GLUTATHIONE REDUCTASE: MUTATION, CLONING AND SEQUENCE ANALYSIS OF THE GENE IN *E. coli*

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Summary

A mutation in the *Escherichia coli* gene *(gor)* for glutathione reductase (EC 1.6.4.2) has been identified and mapped. Mutants deficient in glutathione reductase are not impaired in growth, showing that *E. coli* has no absolute need for this enzyme. A double mutant *(gor trx*B*)* of *E. coli* has been constructed that is simultaneously deficient in glutathione reductase and thioredoxin reductase (EC 1.6.4.5). The double mutant is severely crippled in growth, implying that these enzymes provide the major, but not necessarily the only routes for coupling NADPH with the reduction of ribonucleoside diphosphates required for DNA biosynthesis. The *gor* gene has been cloned and partially sequenced as a prelude to the study of the enzyme by means of protein engineering.

Introduction

Glutathione reductase (EC 1.6.4.2) is a widespread enzyme which catalyses the reduction of oxidized glutathione by NADPH:

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

It is a member of an important family of flavoprotein oxidoreductases that includes dihydrolipoamide dehydrogenase (EC 1.6.4.3), thioredoxin reductase (EC 1.6.4.5) and, a recent addition, mercuric reductase. Dihydrolipoamide dehydrogenase is well-known and widely studied as a component of the 2-oxo-acid dehydrogenase multienzyme complexes (1,2,3) in which it acts to re-

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oxidize the dihydrolipoic acid residues of the lipoate acyltransferase components in an NAD^+-dependent reaction:

$$Lip(SH)_2 + NAD^+ \longrightarrow LipS_2 + NADH + H^+$$

Thioredoxin reductase catalyses the $NADP^+$-linked reduction of a disulphide bridge in the small protein, thioredoxin, that acts in turn as a source of reducing power in the conversion of ribonucleotides to deoxyribonucleotides (4):

$$TrS_2 + NADPH + H^+ \longrightarrow Tr(SH)_2 + NADP^+$$

And mercuric reductase in bacteria is part of a plasmid-encoded system for the detoxification of mercuric ions, catalysing the following reaction (5):

$$Hg^{2+} + NADPH \longrightarrow Hg^\circ + NADP^+ + H^+$$

All these enzymes are dimers with an M_r of about 105 000 and all possess a disulphide bridge in each subunit which is alternately oxidized and reduced as part of the catalytic mechanism (for review see 6). This common feature has served as a focal point for amino acid sequence studies. As shown in Table 1, the sequences around the redox-active disulphide bridges of dihydrolipoamide dehydrogenase, glutathione reductase and mercuric reductase are highly homologous, implying that they have arisen by divergent evolution from a common ancestor (6,7,8,9,10,11). On the other hand, the bridge size and the amino acid sequence surrounding it in thioredoxin reductase are sufficiently different for it to be likely that this enzyme has arisen by convergent evolution towards a common mechanism (7).

The three-dimensional structure of human glutathione reductase is known from its complete amino acid sequence (10) and X-ray crystallographic analysis at 2 Å resolution (13), and this has significantly sharpened our appreciation of the reaction mechanism (14). From other lines of enquiry, the *lpd* gene of *Escherichia coli*, encoding dihydrolipoamide dehydrogenase (15), and the *mer*A gene of transposon Tn 501 from *Pseudomonas aeruginosa* encoding mercuric reductase (16), have been cloned and their nucleotide sequences determined. This has enabled the complete primary structures

Table 1: Amino acid sequences around redox-active disulphide bridges

Dihydrolipoamide dehydrogenase[a]	
(a) Pig heart(6,7,8)	T V C I E K B Z T L G G T C L N V G C I P S K
(b) *E. coli*(6,7)	Y N T L G G V C L N V G C I P S K
(c) *B. stearothermophilus*(9)	G N L G G V C L N V G C I P S K
Glutathione reductase[a]	
(a) Human erythrocytes(10)	A A V V E S H K L G G T C V N V G C V P K K
(b) Yeast(6)	L G G T C V N V G C V P K
Mercuric reductase[a]	
P. aeruginosa(11)	G T I G G T C V N V G C V P S K
Thioredoxin reductase	
E. coli(6,12)	A C A T C D G F

[a]Sequences of tryptic peptides

of the corresponding enzymes to be inferred, but further work is somewhat hampered by the lack of complementary X-ray diffraction studies.

