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Disulfide bond formation in the *Escherichia coli* cytoplasm: an *in vivo* role reversal for the thioredoxins

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Cytoplasmic proteins do not generally contain structural disulfide bonds, although certain cytoplasmic enzymes form such bonds as part of their catalytic cycles. The disulfide bonds in these latter enzymes are reduced in *Escherichia coli* by two systems; the thioredoxin pathway and the glutathione/glutaredoxin pathway. However, structural disulfide bonds can form in proteins in the cytoplasm when the gene (*trxB*) for the enzyme thioredoxin reductase is inactivated by mutation. This disulfide bond formation can be detected by assessing the state of the normally periplasmic enzyme alkaline phosphatase (AP) when it is localized to the cytoplasm. Here we show that the formation of disulfide bonds in cytoplasmic AP in the *trxB* mutant is dependent on the presence of two thioredoxins in the cell, thioredoxins 1 and 2, the products of the genes *trxA* and *trxC*, respectively. Our evidence supports a model in which the oxidized forms of these thioredoxins directly catalyze disulfide bond formation in cytoplasmic AP, a reversal of their normal role. In addition, we show that the recently discovered thioredoxin 2 can perform many of the roles of thioredoxin 1 *in vivo*, and thus is able to reduce certain essential cytoplasmic enzymes. Our results suggest that the three most effective cytoplasmic disulfide-reducing proteins are thioredoxin 1, thioredoxin 2 and glutaredoxin 1; expression of any one of these is sufficient to support aerobic growth. Our results help to explain how the reducing environment in the cytoplasm is maintained so that disulfide bonds do not normally occur.

Keywords: disulfide bond/oxidative stress/protein folding/thiol-disulfide oxidoreductase/thioredoxin

Introduction

The formation of structural disulfide bonds in *Escherichia coli* appears to be strictly segregated according to subcellular compartment. In the periplasm, disulfide bonds are actively formed in many proteins by the Dsb system (Rietsch and Beckwith, 1998). In the cytoplasm, the only disulfide bonds known to be present in proteins are formed in enzymes like ribonucleotide reductase during their catalytic cycles or in the oxidative response transcription factor OxyR during its regulatory cycle. The reduced forms of these proteins are regenerated via the action of the thioredoxin and glutathione/glutaredoxin pathways (Åberg *et al*., 1989; Zheng *et al*., 1998).

In the thioredoxin pathway, thioredoxin reductase (the product of the *trxB* gene) uses the reducing potential of NADPH to maintain thioredoxin 1 (the product of the *trxA* gene) in the reduced state, so that thioredoxin 1 in turn can reduce substrate proteins such as ribonucleotide reductase (Figure 1). The glutathione/glutaredoxin system also uses the reducing potential of NADPH in this case to reduce glutathione via the enzyme glutathione oxidoreductase. Glutathione is then able to reduce the three glutaredoxins (glutaredoxin 1, 2 and 3) (Holmgren, 1989). Only glutaredoxin 1 is able to reduce ribonucleotide reductase efficiently *in vitro*, whereas glutaredoxin 3 has a modest ability to reduce this enzyme (Åslund *et al*., 1994). Although glutaredoxins 2 and 3 are less efficient at reducing protein disulfides, they are active in reducing mixed disulfides of glutathione. Such mixed disulfides are generated and must be resolved during the catalytic cycle of enzymes such as arsenate reductase (Liu and Rosen, 1997). In *E.coli*, either the thioredoxin or the glutaredoxin pathway can be disabled by mutation without serious detriment to the cell. Nevertheless, if both pathways are disrupted, aerobic growth is almost completely eliminated, suggesting overlap between these two systems for the reduction of essential substrates such as ribonucleotide reductase (Prinz *et al*., 1997). However, for some substrates, such as methionine sulfoxide reductase, an apparent specificity towards thioredoxin 1 was deduced from the phenotype of *trxA* mutants (Russel and Model, 1986).

