proteolysis compared with strain NA33 was observed (data not shown).

A method to purify the unproteolyzed form of the enzyme from *E. coli* strain NA33 was therefore devised, using proteinase inhibitors as an added precaution. The method is essentially that described by Deonarain et al. (1989) for the wild-type enzyme, with the following modifications: the lysis buffer contained, in addition, 2 mM PMSF, 1 mM benzamidine, 0.1 μg/mL aprotinin, 0.1 mM TLCK, and 10 mM EDTA; during ion-exchange chromatography on DE-52, the KCl concentration was lowered from 100 to 70 mM, since the former would elute the more basic "arm" protein; 0.2 M KCl was insufficient to elute the "arm" protein from the Procion He-7B affinity column (Scrutton et al., 1987), and a concentration of 1 M KCl was found to be necessary. A new step was also included, that of chromatofocusing on a Pharmacia Mono-P column, using a pH 4–5 gradient. The "start" buffer was 25 mM *N*-methylpiperizine, pH 5.7, and the column was developed with Polybuffer 74, pH 4. The "arm" protein eluted at a pH of 4.7, compared with the wild-type enzyme which elutes at pH 4.3. The pH of eluted samples was immediately raised to around 7.5 by the addition of a few drops of 2.5 M K_2HPO_4. The sample from the Mono-P column was then applied to a Pharmacia Superose 12 gel filtration column equilibrated with 100 mM potassium phosphate buffer, pH 7.5, to achieve buffer exchange and a final purification. The pure enzymes were stored at −20 °C in 15% glycerol. Nondenaturing polyacrylamide gel electrophoresis of the purified "arm" protein showed it to be unproteolyzed and migrating much more slowly than the wild-type enzyme (Figure 3, lane 4). This was confirmed by N-terminal sequence analysis. A typical purification, as monitored by SDS–polyacrylamide gel electrophoresis, is shown in Figure 4.

Limited Proteolysis of the N-Terminal "Arm". Factor Xa was found to be unable to remove the engineered "arm" (Figure 5), presumably because the final arginine residue is inaccessible to the 50-kDa proteinase. However, trypsin did selectively remove the "arm" by cleaving at the various arginine residues within the N-terminal extension (Figure 5). N-Terminal sequence analysis of the "arm-less" enzyme revealed that its N-terminal sequence was Thr-Lys-His-Tyr-Asp-Tyr-

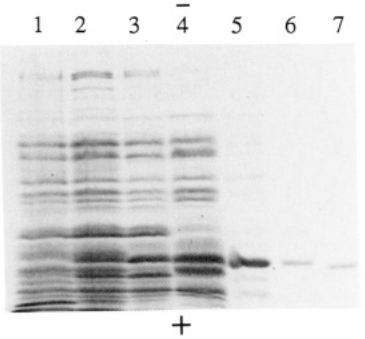

FIGURE 4: Examination of protein samples by means of SDS–polyacrylamide gel electrophoresis at successive stages of the purification of the "arm" glutathione reductase from *E. coli* strain NA33 in the presence of proteinase inhibitors. Lane 1, untransformed *E. coli* strain NA33; lanes 2–6, *E. coli* strain NA33 transformed with pKGR4-Arm: lane 2, cell-free extract; lane 3, 40–80% ammonium sulfate fraction; lane 4, protein eluted from DE-52; lane 5, protein eluted from the Procion Red He-7B affinity column; lane 6, pooled fractions from the Mono-P chromatofocusing column. Lane 7, purified wild-type glutathione reductase. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

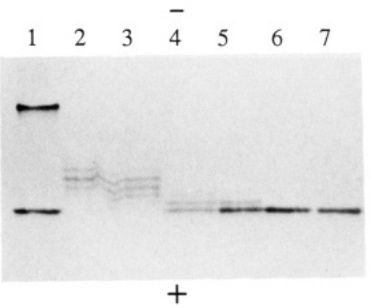

FIGURE 5: Nondenaturing polyacrylamide gel electrophoretic analysis of "arm" glutathione reductase incubated with trypsin. Lane 1, untreated wild-type and "arm" glutathione reductases; lanes 2–6, digestion of "arm" protein with 1:5000 (w/w) trypsin at times 1, 5, 30, 120, and 960 min, respectively; lane 7, wild-type glutathione reductase treated with trypsin for 12 h. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Ile-, the same as that found for purified wild-type glutathione reductase. No cleavage of the polypeptide backbone elsewhere in the protein was detected.