In the present paper we describe our own recent efforts to identify mutations in the gene *(gor)* for *g*lutathione *o*xido*r*eductase of *E. coli*, and to clone and sequence the gene as a preamble to a deeper analysis of the mechanism of flavoprotein oxidoreductases by means of the newer techniques of protein engineering.

Isolation and mapping of the *gor* gene of *E. coli*

Glutathione is not an essential metabolite in *E. coli*, for mutants unable to synthesize it are unaffected in growth (17,18). Thus we suspected that mutants unable to reduce oxidized glutathione might also not be impaired in growth. A method for screening *E. coli* colonies for glutathione reductase *in situ* on agar plates was therefore developed (19).

This is based on the reaction of almost colourless 5,5'-dithiobis-(2-nitrobenzoic acid)(Nbs_2) with reduced glutathione to give the strongly yellow-coloured Nbs^- anion, and consisted of pouring a soft agar overlay containing NADPH, Nbs_2 and oxidized glutathione onto agar plates of *E.coli* colonies previously lysed by exposure to chloroform vapour:

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

$$2GSH + Nbs_2 \longrightarrow GSSG + 2Nbs^- + 2H^+$$

Only colonies having glutathione reductase can produce reduced glutathione, so only those colonies turn yellow.

This method was used to screen colonies of *E. coli* mutated by bacteriophage Mu insertion (19). Mu is a bacteriophage which can lysogenize *E. coli*; it does so by inserting itself pseudo-randomly into the chromosome of the cell. If the insertion point is within a gene, a complete block of normal transcription and translation ensues, i.e. a complete block of gene function. The Mu phage used was Mu*cts*Ap. Stocks of this Mu phage are easily produced because it has a temperature-sensitive repressor and thus at elevated temperatures it is forced to enter the lytic phase of its life-cycle. *E. coli* cells containing inserted phage are easy to select because they are ampicillin-resistant; and the ampicillin-resistance also makes the insertions easy to map because it is a readily-scorable trait.

Of 15 000 colonies screened in two independent experiments, eight were obtained that could not reduce glutathione. Analysis of cell-free extracts showed that these mutants truly lacked glutathione reductase.

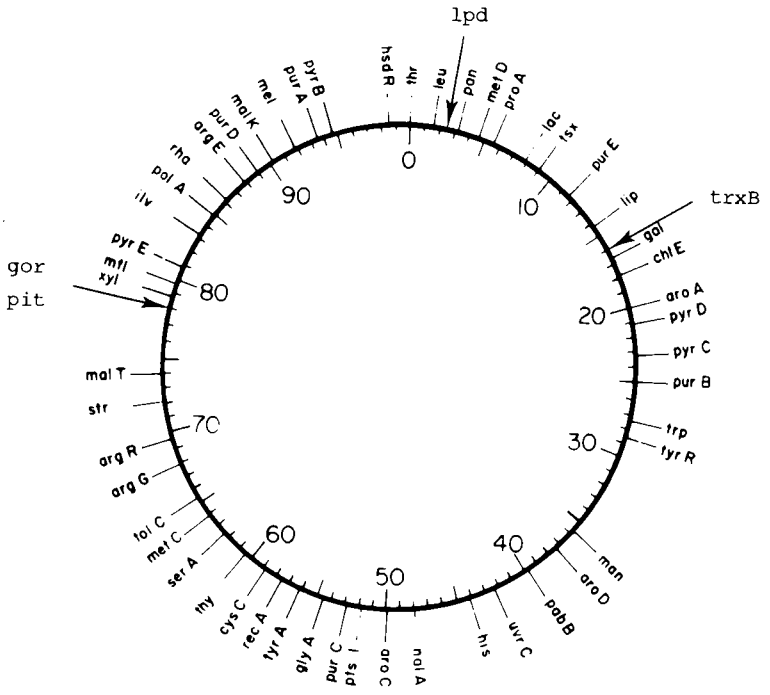

Fig. 1. Linkage map of *E. coli* K-12. The position of the genes for glutathione reductase *(gor)*, dihydrolipoamide dehydrogenase *(lpd)*, thioredoxin reductase *(trx*B) and an inorganic phosphate transport system *(pit)* are shown. The map positions are those listed by Bachmann *et al.* (20,21) except for *trx*B which has been mapped only approximately (22) and was not included.