Structural disulfide bonds do not ordinarily form in cytoplasmic proteins (Pollitt and Zalkin, 1983; Derman and Beckwith, 1991). However, such bonds can form in cells that are defective for certain components of these reducing pathways (Derman *et al*., 1993a; Prinz *et al*., 1997). The formation of structural disulfide bonds in the cytoplasm of *E.coli* was assessed in strains in which the normally periplasmic enzyme alkaline phosphatase (AP) was localized to the cytoplasm by deleting its signal sequence (ΔssAP). AP is useful for this purpose since it contains two disulfide bonds that are required for it to fold into an active conformation (Sone *et al*., 1997).

We have found that strains mutant for the enzyme thioredoxin reductase (*trxB* mutants) express high levels of active cytoplasmic ΔssAP (Derman *et al*., 1993a). We considered two hypotheses to explain the oxidative properties of the *trxB* strain. According to the first hypothesis, the absence of reducing potential in the thioredoxin pathway (due to the loss of thioredoxin reductase) results in a failure to reduce ΔssAP that is spontaneously oxidized. According to the second hypothesis, oxidized thioredoxin accumulates in the *trxB* cytoplasm and can act to promote disulfide bond formation in ΔssAP. However, the properties of a *trxA* mutant and a double *trxA*, *trxB* mutant were not

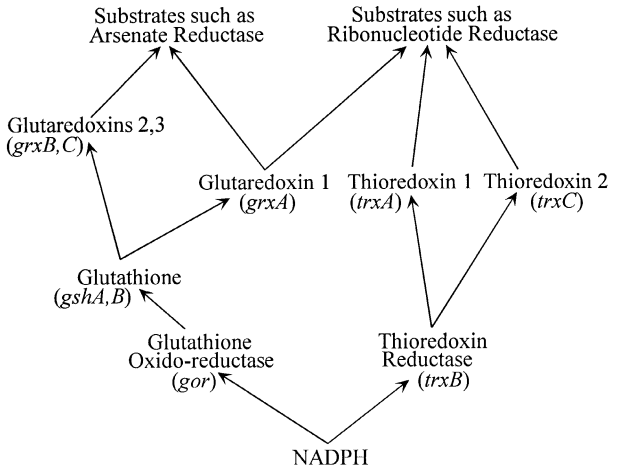

Fig. 1. The disulfide-reducing pathways in the *E.coli* cytoplasm. Arrows represent the path of reduction of disulfide bonds. Gene names are given in parentheses. Glutathione is a tripeptide synthesized by the products of the *gshA* and *gshB* genes.

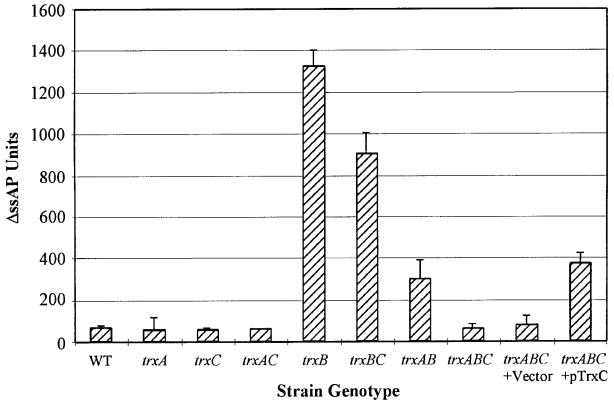

Fig. 2. Alkaline phosphatase activity in the cytoplasm of strains mutated in components of the thioredoxin pathway. Cells were immediately treated with 100 mM iodoacetamide to prevent formation of disulfide bonds during assay preparation. The activity in a wild-type strain is due to ΔssAP that reaches the periplasm (Derman *et al*., 1993). Re-expression of TrxC was accomplished by introducing pEJS80 (identified as *trxABC* + pTrxC). The same strain with the vector pAM238 without the *trxC* gene (identified as *trxABC* + Vector) served as a control. Error bars represent the standard deviation of duplicate cultures.

compatible with either hypothesis. These findings led us to suggest that there might be a hitherto undiscovered thioredoxin in *E.coli* (Derman *et al*., 1993a), a suggestion reinforced by subsequent studies in this laboratory (Prinz *et al*., 1997). Based on this reasoning, we sought and found an open reading frame in the *E.coli* genome sequence that could code for a new thioredoxin homolog, and initiated *in vivo* genetic studies on its function. During the course of this work, the purification of this gene (*trxC*) product, termed thioredoxin 2, and the demonstration of its function *in vitro* as a thioredoxin, was reported (Miranda-Vizuete *et al*., 1997).