Properties of the "Arm" Enzyme before and after Limited Proteolysis. A prerequisite for the experiments envisaged with the modified enzyme is that it should have functional characteristics identical to those of the wild-type enzyme. In the future construction of hybrid enzymes (Deonarain et al., 1992), it would also be highly desirable for the N-terminal "arm" to be removable without effect on the enzyme dimer. The specific catalytic activity of the "arm" enzyme before and after trypsin digestion was found to be similar to that of the wild-type enzyme. A detailed kinetic analysis of the wild-type and "arm" proteins further revealed that the kinetic parameters were essentially the same for each enzyme (Table I). Other properties (e.g., thermal stability and flavin absorption spectra) were also found to be unchanged (data not shown).

NADH-Induced Inactivation of the "Arm" Enzyme. A measured volume of concentrated enzyme (10 μM enzyme-bound FAD) was added to a large volume of buffer (50 mM Tris-HCl, pH 7.5, and 0.5 mM EDTA) to give a final enzyme concentration of 400 nM, and NADH was added to a final concentration of 150 μM. The final volume was 100 mL. The NADH-induced inactivation of the wild-type enzyme was monitored by measuring the catalytic activity of samples (5 μL) as a function of time, the assays being carried out under saturating conditions of substrate (Scrutton et al., 1987). After

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

enzyme	sp act. (units/mg)	K_m(GSSG) (μM)	K_m(NADPH) (μM)	k_{cat} (min^{-1})
wild type	334	97 ± 12	38 ± 4	36000 ± 2600
"arm"	330	81 ± 7	57 ± 6	40400 ± 2790
"arm" (after tryptic cleavage)	332	ND[a]	ND	ND

[a] ND, not determined.

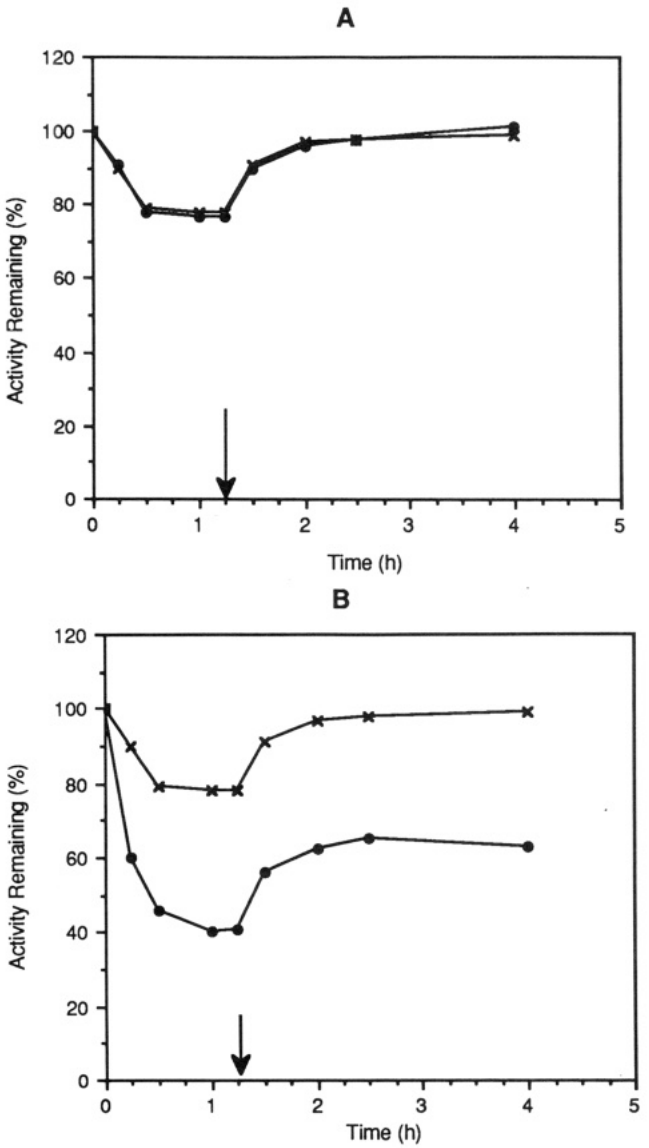

FIGURE 6: Inactivation of wild-type and "arm" glutathione reductases with NADH. (A) Wild-type (●) and "arm" (×) enzymes incubated separately with NADH (150 μM). The enzyme concentrations were 400 nM. (B) An equimolar mixture [total enzyme concentration 400 nM (●) or 50 nM (×)] of wild-type and "arm" proteins incubated with 150 μM NADH. Other details are given in the text. The arrow indicates the point at which the incubations were made 1 mM with respect to GSH.

