Flavoprotein disulphide oxidoreductases: protein engineering of glutathione reductase from *Escherichia coli*

RICHARD N. PERHAM, ALAN BERRY
and NIGEL S. SCRUTTON
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

Introduction

The enzymes glutathione reductase (EC 1.6.4.2), thio-
reductase (EC 1.6.4.5), dihydrolipoamide dehydro-
genase (EC 1.8.1.4 formerly EC 1.6.4.3) and mercuric reductase are all members of a family of enzymes, the flavop-
rotein disulphide oxidoreductases. Glutathione reductase catalyses the reduction of oxidized glutathione (GSSG) by NADPH:

\[ \text{GSSG} + \text{NADPH} + H^+ \rightleftharpoons 2\text{GSH} + \text{NADP}^+ \]

Reduced glutathione (GSH) plays a crucial role in ensuring that other thiol groups remain reduced within the cell and is particularly important in the biosynthesis of DNA [for a review, see Holmgren (1985)].

Dihydrolipoamide dehydrogenase is an essential component of the 2-oxo acid dehydrogenase multi-enzyme complex (Reed, 1974; Perham, 1983) in which it acts to oxidize the dihydrolipoic acid residues of the lipoate acyltransferase components in an NAD^+-dependent reaction:

\[ \text{LipSH}_2 + \text{NAD}^+ = \text{LipS}_2^- + \text{NADH} + H^+ \]

Mercuric reductase in bacteria is part of a plasmid-encoded system for the detoxification of mercuric ions, catalysing the following reaction (Fox & Walsh, 1982):

\[ \text{Hg}^{2+} + \text{NADPH} = \text{Hg}^- + \text{NADP}^+ + H^+ \]

All these enzymes are dimers with an *M* of about 105 000 and each possesses a redox-active disulphide bridge which is essential to the catalytic mechanism [reviewed by Williams (1976)]. Considerable homology, consistent with their evolution from a common ancestor, exists between the enzymes glutathione reductase, dihydrolipoamide dehydrogenase and mercuric reductase, as shown by studies of the amino acid sequences around their redox-active disulphides (Perham et al., 1978; Williams et al., 1982; Packman & Perham, 1982; Krahth-Siegel et al., 1982; Fox & Walsh, 1983).

The *Ipd* gene of *Escherichia coli* encoding dihydrolipo-
amide dehydrogenase (Stephens et al., 1983), the *merA* gene of the transposon Tn501 from *Pseudomonas aeruginosa*, encoding mercuric reductase (Brown et al., 1983), and most recently the gor gene of *E. coli* encoding glutathione reductase (Greer & Perham, 1986) have been cloned and their nucleotide sequences determined. This has enabled the complete amino acid sequence of these enzymes to be inferred and their structures compared. However, the best structural information for any of these enzymes is that available for human glutathione reductase. The amino acid sequence is known (Krauth-Siegel et al., 1982) and the X-ray crystallographic structure (Thieme et al., 1981) has recently been refined to 1.54 Å resolution (Karpplus & Schulz, 1987). These studies have led to a detailed appreciation of the reaction mechanism for glutathione reductase (Pai & Schulz, 1983).

The homology between the primary structures of the *E. coli* dihydrolipoamide dehydrogenase and human glutathione reductase is indicative of closely similar three-dimen-
sional structures, a conclusion borne out by a direct attempt to fit the amino acid sequence of the *E. coli* dihydrolipo-
amide dehydrogenase to the three-dimensional structure of human glutathione reductase (Rice et al., 1984). The differ-
ence in coenzyme specificity (the former uses NAD, the latter NADP) is explicable in terms of additional positively charged side-ehains in the coenzyme binding pocket of glutathione reductase, complementary to the additional negatively charged phosphate group of its coenzyme (Rice et al., 1984). Similarly, it is obvious that many of the features of the catalytic mechanism envisaged for glutathione reductase (Pai & Schulz, 1983) must be shared by dihydrolipoamide dehydrogenase and other flavoprotein disulphide oxido-
reductases.

The cloning and sequence analysis of the *E. coli* gor gene and the strong homology between the human and *E. coli* glutathione reductases (Greer & Perham, 1986) now makes it possible to test some of these predictions and to learn more about the reaction mechanism of the enzyme by the methods of protein engineering.

Expression and site-directed mutagenesis of glutathione reductase

As a first step in this direction, the *E. coli* gor gene has been subcloned and expressed in a plasmid vector under the control of the strong tac promoter (Scrutton et al., 1986, 1987). This has allowed the level of glutathione reductase activity in *E. coli* cells to be raised more than 200-fold, which has in turn facilitated the purification of the enzyme in amounts necessary for protein chemical and X-ray crystallo-
graphic analysis. The purified enzyme was shown to have all the properties previously reported for *E. coli* glutathione reductase (Scrutton et al., 1987).