In this paper, we utilize null mutations in the *trxA* and *trxC* genes to investigate the mechanism by which disulfide bond formation in ΔssAP occurs in the absence of thioredoxin reductase. We show that the two thioredoxins (thioredoxin 1 and 2) are both necessary for AP activity in the cytoplasm. We propose that the oxidized thioredoxins that accumulate in a *trxB* mutant can actively promote disulfide bond formation in appropriate substrate proteins. Further, we investigate the role of thioredoxin 2 as a disulfide-reducing protein *in vivo*, and show that it can fulfill many of the roles of thioredoxin 1. We also describe a strain that can be used to identify disulfide-reducing proteins from *E.coli* and other organisms.

Results

When AP is expressed in the cytoplasm of cells missing the enzyme thioredoxin reductase, substantial amounts (25–50%) of AP accumulate with its formed structural disulfide bonds. We have previously suggested that this phenomenon was due to the alteration of the oxidation state of thioredoxin and of a second, hitherto unknown, thioredoxin (Derman *et al*., 1993a; Prinz *et al*., 1997). Thus, we initiated genetic studies on the newly discovered open reading frame (now termed *trxC*) that appeared to encode a thioredoxin homolog.

Thioredoxins 1 and 2 are required for cytoplasmic disulfide bond formation in a trxB strain

To study the role of the second thioredoxin, thioredoxin 2, we generated a null mutation by deleting the coding region of *trxC* between the first and last four codons. This strategy was chosen due to the lower probability of affecting the expression of downstream genes compared with generating an insertion or replacement construct. The deletion strain was constructed in the presence of a complementing *trxC* plasmid in case the deletion was lethal. Cells deleted for *trxC* proved viable, and mutants containing this deletion were then used to investigate the mechanism of cytoplasmic disulfide bond formation. We again used the enzymatic activity of ΔssAP as a reporter of disulfide bond formation in the cytoplasm. As observed previously, a wild-type strain exhibits low AP activity, and a *trxB* strain yields high activity (Figure 2). Unlike the *trxB* strain, where nearly all the AP activity is in the cytoplasm, the low level of activity in a wild-type strain is due to the small amount of AP that gets exported to the periplasm despite the lack of a signal sequence (Derman *et al*., 1993a,b).

The first hypothesis outlined in the Introduction assumes that the AP activity in the *trxB* mutant is a result of the failure to reduce AP due to the absence of the reduced forms of the two thioredoxins. To test this hypothesis, we constructed the double mutant *trxA*, *trxC* strain which would eliminate the thioredoxins as reductants from the cytoplasm. However, the deletion of both *trxA* and *trxC* simultaneously gave no increase in AP activity relative to the wild-type strain. The second hypothesis proposes that the thioredoxins, which accumulate in the oxidized form in a *trxB* mutant, are capable of actively promoting disulfide bond formation in ΔssAP. This hypothesis predicts that we should be able to eliminate the activation of AP in the *trxB* mutant by generating the triple mutant *trxA*, *trxB*, *trxC*. This prediction proved correct. Deletion of both thioredoxins in a *trxB* strain expressing ΔssAP decreases the AP activity from the high level seen in a *trxB* strain to about the level of the control $TrxB^+$ strain. The low AP activity of the *trxA*, *trxB*, *trxC* strain clearly shows that the thioredoxins must be present for disulfide bond formation in a *trxB* strain, suggesting an important