inactivation, reactivation was achieved by adding GSH to a final concentration of 1 mM. A typical profile of inactivation/reactivation is illustrated in Figure 6A and is similar to that described previously for the wild-type enzyme (Arscott et al., 1989). An identical profile was obtained for the "arm" enzyme inactivated and reactivated under the same conditions (Figure 6A).

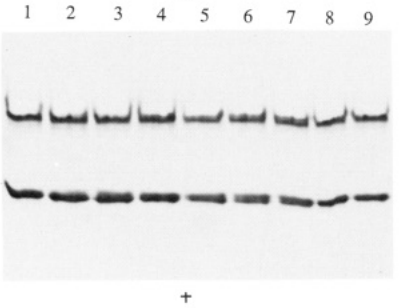

FIGURE 7: Nondenaturing polyacrylamide gel electrophoresis of the mixture of wild-type and "arm" glutathione reductases before and after NADH inactivation and reactivation. Lanes 1–4, samples taken from the experiment with 400 nM total enzyme concentration; lanes 5–8, samples taken from the experiment with 50 nM total enzyme concentration. Lanes 1 and 5, enzyme mixture untreated with NADH; lanes 2 and 6, as lanes 1 and 5, but the enzyme mixture was exposed to 1 mM GSH after 75-min incubation; lanes 3 and 7, samples inactivated by treatment with 150 μM NADH for 75 min; lanes 4 and 8, as lanes 3 and 7, respectively, but the enzyme mixture was reactivated by exposure to 1 mM GSH and the incubation continued for an additional 165 min; lane 9, wild-type and "arm" glutathione reductases.

To look for subunit exchange during the inactivation and reactivation of the enzyme, an equimolar mixture of wild-type and "arm" glutathione reductases (final total concentrations of 400 and 50 nM) was incubated with NADH as described above. After inactivation (Figure 6B), reactivation was achieved by adding 1 mM GSH as before. Samples of the protein were concentrated in a Centricon concentrator, and analyzed by nondenaturing polyacrylamide gel electrophoresis at various stages of the process (Figure 7). No hybrid of wild-type and "arm" glutathione reductases (identifiable as a band migrating between the bands of wild-type and "arm" enzymes in the gel) could be detected, despite heavy loadings. It would appear, therefore, that monomers of glutathione reductase are not produced as part of the inactivation process induced by NADH.

Discussion

It is now possible by means of in vitro mutagenesis to redesign proteins to satisfy preconceived requirements. The addition of five arginine residues onto the C-terminus of recombinant human urogastrone, an inhibitor of gastric acid secretion, imparted sufficient additional positive charge to the protein to enable a rapid and inexpensive purification procedure based on ion-exchange chromatography to be devised (Sassenfeld & Brewer, 1984). We have successfully adapted this approach to modify the chromatographic and electrophoretic properties of *E. coli* glutathione reductase to facilitate the study of subunit exchange and the creation of hybrid enzymes. In human glutathione reductase, there exists an N-terminal segment (about 18 residues) of protein with no defined structure (Karplus & Schulz, 1987; Karplus et al., 1989) which is absent from the glutathione reductase of *E. coli* (Greer & Perham, 1986). We conjectured, therefore, that the addition of an arginine-rich sequence at the N-terminus of *E. coli* glutathione reductase might achieve our purpose without affecting the catalytic activity of the enzyme. To permit the option of removing the basic N-terminal segment after purification of the enzyme, we additionally incorporated a cleavage site for the proteinase factor Xa (Figures 1 and 2).

The fact that the N-terminal regions of many proteins are exposed in crystal structures makes them an attractive target for extension, especially if, as with glutathione reductase (Pai & Schulz, 1983; Karplus & Schulz, 1987; Karplus et al., 1989), the C-terminal region is involved in interactions important for catalysis. However, extra nucleotide sequence at the 5′ end of the gene may affect the initiation of translation, as noted here, and the C-terminal end of the polypeptide may generally be more favorable for modification. The principle, of course, is unaffected.

