Volume 241, number 1,2, 46–50 FEB 06526 December 1988

Engineering of an intersubunit disulphide bridge in glutathione reductase from *Escherichia coli*

Nigel S. Scrutton, Alan Berry and Richard N. Perham

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England

Received 15 September 1988

By site-directed mutagenesis, Thr-75 was converted to Cys-75 in the glutathione reductase (EC 1.6.4.2) of *Escherichia coli*. This led to the spontaneous formation of an intersubunit disulphide bridge across the 2-fold axis of the dimeric enzyme. The disulphide bridge had no deleterious effect on the catalytic activity, but nor did it increase the thermal stability of the enzyme, possibly because of local conformational flexibility on the dimer interface. The T75C mutant, like the wild-type enzyme, was inactivated by NADPH, proving that this inactivation cannot be due to simple dissociation of the dimer.

Glutathione reductase; Protein engineering; Site-directed mutagenesis; Disulfide bridge; Thermal stability; NADPH

1. INTRODUCTION

The folding of protein monomers and their assembly into active enzyme multimers is a fundamental part of protein biosynthesis. However, comparatively little is known of the detailed mechanisms involved in such macromolecular assembly [1]. A closely related problem is that of the role of disulphide bridges in the stabilization of protein conformation [2]. Both problems can be studied by the techniques of site-directed mutagenesis, but attempts at engineering disulphide bridges into proteins have mainly been confined to intrachain bridges and have met with varying degrees of success [3–5]. The only reported example of an engineered disulphide bridge between subunits is for the N-terminal domain of the dimeric λ-repressor [6].

Glutathione reductase (EC 1.6.4.2) is a member of the growing family of flavoprotein disulphide oxidoreductases which includes dihydrolipoamide dehydrogenase, mercuric reductase and trypanothione reductase ([7] and refs therein). All these enzymes are homodimers with an apparent M_r of about 105000. The amino acid sequence of *Escherichia coli* glutathione reductase has been deduced from the DNA sequence of the cloned *gor* gene [8] and found to show extensive homology with the human enzyme, the structure of which has been solved by X-ray crystallography to 1.54 Å resolution [9]. Two major differences between the proteins are apparent [8]: first, the *E. coli* enzyme lacks the mobile N-terminal segment of 17 residues present in the human enzyme [9,10] and second, the human enzyme contains an intersubunit disulphide bridge between residues Cys-90 and Cys-90′ [10].

Glutathione reductase of *E. coli* is an excellent vehicle for the study of structure-function relationships by site-directed mutagenesis [11]. We have started an investigation into subunit assembly by studying the effects of replacing Thr-75 in the *E. coli* enzyme (the position equivalent to Cys-90 in the human enzyme) with a cysteine residue, thereby conferring the potential to form an intersubunit disulphide bridge. The active site of glutathione reductase involves side chain contributions from both subunits of the protein [9,10]. Re-

Correspondence address: R.N. Perham, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

cent kinetic experiments on glutathione reductase [12] have suggested that the inactivation of the *E. coli* enzyme induced by NADPH [13] could be due to an enforced dissociation of the protein dimer, with accompanying disruption of the active site. If the mutagenic change of Thr-75 to Cys-75 produced a mutant protein containing an intersubunit disulphide bridge, it was hoped that this might also allow a test of this potential mechanism for the NADPH-induced inactivation.

2. MATERIALS AND METHODS

2.1. *Genetical manipulation, mutagenesis and DNA sequencing*

The *E. coligor* gene (non-coding strand) was subjected to site-directed mutagenesis by standard procedures in a derivative of bacteriophage M13K19 [14], as described elsewhere [11]. The mutagenic oligonucleotide 5′-TGGTTTTGATACCTGTATC-AATAAAT-3′ (T75C) was kindly synthesized by Dr A. Northrop, Institute of Animal Physiology & Genetic Research, Babraham, Cambridge, on a Biosearch model 8600 DNA synthesizer. The whole of the mutated gene was sequenced [11] to ensure that no other mutations had been introduced. The mutant gene was excised by restricting the bacteriophage RF with *Eco*RI and *Hin*dIII, and the appropriate fragment was subcloned into the expression vector pKK223-3 cut with the same enzymes [7]. The construct was transformed into *E. coli* strain SG5, which carries a chromosomal deletion of the *gor* gene [7,8].