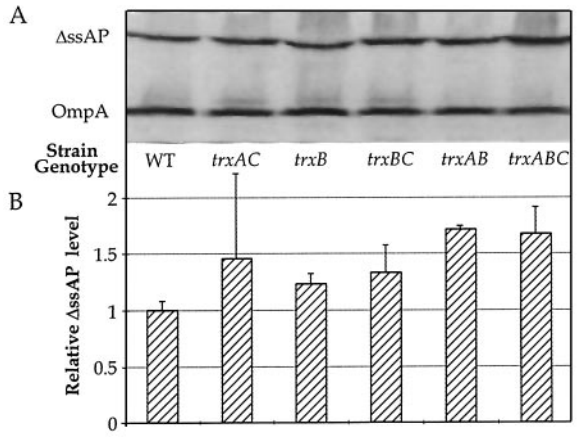

Fig. 3. Expression of ΔssAP relative to an OmpA control. Strains mutated for various elements of the thioredoxin pathway were pulse-labeled with [^{35}S]methionine and the extracts immunoprecipitated with antibody to AP and OmpA. (**A**) Samples were separated on an SDS–PA gel, and visualized on a Bio-Rad Molecular Imager. (**B**) The number of counts in each band was quantitated using Bio-Rad's ImageQuant software, and the amount of ΔssAP was normalized to the amount of OmpA, then reported relative to the wild-type strain. Error bars represent the standard deviation of duplicate cultures.

role for them in the oxidation of AP. Furthermore, a mutation in either of the thioredoxins alone, combined with *trxB*, causes the resultant strain to show a decreased level of activity relative to a *trxB* strain, with the decrease in activity being more pronounced with the *trxA* mutation than with the *trxC* mutation.

To ensure that the differences in activity of AP are due to a failure of the protein to become active rather than to a change in the level of expression of AP, we pulse-labeled and immunoprecipitated AP. Quantitation showed that the level of AP produced, normalized to the amount of OmpA, is comparable between the strains, indicating that the differences in activity are due to the level of activity of the protein, rather than the level of expression (Figure 3).

To verify that the change in phosphatase activity was due to the deletion of the *trxC* gene, assays were performed in a *trxA, trxB, trxC* strain in the presence of a plasmid containing *trxC* and also in the presence of the equivalent vector as a control (Figure 2). The activity in the strain with the *trxC* plasmid increases to the level of a *trxA, trxB* strain, while the strain with the vector alone is not altered in phosphatase activity. Similarly, when these plasmids are introduced into a *trxB, trxC* strain, the strain with the *trxC* plasmid showed increased activity, while the strain with the vector alone did not (data not shown). These results indicate that the changes in activity are due to the deletion of the *trxC* gene.

The role of thioredoxin 2 in the reducing pathways of E.coli

The role of thioredoxin 2 as an oxidant in the *trxB* background is unlikely to reflect the function of this protein in the ordinarily reducing environment of a wild-type cytoplasm. We wished to determine whether thioredoxin 2 contributes to the important reducing processes normally taking place in the cytoplasm. To do this, we have constructed a number of multiple mutants lacking various combinations of the important disulfide-reducing components and determined the viability of these mutants.

Table I. Viability of strains mutated in components of the thioredoxin pathway

Relevant features	(strain)	Growth on NZ			
		+ DTT	– DTT	+ arabinose	– arabinose
trxA gshA	(Aegis281)	+[a]	+	N/A	N/A
trxA trxC gshA	(Aegis282)	+	–	N/A	N/A
trxA grxA	(Aegis283)	+	+	+	+
trxA trxC grxA + TrxC	(Aegis284)	–	–	+	–
trxA trxC grxA + NrdH	(FÅ295)	–	–	+	–

Relevant features	(strain)	Growth on M63	
		+arabinose	–arabinose
trxA grxA	(FÅ47)	–[b]	–
trxA grxA + NrdH	(FÅ48)	+	–
trxA grxA + TrxA	(FÅ50)	++++	+
trxA grxA + TrxC	(FÅ51)	+++	–
trxA grxA + GrxA	(FÅ120)	++++	–