One of these strains was selected and the *gor* lesion was mapped by transduction with bacteriophages P1 and T4GT7 as vectors: it was located between 77 and 78 min on the *E. coli* genome (Fig. 1). As suspected initially, the *gor* mutation caused no defect in growth (19).

Properties of a *gor-trx*B double mutant

Deoxyribonucleotides in *E. coli* are synthesized by reduction of the

corresponding ribonucleotides in the presence of the enzyme ribonucleotide diphosphate reductase (23). An essential cofactor for ribonucleotide reductase is either thioredoxin or glutaredoxin, small proteins (M_r 12 000) containing an intrachain disulphide bridge (4). Thioredoxin is reduced by NADPH in a reaction catalysed by thioredoxin reductase, an enzyme encoded by gene *trx*B (24); glutaredoxin requires glutathione/glutathione reductase for its reduction (Fig. 2).

The thioredoxin and glutaredoxin systems function in parallel (4): the simultaneous presence of both thioredoxin reductase and glutathione reductase is not required since mutants defective in thioredoxin reductase (*trx*B) (24) and glutathione reductase (*gor*) (19) are individually viable. The obvious question is whether a *gor*-*trx*B double mutant is also viable - that is, whether reducing power must reach ribonucleotide reductase *via* one or other of these enzymes, or whether yet another pathway exists.

Combination of the *gor* and *trx*B mutations in the same *E. coli* cell is not a trivial operation because neither of these mutations is itself directly selectable. Fortunately, the *gor* mutation we describe above is selectable because of the Mu*cts*Ap used to block gene function. The obvious approach is to transduce the *gor* :: Mu*cts*Ap construct from this mutant into a strain already known to contain the *trx*B mutation. This recipient strain must be lysogenic for bacteriophage Mu so that there is Mu repressor in the cell - otherwise the incoming Mu in the *gor* :: Mu*cts*Ap construct will simply enter

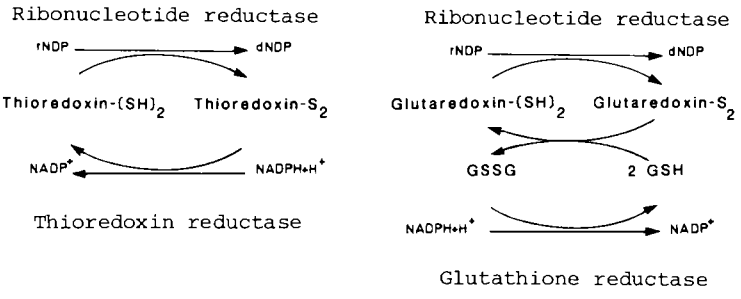

Fig. 2. Accepted pathways for the biosynthesis of deoxyribonucleotides

the lytic phase and kill the potential double mutant even before it can form. The *trx*B strain described by Fuchs (24) was used as the starting point for the manipulations. It was first made lysogenic for Mu*cts*Km, a Mu phage bearing a gene for kanamycin-resistance. This new strain was then used as the recipient in a bacteriophage P1-mediated transduction using a *gor* :: Mu*cts*Ap donor and selecting for ampicillin-resistant progeny. There are two possible outcomes of such a transduction; the incoming Mu may recombine with the Mu already inserted in the chromosome of the recipient, leaving the *gor* gene of the recipient intact, or the incoming *gor* gene may recombine with the *gor* gene of the recipient, rendering the recipient glutathione reductase-negative. Thus two classes of ampicillin-resistant recombinants are expected in roughly a one-to-one ratio. This in fact is what was found.

One class of recombinants grew much like the recipient strain whereas the second class grew much more slowly. As expected, the first class still possessed glutathione reductase but the second class lacked it. They also still lacked thioredoxin reductase, as shown in Table 2. These *gor-trx*B double mutants grow poorly relative to the parent strains, but they do grow. The requirement for either of these two enzymes is therefore not, in *E. coli*, an absolute one.