2.2. *Growth of cells and purification and assay of enzymes*

Wild-type and mutant (T75C) glutathione reductases were purified from *E. coli* SG5 transformed with the appropriate expression plasmid as described elsewhere [7,11], except that 2-mercaptoethanol was omitted from all the buffers. Glutathione reductase activity was estimated at 30°C by following the GSSG-dependent oxidation of NADPH [7].

2.3. *SDS-polyacrylamide gel electrophoresis and immunoblotting*

SDS-polyacrylamide gel electrophoresis was carried out in 10% polyacrylamide slab gels and protein bands were visualised by staining with Coomassie brilliant blue [7]. Antibodies against *E. coli* glutathione reductase were raised in female New Zealand white rabbits [15] and antiserum was stored at −70°C until required. Proteins were transferred electrophoretically from SDS-polyacrylamide gels onto nitrocellulose filters [16,17] and immunoblots were prepared by the method of Hawkes et al. [18] as modified by Johnson et al. [19].

3. RESULTS

3.1. *Formation of an intersubunit disulphide bridge*

The wild-type and mutant (T75C) enzymes were purified from extracts of *E. coli* SG5 cells over-expressing (about 200-fold) the wild-type or mutated *gor* gene from the plasmid vector pKK223-3 [7]. Samples of the mutant and wild-type enzymes were submitted to SDS-polyacrylamide gel electrophoresis in the presence and absence of 0.1 M DTT and 2 mM 2-mercaptoethanol in the sample and running buffers, respectively (fig.1A). In the absence of reducing agents, the mutant glutathione reductase migrated with an apparent M_r of about 100000 whereas the wild-type enzyme migrated with an apparent M_r of 49000, as expected for the enzyme subunit which has an M_r of 48717 deduced from the DNA sequence of the *gor* gene [8]. Under reducing conditions both the wild-type enzyme and the T75C mutant migrated with the apparent M_r (49000) of the monomer. These results suggested that the T75C mutant enzyme possesses an intersubunit disulphide link which had formed spontaneously in the absence of any added oxidizing agent. An immunoblot with rabbit anti-glutathione reductase antibody confirmed that the higher molecular mass protein observed for the T75C mutant in the absence of reducing agents was glutathione reductase (not shown). Gel filtration through Superose 12 equilibrated with 0.1 mM potassium phosphate buffer, pH 7.5, indicated that both the wild-type enzyme and T75C mutant migrated as dimers (apparent M_r 94000) under non-reducing conditions. These results demonstrate conclusively that conversion of Thr-75 to a cysteine residue has led to the spontaneous formation of an intersubunit disulphide bridge in the *E. coli* enzyme.

The T75C mutant was found to have a specific catalytic activity of 343 units/mg, which compares favourably with the value (334 units/mg) obtained for the wild-type enzyme under the same saturating conditions of substrates [7]. Thus an intersubunit disulphide bridge can be inserted across the dimer interface of *E. coli* glutathione reductase without detriment to the catalytic activity.