Relevant features	(strain)	Growth on methionine sulfoxide
trxA metE	(A313)	–
trxA metE + TrxA	(A313 + pWP619)	++++
trxA metE + TrxC	(A313 + pEJS33)	+/–
trxA metE + GrxA	(A313 + pFÅ1)	+++
trxA metE + NrdH	(A313 + pFÅ12)	–

[a]Growth on NZ was rated with '+' for any growth and '–' for no growth.
[b]Growth on M63 and methionine sulfoxide was rated by the size of colonies after 2 days of incubation at 37°C.

Cells require either the thioredoxin or glutathione/glutaredoxin pathway for normal growth. For example, *trxB, gshA* double mutants lacking thioredoxin reductase and glutathione (the product of the *gshA* gene is essential for the synthesis of glutathione) are essentially inviable, and only grow in the presence of DTT (Prinz *et al*., 1997). This inviability is presumably due to the complete block of the glutathione/glutaredoxin and thioredoxin pathways. However, a *trxA, gshA* double mutant is viable (Prinz *et al*., 1997). Since this latter double mutant is blocked for the glutathione/glutaredoxin pathway and lacks thioredoxin 1, it appears likely that its viability is due to the presence of thioredoxin 2. To determine whether this was the case, we constructed a triple mutant, *trxA, trxC, gshA*. This strain is inviable and could only be constructed in the presence of DTT (Table I). This finding demonstrates that eliminating both thioredoxins completely disables the thioredoxin reductase/thioredoxin pathway, whereas eliminating only thioredoxin 1 does not.

Glutaredoxin 1, the product of the *grxA* gene, is the most efficient disulfide-reductant in the glutathione/glutaredoxin pathway (Åslund *et al*., 1997). It seemed possible that this protein along with the two thioredoxins are the important contributors to the thiol redox environment of the cytoplasm. To test this possibility, we attempted to construct a triple mutant *trxA, trxC, grxA* strain. We were only able to construct this mutant when one of the missing

disulfide-reducing proteins, such as thioredoxin 2, was expressed from a plasmid, indicating that a *trxA*, *trxC*, *grxA* strain is inviable (Table I). These results demonstrate that any one of the three proteins, thioredoxin 1, thioredoxin 2 or glutaredoxin 1, is capable of supplying the disulfide-reducing capacity to the cell necessary for viability. We anticipate that this activity is required for cell growth because of the need to reduce enzymes such as ribonucleotide reductase.

Surprisingly, the *trxA*, *trxC*, *grxA* strain is not able to grow in the presence of DTT, even though other strains that knock out the reducing capacity of both pathways such as a *trxB*, *gor* strain can. This result shows that DTT can substitute for thioredoxin reductase or glutathione oxidoreductase, but not for the thioredoxins or glutaredoxins.

We examined the role of the thioredoxins under a variety of conditions including growth on minimal media, growth under anaerobic conditions, and growth on methionine sulfoxide, to determine which processes they participate in. A *trxA*, *grxA* strain is unable to grow on minimal media, despite the presence of the chromosomal *trxC* gene (Russel *et al*., 1990). Expression of *trxC* from the arabinose promoter on a plasmid restores the ability of this strain to grow on minimal media, presumably due to a higher level of *trxC* expression (Table I). Under anaerobic conditions, *E.coli* uses an alternative ribonucleotide reductase that is independent of disulfide reduction (Mulliez *et al*., 1995). A *trxB*, *gor* strain is able to grow anaerobically, suggesting that the disulfide-reducing pathways are not essential under these conditions (Prinz *et al*., 1997). We find that a *trxA*, *trxC*, *grxA* strain is also capable of anaerobic growth (data not shown), confirming that the essential aerobic substrates are either replaced, not needed or reduced in another way in the absence of oxygen. One of the few substrates that were thought to be specifically acted on by thioredoxin 1 is the enzyme methionine sulfoxide reductase (MsrA) (Russel and Model, 1986). To determine how stringent the requirement is for thioredoxin 1, we overproduced several disulfide-reducing proteins in a *trxA* strain and tested for the ability to use methionine sulfoxide as the sole source of methionine. Increasing the expression of glutaredoxin 1 allowed growth, indicating that at higher levels of expression, it can reduce methionine sulfoxide reductase (Table I). Expression of thioredoxin 2 allowed only very poor growth.