Table 2: Levels of glutathione reductase, thioredoxin reductase and dihydrolipoamide dehydrogenase activities in mutated *E. coli* cells

Strain	Mutations	Cell-free extract			
			Enzyme activities (nmol/min/mg prot)		
		Prot.concn (mg/ml)	GR	TR	DLD
JF202	$gor^+, trxB^-, lpd^+$	4.1	104	n.d.	600
SG2	$gor^-, trxB^+, lpd^+$	5.4	n.d.	39	800
14a	$gor^-, trxB^-, lpd^+$	3.4	n.d.	n.d.	790

n.d. = not detectable

Cloning the *E. coli* gene for glutathione reductase

The *gor* mutation described above is not ideal for cloning work because of the presence of the Mu*cts*Ap, which makes the strain both temperature-sensitive and ampicillin-resistant. This makes difficult the use of normal transformation methods and common plasmids. A successful attempt was made to remove this impediment by deleting the *gor* gene and the Mu phage inserted in it from the *E. coli* chromosome. The strategy adopted for this was as follows. The *gor* gene is known to map close to the *pit* locus (Fig. 1) which encodes one of the two *E. coli* transport systems for inorganic phosphate and in particular is the system largely responsible for the uptake of arsenate ion (25). Strains in which the *pit* locus is mutated are thus relatively insensitive to arsenate in the growth medium. Growth of the *gor* :: Mu*cts*Ap mutant on arsenate-containing medium at elevated temperature will presumably select for cells which are not temperature-sensitive or arsenate-sensitive. Among these cells should be *pit-gor* deletion mutants.

The arsenate medium used for the initial screen provided only a weak counter-selection against cells with a functional *pit* locus. Strains isolated from this test were checked on ampicillin-containing medium for loss of the Mu phage. One strain was ampicillin-sensitive, though when tested on the original selection medium it proved much more arsenate-sensitive than its parent. However, it was sensitive to bacteriophage Mu and, when a cell-free extract was assayed, it was found to be glutathione reductase-negative. It presumably contains a deletion of the *gor* gene and neighbouring DNA which also renders the cells arsenate-sensitive. The isolation of this strain off a selective medium to which it is extremely sensitive must be regarded as highly fortuitous.

As we have seen, the *gor* mutation in *E. coli* has no known phenotype and there is no growth test that can be used to select for glutathione reductase-containing cells, an otherwise obvious route for cloning the gene from a gene bank. We could of course apply in reverse the direct colony assay used to detect the original *gor* mutants (see above) but that was judged to be too tedious. However, the arsenate-sensitivity of the

Δ*gor* mutant is probably due to the deletion of a portion of the genome close to the *gor* gene. We argued that if it were possible to clone sections of DNA conferring arsenate-resistance back into this strain, the *gor* gene might be cloned simultaneously if the map distance between it and and arsenate-resistance locus was small enough. For these experiments a counter-selection medium for the Δ*gor* mutant was developed on which it was possible to select against the mutant in favour of its *gor* :: Mu*cts*Ap parent or a wild-type *E. coli*. Reversion of the arsenate-sensitivity of the Δ*gor* mutant to arsenate-resistance was undetectable and therefore did not interfere with the selection.

By this means, the Δ*gor* recipient strain was used to screen the Clarke and Carbon (26) bank of recombinant plasmids containing *E. coli* DNA for arsenate-resistance. Of the arsenate-resistant clones isolated during the screen, ten were tested by colony assay for the presence of glutathione reductase. All of the strains appeared glutathione reductase-positive. After screening the cells for plasmid, the strain containing the smallest plasmid was used for the isolation of plasmid DNA by a rapid boiling method (27). This DNA preparation transformed the Δ*gor* mutant to arsenate-resistance; and when a cell-free extract was assayed it was clear that the cells had also been rendered glutathione reductase-positive.