3.2. *Thermal stability and kinetic inactivation*

We were interested to know if the presence of the new cystine 75-75′ link had any effect on the thermal stability of *E. coli* glutathione reductase. The wild-type and T75C mutant enzymes were held for 10 min at various temperatures before assay but no differences were observed in the thermal

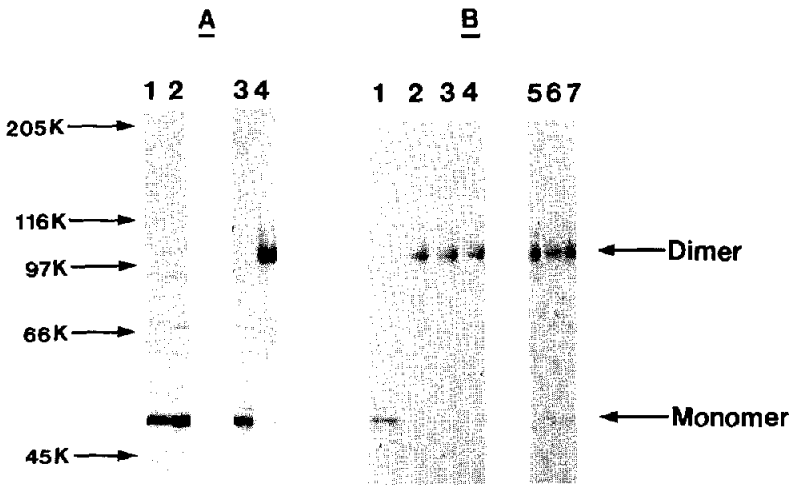

Fig.1. SDS-polyacrylamide gel electrophoresis of wild-type and T75C mutant *E. coli* glutathione reductase. The molecular mass markers used were: 205K (K = kDa), myosin; 116K, β-galactosidase; 97K, phosphorylase *b*; 66K, bovine serum albumin; and 45K, ovalbumin. (A) 10% polyacrylamide gels were run in the presence (reducing conditions) or absence (non-reducing conditions) of 2-mercaptoethanol in the running buffer [7]. Samples (10 μg) were applied to the gel in sample buffer containing (reducing conditions) or not containing (non-reducing conditions) DTT. Lanes: 1, wild-type enzyme, reducing conditions; 2, T75C mutant, reducing conditions; 3, wild-type enzyme, non-reducing conditions; 4, T75C mutant, non-reducing conditions. (B) Samples of wild-type enzyme or T75C mutant were incubated at various temperatures for 10 min in 0.1 M potassium phosphate buffer, pH 7.0, in the absence of NADPH or in the presence of 5 mM NADPH. Electrophoresis was carried out under non-reducing conditions, as described in A. Lanes: 1, wild-type enzyme, plus NADPH, 50°C; 2, T75C mutant, minus NADPH, 22°C; 3, T75C mutant, minus NADPH, 37°C; 4, T75C mutant, minus NADPH, 50°C; 5, T75C mutant, plus NADPH, 22°C; 6, T75C mutant, plus NADPH, 37°C; 7, T75C mutant, plus NADPH, 50°C.

stability profiles (fig.2). Similarly, both enzymes lost activity at the same rate when incubated at temperatures between 67°C and 70°C (not shown). Thus, the covalent intersubunit link engineered in the T75C mutant appears not to confer any significant increase on the thermal stability of the enzyme.

3.3. *NADPH-induced inhibition*

In the presence of 5 mM NADPH, the wild-type enzyme was rapidly inactivated at temperatures above 25°C (fig.3). Under the same conditions, the T75C mutant enzyme exhibited almost exactly the same inactivation as the wild-type enzyme. In both cases, full catalytic activity could be recovered by incubating the NADPH-inactivated enzymes with GSSG for 2–3 h. To confirm that monomerization of the T75C mutant had not occurred, samples previously incubated with NADPH at 25°C, 30°C and 50°C were electrophoresed in an SDS-polyacrylamide gel under non-reducing conditions. The inactivated protein migrated as a dimer with