Given the apparent non-specificity of the thiol-disulfide oxidoreductases, we wondered if the *trxA*, *trxC*, *grxA* triple mutant strain could be used to address the function of other disulfide-reducing proteins. Indeed, we found that *nrdH* (a normally cryptic *E.coli* gene encoding a potent disulfide-reductant; Jordan *et al*., 1997), when expressed from a plasmid, was able to support the growth of the triple mutant. However, a plasmid expressing *grxC*, in addition to the chromosomal expression of glutaredoxin 3, was not able rescue this strain, despite the fact that glutaredoxin 3 has a low but significant activity reducing ribonucleotide reductase *in vitro* (Åslund *et al*., 1994). This suggests either that the reducing potential of glutaredoxin 3 is not low enough to reduce this enzyme *in vivo*, or that some other essential enzyme, which glutaredoxin 3 cannot act on, is responsible for the inviability.

Discussion

We initiated this series of studies to determine why stable structural disulfide bonds were absent from proteins in the cytoplasm of cells (Derman *et al*., 1993a). We began with the presumption that the reducing potential of cytoplasmic thiol redox components was required to prevent disulfide bonds from forming. To pursue this question, we have assessed a variety of mutant cells for their ability to alter the cytoplasmic environment so that such bonds can form. The results presented here suggest that our starting presumption was incorrect. Thus, it is the presence of an active thiol oxidant—a promoter of disulfide bond formation—that causes such bonds to form. This conclusion is based on the properties of mutations lacking components of the thioredoxin reductase/thioredoxin pathway.

Mutants lacking the enzyme thioredoxin reductase accumulate high levels of the disulfide-bonded active form of AP when this normally periplasmic protein is retained in the cytoplasm. Here we show that this effect is not due to the elimination of the reduced forms of thioredoxin 1 and 2, as a *trxA*, *trxC* double mutant, lacking both these proteins, exhibits little or no AP activity. Rather, we propose that the disulfide bond formation that occurs in the *trxB* mutant is due to the action of the oxidized forms of these thioredoxins. That is, the oxidized products of the *trxA* and *trxC* genes are catalyzing the formation of disulfide bonds in substrates such as AP.

It may appear surprising that thioredoxins, which are potent reductants, could also act as oxidants under certain conditions. However, such an activity of these proteins is not unexpected. While the redox potential of the disulfide bond in thioredoxin 1 is the lowest of the known thiol-disulfide oxidoreductases (–270 mV at pH 7.0), it is still considerably higher than that of the structural disulfide bonds in folded proteins such as bovine pancreatic trypsin inhibitor (BPTI; Creighton and Goldberg, 1984). This difference in redox potential makes the net transfer of the disulfide bond from thioredoxin to proteins such as alkaline phosphatase a favorable reaction. Such a process has been demonstrated *in vitro*; oxidized thioredoxin can transfer disulfide bonds to reduced ribonuclease (Pigiet and Schuster, 1986; Lundström *et al*., 1992). Thus, our explanation for the accumulation of the disulfide-bonded form of AP in the *trxB* mutant is consistent with the *in vitro* properties of thioredoxins.