The Clarke and Carbon bank strain carrying this plasmid was isolated and plasmid DNA prepared (28). On agarose gel electrophoresis this DNA preparation appeared homogeneous and, as expected, it could transform the Δ*gor* mutant to arsenate-resistance. Assay of cell-free extracts showed that the arsenate-resistant transformants were also glutathione reductase-positive. Analysis of linearized plasmid DNA on agarose gel electrophoresis showed the plasmid to be about 16.5 kilobase pairs in length. It proved difficult to remove the inserted *E. coli* chromosomal DNA either whole or piecemeal by digestion with restriction enzymes, so random-sheared pieces of whole plasmid DNA were cloned into bacteriophage m13mp9 and these clones were then sequenced using the dideoxy-chain termination method (29,30). Because of the strong homology expected between human and *E. coli* glutathione reductase, it was hoped that some of these clones would be recognized as

E. coli LysMetGlyAlaThrLysLysAspPheAspAsnThrValAlaIle
AAGATGGGGGCAACCAAAAAAGACTTCGACAATACCGTCGCCATT

Human LysMetGlyAlaThrLysAlaAspPheAspAsnThrValAlaIle
452 460

E. coli HisProThrAlaAlaGluGluPheValThrMetArgTER
CACCCAACGGCGGCAGAAGAGTTCGTGACAATGCGTTAAATGTTA

Human HisProThrSerSerGluGluLeuValThrLeuArgTER
467 470 478

Fig. 3. DNA sequence (tentative) of the 3'-terminal region of the *E. coli gor* gene. The amino acid residues overscored are identical with those of human erythrocyte glutathione reductase, the numbering of which is taken from ref. 10.

part of the *gor* gene. This turned out to be the case. For example, a clone containing DNA encoding the carboxy-terminus of glutathione reductase was isolated from among those sequenced (Fig. 3). From this clone, a radioactive probe to this portion of the gene was made and used to probe Southern blots of plasmid DNA cut with various restriction enzymes. In this way, the *gor* gene was localized to one end of the chromosomal DNA insert carried in the plasmid (Fig. 4). This section of the DNA was isolated and the complete DNA sequence of the *E. coli gor* gene should soon be available.

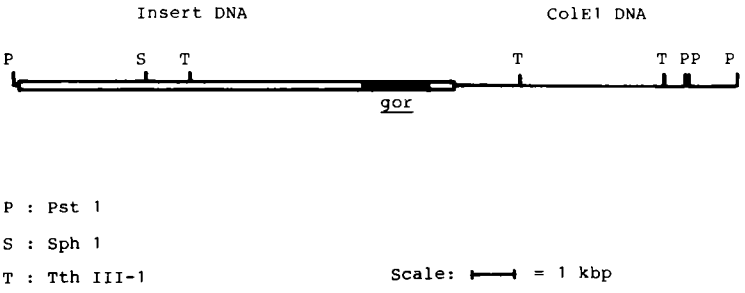

Fig. 4. Restriction map of the Col E1 plasmid carrying the *E.coli gor* gene

Discussion

Several interesting conclusions can be drawn from the results we have obtained thus far. First, glutathione reductase is not itself an essential enzyme in *E. coli*, though a cell devoid of glutathione reductase and thioredoxin reductase is crippled in growth. The existence of twin pathways for ribonucleotide reduction via glutaredoxin and thioredoxin can be viewed as valuable protection for this essential metabolic pathway. The crippled growth of the *gor*, *trx*B double mutant would imply that glutathione reductase and thioredoxin reductase provide the major routes for coupling NADPH with the reduction of ribonucleoside diphosphates. In the absence of these enzymes, thioredoxin and glutaredoxin may be entering a more general network of disulphide oxidoreductions in the cell. An entertaining possibility in the context of flavoprotein dehydrogenases is the involvement of the NAD^+-linked 2-oxo acid dehydrogenase complexes and dihydrolipoamide dehydrogenase, since free dihydrolipoic acid can readily reduce thioredoxin (A. Holmgren and R.N. Perham, unpublished work).

It is clear from the C-terminal sequence of the *E. coli* enzyme (Fig. 3) that the histidine residue assigned a specific role as proton donor in the mechanism of the human enzyme (14) is present as part of a highly-conserved amino acid sequence. A comparable histidine residue has been implicated in the mechanism of dihydrolipoamide dehydrogenase (31) but is lacking in the related enzyme mercuric reductase (16). The cloning and complete nucleotide sequence of the *gor* gene will open the way to a sophisticated analysis of the role of this histidine and other residues in the mechanism by means of protein engineering.

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