Proteolysis of the N-terminal extension was found to occur either in vivo or during the purification of the modified glutathione reductase from the *gor* deletion strain of *E. coli*, strain NS3, to the extent that four of the five N-terminal arginine residues were completely lost (Figure 3). Future design strategies might attempt to induce resistance to proteinases, for example, by inserting occasional proline residues. In the present experiments, proteolysis was suppressed by expressing the "arm" protein in a proteinase-deficient host and by adding a variety of proteinase inhibitors during the preparation of the enzyme.

As expected, the chromatographic and electrophoretic properties of the "arm" enzyme were markedly different from those of the wild-type enzyme. Early elution from DE-52 was observed as predicted. However, somewhat surprisingly, the "arm" protein was found to bind much more tightly than the wild-type enzyme to the Procion Red He-7B affinity column. This permitted the facile separation of the "arm" protein from any small amount of wild-type enzyme encoded by the chromosomal *gor* gene in *E. coli* strain NA33. A difference in elution for the wild-type and "arm" enzymes was also noted in chromatofocusing, reflecting the difference in their isoelectric points. The hoped-for large difference in the mobilities of the wild-type and "arm" enzymes when subjected to nondenaturing polyacrylamide gel electrophoresis was readily apparent (Figure 3).

Removal of the N-terminal "arm" was not achieved by digestion with factor Xa, despite the presence of the necessary cleavage site, which suggests that the site may be poorly accessible to this proteinase. However, trypsin was able to cleave the "arm" off completely (Figure 5), leaving an N-terminal sequence identical to that of the wild-type protein. Both the cleaved and uncleaved enzymes possessed kinetic parameters indistinguishable from those of the wild-type enzyme (Table I), thereby proving that the "arm" offers no impediment to catalysis. Moreover, removal of the "arm" is evidently unnecessary for the analysis of the kinetic properties of hybrid enzymes containing the modified subunit.

Given all these favorable properties, we were able to make use of the "arm" enzyme to study subunit exchange in the glutathione reductase dimer. No dissociation followed by reassociation of glutathione reductase was observed when equimolar mixtures (400 or 50 nM) of the wild-type and "arm" protein were incubated at ambient temperature for over 4 h. This lack of subunit exchange no doubt reflects the many interactions across the subunit interface observed in the crystallographic structure of the highly similar human enzyme (Karplus & Schulz, 1987). Subunit dissociation has been proposed as a possible mechanism for the inactivation of the enzyme induced by NADPH or NADH (Arscott et al., 1989). The kinetics of inactivation were found to depend inversely on enzyme concentration, which is consistent with dissociation of the dimer. Second-order reactivation kinetics were observed during the addition of reduced glutathione, which is consistent with the re-formation of the dimer from monomers. To test this model, we carried out inactivation–reactivation experiments under conditions identical to those described by Arscott et al. (1989) with equimolar concentrations of the wild-type and "arm" enzymes. The profiles of inactivation–reactivation

(Figure 6) indicate that if dissociation is the mechanism of inactivation, then statistically about 10% of the enzyme sample should be present as the heterodimer after reactivation. No evidence of the heterodimer being formed was obtained by nondenaturing gel electrophoresis (Figure 7), even with heavily overloaded samples (80 μg of total protein). This casts serious doubt on any explanation of the NADPH-induced inactivation of glutathione reductase that depends on dissociation of the enzyme dimer. Perhaps a more attractive explanation may be found in chelation of metal ions at the redox-active thiol groups in the glutathione-binding site, as propounded by Pienado et al. (1991).

In conclusion, our results have demonstrated the feasibility of creating benign alterations to the surface charge of enzymes by modification of the N-terminal (and mutatis mutandis, the C-terminal) sequences of the proteins. This has proved valuable in a study of subunit exchange in which hybrid species can be detected by means of nondenaturing polyacrylamide gel electrophoresis. It would clearly be desirable to extend this technique to the creation of hybrid enzymes in vivo, which would allow structures to be generated without the need for subunit dissociation or unfolding in vitro. This is described in the following paper (Deonarain et al., 1992).

ACKNOWLEDGMENTS

We thank Dr. J. McCormick for performing the N-terminal sequence analysis, Dr. A. Berry for valuable discussion, and Mr. N. Hinchliffe for skilled technical assistance.

Registry No. NADH, 58-68-4; NADPH, 53-57-6; GSSG, 27025-41-8; glutathione reductase, 9001-48-3.