These findings raise a number of interesting points: first, they further support the conclusion that a catalytic system is required for disulfide bond formation in proteins to occur with significant efficiency. For example, when the DsbA or DsbB proteins are absent from the periplasm, the oxidation of cysteines in exported proteins proceeds quite slowly (Bardwell *et al*., 1991). One of the potential roles considered for the two major disulfide-reducing pathways in the cytoplasm is that they reduce any incidental disulfide bonds that form in cytoplasmic proteins. However, as we have shown that in ΔssAP these bonds do not form in the cytoplasm of either a wild-type strain or a *trxA*, *trxB*, *trxC* strain, we conclude that disulfide bond formation in the cytoplasm does not occur very frequently unless it is catalyzed. This could mean that for most cytoplasmic proteins there is little need for

the thioredoxin and glutathione/glutaredoxin pathways in the maintenance of reduced thiols under laboratory growth conditions. Secondly, folded and oxidized AP is not able to be reduced by the cytoplasmic reducing pathways, as disulfide bonds accumulate in AP in the cytoplasm of the *trxB* strain, despite the presence of the glutathione/glutaredoxin pathway. Similarly, in certain double mutants of the glutathione/glutaredoxin pathway (e.g. *gor, grxA*, assayed on NZ medium), high levels of cytoplasmic AP activity can be achieved, even though the thioredoxin pathway is intact (F.Åslund, unpublished data). Therefore, the disulfide-reductants do not appear to significantly affect the formation of structural disulfide bonds in cytoplasmic AP.

Our results also provide another example where the role of a thiol-disulfide oxidoreductase is altered by a change in the environment in which it is expressed. The protein disulfide bond isomerase, DsbC, acts as an oxidant in the *E.coli* periplasm, when the protein required for DsbC reduction, DsbD, is eliminated (Missiakas *et al*., 1995; Rietsch *et al*., 1996). More recently, we have shown that thioredoxin 1 can also act as an oxidant when it is exported to the oxidizing environment of the *E.coli* periplasm (Debarbieux and Beckwith, 1998). In this paper, we present evidence suggesting that both thioredoxins 1 and 2, whose normal role is to act as reductants, can act as oxidants in a *trxB* mutant.

In fact, it may be that under certain growth conditions or in some of the environments wild-type *E.coli* encounter, accumulation of oxidized thioredoxins does take place. Certain stress conditions, such as exposure to hydrogen peroxide, generate an increased oxidizing environment within the cytoplasm that results in the oxidation of thioredoxin 1 (F.Åslund, unpublished data). This accumulation of oxidized thioredoxins could be dangerous to the cell, as we have shown that they can induce disulfide bond formation in other proteins. Therefore, the cell responds to this challenge by activation of the regulatory protein OxyR, which increases the expression of some components of the reducing machinery (Zheng *et al*., 1998). This probably helps to reduce any aberrant disulfide bonds that may occur in cytoplasmic proteins. Another potential target of such oxidative stress could be the precursor forms of secreted proteins, before they are translocated across the membrane. However, we consider oxidation of these precursors to be unlikely, as the rate of secretion is much higher than that of thioredoxin-catalyzed disulfide bond formation. For example, secretion of a protein such as AP (with its signal sequence) is complete in $<$30 s (Michaelis *et al*., 1986), while cytoplasmic disulfide bond formation, even in a *trxB* mutant, takes as long as 10 min (Derman *et al*., 1993a).

In other organisms, there may be another mechanism at work to prevent disulfide bond formation by oxidized thioredoxins. Mammalian thioredoxins and glutaredoxins contain conserved cysteine residues other than those in the active site. These non-active site cysteines form disulfide bonds that inactivate thioredoxin upon oxidation (Ren *et al*., 1993). We propose that these bonds may play an autoregulatory role to prevent the disulfide-reducing proteins from promoting disulfide bond formation.

With the availability of a *trxC* null mutant, we have also been able to address a number of other questions concerning the disulfide-reducing components of the *E.coli* cytoplasm. We have obtained evidence for an *in vivo* role for the newly discovered thioredoxin 2, the product of the *trxC* gene. By examining the phenotype of *trxC* mutations in combination with other mutations in the thioredoxin and glutathione/glutaredoxin systems, we show that the loss of both thioredoxins 1 and 2 completely blocks the thioredoxin pathway. Since a *trxA, trxC, grxA* strain is not viable unless complemented by one of the three genes, we conclude that these three are the only (sufficiently expressed) proteins able to perform the disulfide-reductive functions essential for aerobic growth of *E.coli*. Furthermore, any one of these proteins expressed by itself is capable of supporting growth.