The active site of human glutathione reductase is shown in schematic form in Fig. 1. When comparison is made with the amino acid sequence of the *E. coli* enzyme (Greer & Perham, 1986), the conservation of amino acid residues important in the catalytic mechanism (Pai & Schulz, 1983) is almost perfect. Only one residue is changed: His-219 in the human enzyme is replaced by a lysine (Lys-199) residue. The difference in residue number is a result of the omission from the *E. coli* enzyme of the first 17 amino acids of the human enzyme.

Given the high degree of homology between the two enzymes and the very good X-ray structure of the human enzyme, we have designed and made several site-directed mutations in *E. coli* glutathione reductase. Thus, we have converted both active-site cysteine residues (Cys-42 and Cys-47) into serine residues and have also begun investigations into the role of His-439 (corresponding to His-467 in the human enzyme) as a H⁺ donor during the catalytic cycle of the enzyme. Of particular interest for our present purposes are the properties of enzymes in which we have mutated residue Tyr-177. This residue is the counterpart of Tyr-197 in the human enzyme (Greer & Perham, 1986), which lies in the NADPH-binding pocket and shields the isalloxazine ring of the flavin in the absence of NADPH. On binding NADPH, the tyrosine side-chain moves to allow the nicotinamide ring of the NADPH to come close to the flavin for electron transfer (Pai & Schulz, 1983) and it has been postulated (Rice et al., 1984) that this residue acts as a 'lid' in the NADPH pocket to prevent loss of electrons from the flavin moiety. Two mutations in position 177 have been made to date, one (Y177F) of tyrosine to phenylalanine, and the other (Y177S) of tyrosine to serine.

**Tyrosine-177 and the kinetic mechanism of glutathione reductase**

Mutagenesis was carried out in a derivative of bacteriophage M13K19 carrying the non-coding strand of the *gor* gene. The mutagenic oligonucleotides 5'-CGCGGGTTTCCATCGCCG-3' (Y177F) and 5'-CGCGGGTTCCATCGCCG-3' (Y177S) were annealed to single-stranded template DNA and the EcoB/K double-primer selection procedure described by Carter et al. (1985) was used to produce mutants. All putative mutants were screened by hybridization with mutagenic oligonucleotide and the mutations were confirmed by dideoxy sequencing. The mutant genes were then subcloned into the expression vector pKK223-3 (Scrutton et al., 1987) and were transformed into a strain (SG5) of *E. coli* carrying a chromosomal deletion of the *gor* gene (Greer & Perham, 1986).

The mutant and wild-type enzymes were purified (Scrutton et al., 1987). Mutant Y177F was almost as active as the wild-type enzyme.

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**Fig. 1. The active site of human glutathione reductase**

The catalytic centre of human glutathione reductase (Rice et al., 1984), showing both substrates and the FAD cofactor. Some amino acid residues thought important for catalysis/binding are also shown. Tyr-197 (which is thought to function as a moveable 'lid' for the flavin) is shown in the position it adopts when NADPH is not bound to the enzyme.
Table 1. Kinetic analysis of Tyr-177 mutants