REFERENCES

Arscott, L. D., Drake, D. M., & Williams, C. H., Jr. (1989) *Biochemistry 28*, 3591–3598.

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

Biggin, M. D., Gibson, T. G., & Hong, G. F. (1983) *J. Mol. Biol. 144*, 103–116.

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

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

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

Deonarain, M. P., Scrutton, N. S., Berry, A., & Perham, R. N. (1990) *Proc. R. Soc. London, B 241*, 179–186.

Deonarain, M. P., Scrutton, N. S., & Perham, R. N. (1992) *Biochemistry* (following paper in this issue).

Distefano, M. D., Moore, M. J., & Walsh, C. T. (1990) *Biochemistry 29*, 2703–2713.

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

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

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

Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237–271.

Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 701–729.

Karplus, P. A., Pai, E. F., & Schulz, G. E. (1989) *Eur. J. Biochem. 178*, 693–703.

Krauth-Siegel, R. L., Blatterspiel, R., Saleh, M., Schulz, G. E., Schirmer, R. H., & Untucht-Grau, R. (1982) *Eur. J. Biochem. 121*, 259–267.

Krauth-Siegel, R. L., Enders, B., Henderson, G. B., Fairlamb, A. H., & Schirmer, R. H. (1987) *Eur. J. Biochem. 164*, 123–128.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lederberg, J., & Zinder, N. (1948) *J. Am. Chem. Soc. 70*, 4267–4268.

Lowe, C. R., Hans, M., Spibey, N., & Drabble, W. T. (1980) *Anal. Biochem. 104*, 23–28.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321–349.

Packman, L. C., & Perham, R. N. (1982) *FEBS Lett. 139*, 155–158.

Pai, E. F., & Schulz, G. E. (1983) *J. Biol. Chem. 258*, 1751–1757.

Pai, E. F., Karplus, P. A., & Schulz, G. E. (1988) *Biochemistry 27*, 4465–4474.

Patel, M. S., & Roche, T. E. (1990) *FASEB J. 4*, 3224–3233.

Perham, R. N. (1991) *Biochemistry 30*, 8501–8512.

Perham, R. N., Harrison, R. A., & Brown, J. P. (1978) *Biochem. Soc. Trans. 6*, 47–50.

Pienado, J., Florindo, J., Garcia-Alfonso, C., Marinez-Galisteo, E., Llobel, A., & Lopez-Barea, J. (1991) *Mol. Cell. Biochem. 101*, 175–187.

Rice, D. W., Schulz, G. E., & Guest, J. R. (1984) *J. Mol. Biol. 143*, 161–178.

Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H., & Roe, B. A. (1980) *J. Mol. Biol. 143*, 167–178.

Sassenfeld, H. M., & Brewer, S. J. (1984) *Bio Technology 2*, 76–81.

Schirmer, R. H., Krauth-Siegel, R. L., & Schulz, G. E. (1989) in *Glutathione: Chemical, Biochemical and Medical Aspects. Part A* (Dolphin, D., Poulson, R., & Avramovic, O., Eds.) pp 553–596, John Wiley, New York.

Schulze, E., Benen, J. A. E., Westphal, A., van Berkel, W. J. H., & de Kok, A. (1990) in *Flavins and Flavoproteins* (Curti, B., Ronchi, S., & Zanetti, G. Eds.) pp 569–572, Walter de Gruyter, Berlin.

Scrutton, N. S., Berry, A., & Perham, R. N. (1987) *Biochem. J. 245*, 875–880.

Scrutton, N. S., Berry, A., & Perham, R. N. (1988) *FEBS Lett. 241*, 46–50.

Scrutton, N. S., Berry, A., & Perham, R. N. (1990a) *Nature (London) 343*, 38–43.

Scrutton, N. S., Berry, A., Deonarain, M. P., & Perham, R. N. (1990b) *Proc. R. Soc. London, B 242*, 217–224.

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.

Walsh, C. T., Distefano, M. D., Moore, M. J., Shewchuk, L. M., & Verdine, G. L. (1988) *FASEB J. 2*, 124–130.

Williams, C. H., Jr. (1976) *Enzymes (3rd Ed.) 13*, 89–173.

Williams, C. H., Jr., Arscott, L. D., & Schulz, G. E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2199–2202.

Wong, K. K., Vanoni, M. A., & Blanchard, J. S. (1988) *Biochemistry 27*, 7091–7096.