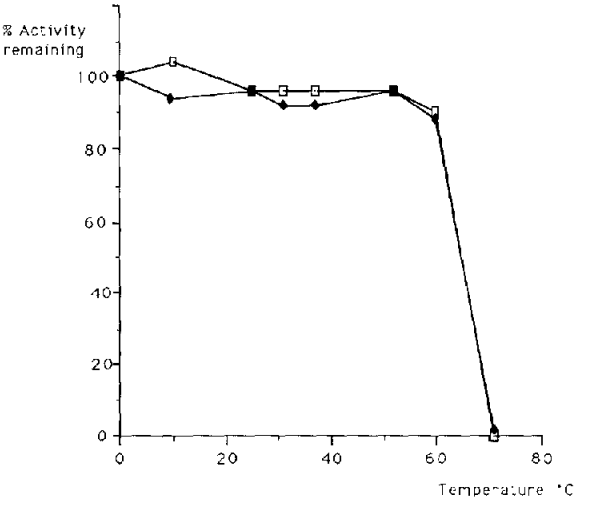

Fig.2. Thermostability of the wild-type and T75C mutant glutathione reductases. The enzymes (0.01 units) were incubated at the desired temperature for 10 min in 40 μl of 0.1 M potassium phosphate buffer, pH 7.0, and were then assayed under conditions of saturating substrates [7]. (□) Wild-type enzyme; (◆) T75C mutant.

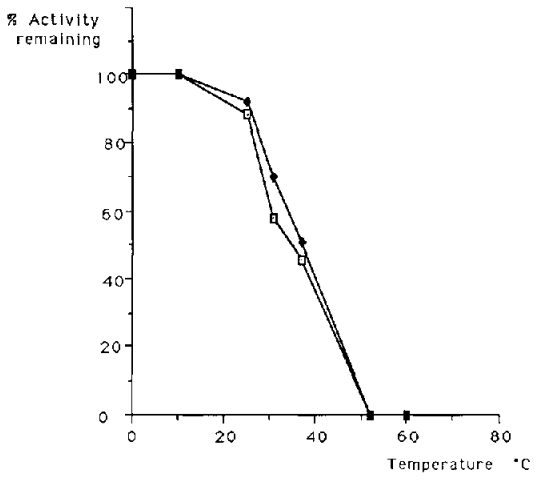

Fig.3. Inactivation of the wild-type and T75C mutant glutathione reductases in the presence of NADPH. The enzymes (0.01 units) were incubated at various temperatures for 10 min in 0.1 M potassium phosphate buffer, pH 7.0, in the presence of 5 mM NADPH and were then assayed under conditions of saturating substrates [7]. (□) Wild-type enzyme; (♦) T75C mutant.

an apparent M_r of about 100000 (fig.1B). It therefore appears that the intersubunit disulphide bridge has remained intact but that prevention of dissociation of the enzyme dimer has not prevented the NADPH-induced inactivation.

4. DISCUSSION

Human glutathione reductase contains an intersubunit disulphide bridge between residues Cys-90 and Cys-90′ [10]. The Cys-90 residue is within the FAD-binding domain and lies on the two-fold axis of symmetry of the protein dimer [9,10]. The lack of an equivalent disulphide bridge in *E. coli* glutathione reductase is one of the few major differences between it and the human enzyme [8]. The mutation of Thr-75 (the corresponding residue in the *E. coli* protein [8]) to a cysteine residue has now been shown to generate an *E. coli* glutathione reductase that contains an intersubunit disulphide bridge, one which was formed without the need for added oxidizing agents. We cannot say whether this disulphide link exists in the dimer in vivo or whether it was formed by oxidation of the pair of juxtaposed thiol groups during the aerobic preparation of the enzyme. However, it had no discernible effect on the catalytic activity of the enzyme in vitro and, as far as we know, is the first intersubunit disulphide bridge to be introduced into an enzyme with full retention of the biological activity. This result is compelling evidence for the close similarity in structure of *E. coli* and human glutathione reductases [8], and gives us confidence that the crystal structure of the human enzyme is a valid model for site-directed mutagenesis experiments on the *E. coli* enzyme [11].