Glutathione reductase was assayed under saturating conditions as described previously (Scrutton et al., 1987). Kinetic parameters were evaluated using a 5 x 5 matrix of substrate concentrations and the data were analysed by least-squares regression analysis as outlined in the text.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>Y177F</th>
<th>Y177S</th>
</tr>
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<tbody>
<tr>
<td><strong>Specific activities (under saturating assay conditions)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Units mg^-1</td>
<td>251.8</td>
<td>246.1</td>
<td>72.3</td>
</tr>
<tr>
<td><strong>Forward reaction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanism</td>
<td>Ping-Pong</td>
<td>Ping-Pong</td>
<td>Ordered Sequential</td>
</tr>
<tr>
<td>$K_m$ NADPH</td>
<td>38.0 ± 4.1 μM</td>
<td>23.9 ± 4.5 μM</td>
<td>30.1 ± 8.0 μM</td>
</tr>
<tr>
<td>$K_m$ GSSG</td>
<td>96.6 ± 11.7 μM</td>
<td>53.3 ± 8.8 μM</td>
<td>1.77 ± 1.54 μM</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>52.1 ± 3.8 nmol min^-1</td>
<td>42.8 ± 5.3 nmol min^-1</td>
<td>46.4 ± 7.9 nmol min^-1</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>36 100 ± 2630 min^-1</td>
<td>31 400 ± 3890 min^-1</td>
<td>8190 ± 1400 min^-1</td>
</tr>
<tr>
<td><strong>Reverse reaction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanism</td>
<td>Ter-Bi Sequential</td>
<td>Ter-Bi Sequential</td>
<td>Ter-Bi Sequential</td>
</tr>
<tr>
<td>$K_m$ NADP^+</td>
<td>120 ± 19.7 μM</td>
<td>72.1 ± 13.4 μM</td>
<td>77.6 ± 8.7 μM</td>
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<tr>
<td>$K_m$ GSH</td>
<td>1270 ± 980 μM</td>
<td>1220 ± 274 μM</td>
<td>697 ± 109 μM</td>
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<tr>
<td>$V_{max}$</td>
<td>39.1 ± 3.3 nmol min^-1</td>
<td>33.0 ± 1.6 nmol min^-1</td>
<td>10.0 ± 0.3 nmol min^-1</td>
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<tr>
<td>$k_{cat}$</td>
<td>3010 ± 254 min^-1</td>
<td>1350 ± 65 min^-1</td>
<td>97.9 ± 2.9 min^-1</td>
</tr>
</tbody>
</table>

![Fig. 2. The branching mechanism of yeast glutathione reductase](image)

The hybrid Bi-Bi Ordered Sequential Ping-Pong mechanism proposed for yeast glutathione reductase (Mannervik, 1973).

Conclusions

A hybrid kinetic mechanism appears to operate for E. coli glutathione reductase, as proposed earlier for the yeast enzyme (Mannervik, 1973). It is particularly impressive that the balance of flux between the Ping-Pong and Ordered Sequential mechanism (Fig. 2), as postulated for the yeast enzyme (Mannervik, 1973). In the wild-type E. coli enzyme, the Ping-Pong route is strongly favoured. However, as we move to the mutants Y177F and Y177S, the flux can evidently be diverted from the Ping-Pong to the Ordered Sequential pathway. Also unexpectedly, since the mutation Y177S is in the NADPH-binding pocket and not near the GSSG-binding site (Thierne et al., 1981; Pai & Schulz, 1983), the $K_m$ for GSSG for the mutant enzyme was about 50 times lower than that observed with the wild-type enzyme. However, this may account for the change in kinetics since high concentrations of GSSG favour the Ordered Sequential pathway over the Ping-Pong pathway for the hybrid mechanism (Mannervik, 1973).
Sequential pathways in this hybrid mechanism can be crucially tipped by mutation of just one amino acid residue, Tyr-177, in the \textit{E. coli} protein. Further work is in hand to characterize these mutants and to investigate the ability of the mutated residue to protect the flavin from electron loss and to study the environment of the flavin in these mutants. Comparable studies of other mutants can be expected to throw light on other aspects of the structure and mechanism of this important class of dehydrogenase.

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\textbf{Dihydrolipoamide dehydrogenase: a 'new' function for an old enzyme?}

MICHAEL J. DANSON

Department of Biochemistry, University of Bath, Bath BA2 7AY, U.K.

Introduction

Dihydrolipoamide dehydrogenase (EC 1.8.1.4) catalyses the \textit{NAD}⁺-dependent oxidation of dihydrolipoamide (Williams, 1976) via a catalytic mechanism which involves the alternate oxidation and reduction of an intrachain disulphide bond and a base (B) on the enzyme (Fig. 1). The enzyme fulfils this function as an integral component of the pyruvate, 2-oxoglutarate and branched-chain 2-oxo acid dehydrogenase complexes (Reed, 1974; Perham, 1975; Petit et al., 1978) and of the glyoxylate enzyme cleavage system (Kikuchi & Hiraga, 1982). These are the established metabolic roles of dihydrolipoamide dehydrogenase but there is accumulating evidence to suggest that the enzyme may serve an as yet undefined additional function and one that is specific to a location in the plasma membrane of the cell. The present communication serves to review the data supporting this possibility.

\textit{Dihydrolipoamide dehydrogenase of the archaeabacteria}

The archaeabacteria are now recognized as a phylogenetically distinct group of organisms, constituting a third evolutionary kingdom in addition to the eubacteria and eukaryotes (reviewed in Fewson, 1986; Woese & Olsen, 1986; Danson, 1988). They comprise three phenotypes: the extreme

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**Fig. 1. \textit{NAD}⁺-dependent oxidation of dihydrolipoamide catalysed by dihydrolipoamide dehydrogenase**