The failure to detect any increase in the thermal stability of the T75C mutant over wild-type enzyme is not as surprising as it may appear, given the nature of the dimer interface of glutathione reductase. In the human enzyme the interface interactions lie in two clearly defined areas of protein-protein contact [9]. The 'upper' area between the two interface domains contributes a much greater proportion of the binding energy and is the most ordered part of the protein. In contrast, the 'lower' area contains the only portion of polypeptide chain (other than the N-terminal segment) where the main chain geometry is not clearly defined in the electron density map. The upper area is therefore much more important for dimer interaction than the lower one [9]. The same is likely to be true for the interface in the *E. coli* enzyme, since the enzymes show 92% sequence identity in the upper interface area compared with 52% identity overall. The addition of the Cys-75/Cys-75′ disulphide bridge in the T75C mutant therefore places a covalent link across the lower interface area and any increase in stability afforded by this interaction is most probably masked by the stronger interactions across the upper contact area.

The results illustrated in figs 1B and 3 conclusively show that dissociation of the T75C dimer does not occur and as such cannot account for the NADPH-induced inactivation of the enzyme. However, given the placing of the new disulphide bridge on the dimer interface discussed above, it is unlikely that the covalent link between the subunits can prevent conformational changes along the dimer interface. Thus, a conformational change induced by NADPH could still physically disrupt the active site geometry, despite the enzyme remaining a dimeric protein. More detailed evaluation of the kinetics of inactivation by NADPH of the T75C mutant, together with X-ray crystallographic analysis, may shed more light on this curious inactivation process.

Acknowledgements: We are grateful to the Science and Engineering Research Council for financial support and a Research Studentship (to N.S.S.) and to St John's College, Cambridge, for the award of a Benefactors' Scholarship (to N.S.S.). A.B. is a Royal Society 1983 University Research Fellow.

REFERENCES

[1] Shaw, W.V. (1987) Biochem. J. 246, 1–17.
[2] Creighton, T.E. (1988) BioEssays 8, 57–63.
[3] Perry, L.J. and Wetzel, R. (1986) Biochemistry 25, 733–739.
[4] Wells, J.A. and Powers, D.B. (1986) J. Biol. Chem. 261, 6564–6570.
[5] Wetzel, R., Perry, L.J., Baase, W.A. and Becktel, W.J. (1988) Proc. Natl. Acad. Sci. USA 85, 401–405.
[6] Sauer, R.T., Hehir, K., Stearman, R.S., Weiz, M.A., Jeitler-Nilsson, A., Suchanek, E.G. and Pabo, C.O. (1986) Biochemistry 25, 5992–5998.
[7] Scrutton, N.S., Berry, A. and Perham, R.N. (1987) Biochem. J. 245, 875–880.
[8] Greer, S. and Perham, R.N. (1986) Biochemistry 25, 2736–2742.
[9] Karplus, P.A. and Schulz, G.E. (1987) J. Mol. Biol. 195, 701–729.
[10] Thieme, R., Pai, E.F., Schirmer, R.H. and Schulz, G.E. (1981) J. Mol. Biol. 151, 763–782.
[11] Berry, A., Scrutton, N.S. and Perham, R.N. (1988) Biochemistry, in press.
[12] Arscott, D.L., Drake, D.M. and Williams, C.H., jr (1987) in: Flavins and Flavoproteins (Edmondson, D.E. and McCormick, D.B. eds) pp.75–78, Walter de Gruyter, Berlin.
[13] Mata, A.M., Pinto, M.C. and Lopez-Barea, J. (1985) Mol. Cell. Biochem. 67, 65–76.
[14] Carter, P., Bedouelle, M., Waye, M.M. and Winter, G. (1985) in: Oligonucleotide Site-Directed Mutagenesis in M13. A Laboratory Manual, Anglian Biotechnology.
[15] Hudson, L. and Hay, F.C. (1976) Practical Immunology, Blackwell Scientific Publications, Oxford.
[16] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[17] Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
[18] Hawkes, R., Niday, E. and Gordon, J. (1982) Anal. Biochem. 119, 142–147.
[19] Johnson, D.A., Gautsch, J.W., Sportsman, J.R. and Elder, J.H. (1984) Gene Anal. Techn. 1, 3–8.