While the cytoplasmic disulfide-reducing proteins appear to overlap in their functions, there are some indications of specificity of function. In particular, genetic studies have suggested that, *in vivo*, thioredoxin 1 is essential for the reduction of methionine sulfoxide reductase. However, we have found that when glutaredoxin 1 is overproduced in a *trxA* mutant, it can substitute for thioredoxin 1 in the reduction of methionine sulfoxide reductase (Russel and Model, 1986). In another example, thioredoxin 2 is able to complement a *trxA, grxA* mutant for growth on minimal medium when the *trxC* gene is overexpressed from a plasmid, but not when expressed from the chromosome. These findings suggest that the specific requirements in these reactions may not be dependent only on a specific binding interaction with the substrate proteins. Rather, any observed specificity of disulfide-reducing proteins deduced from the phenotype of mutant strains may also be dependent on the relative concentration of that protein and its redox potential.

Given this lack of stringent specificity, it seems that *E.coli* could survive with a single reducing system. Organisms of the genus *Mycoplasma* appear to possess a single thioredoxin system, and no glutathione/glutaredoxin system (Fraser *et al*., 1995; Himmelreich *et al*., 1996). This raises the intriguing question of why *E.coli* maintains this plethora of disulfide-reductants. We point out that in the phage T4 genome, where it might be assumed that genetic space is at a premium, there are two glutaredoxins encoded (Gvakharia *et al*., 1996). This, despite the fact that when T4 infects *E.coli*, it is infecting a cell already providing three glutaredoxins and two thioredoxins. *Escherichia coli* is not alone in this regard, as a large number of organisms have more than one thioredoxin, and some substantially more (Wetterauer *et al*., 1992; Rivera-Madrid *et al*., 1995; Lim *et al*., 1996). Part of the answer may be that by co-evolving expression levels with substrate specificity, the cell can respond to different stress situations that are present in various environments. For example, hydrogen peroxide stimulates an OxyR response that results in the specific induction of *grxA* transcription. It could be that each of the thioredoxins and glutaredoxins has a particular role to play under conditions of stress, and that different sources of stress may require different responses. Organisms with less disulfide-reducing variety may not encounter the same sources of oxidative stress, or they may deal with these stresses in novel ways.

Materials and methods

Bacterial strains and growth conditions

Strains and plasmids used are listed in Table II. Cells were generally grown in NZ medium, described previously (Derman *et al*., 1993b), at

Table II. Strains and plasmids used in this research

Strain or plasmid	Relevant genotype and features	Reference or source
Plasmids		
pAID135	pBR-based amp^r *phoA*Δ2-22 under tac promoter	Derman *et al*. (1993a)
pBAD18	pBR-based amp^r with arabinose regulatory region	Guzman *et al*. (1995)
pBAD22	pBR-based amp^r with arabinose regulatory region	Guzman *et al*. (1995)
pBAD24	pBR-based amp^r with arabinose regulatory region	Guzman *et al*. (1995)
pBAD33	pACYC-based cam^r with arabinose regulatory region	Guzman *et al*. (1995)
pWP619	pBAD22 + *trxA* under arabinose promoter	W.Prinz, unpublished
pAM238	pSC101-based spec^r	Gil and Bouché (1991)
pEJS33	pBAD18 + *trxC* under arabinose promoter	this work
pEJS62	pBAD33 + *trxC* under arabinose promoter	this work
pEJS80	pAM238 + *trxC* under its own promoter	this work
pFÅ1	pBAD18 + *grxA* under arabinose promoter	this work
pFÅ12	pBAD24 + *nrdH* under arabinose promoter	this work
pFÅ13	pBAD24 + *grxC* under arabinose promoter	this work
Strains		
CAG18480	*nadB*::Tn10	Singer *et al*. (1989)
A313	*trxA*::kan *metE*::Tn10	Russel and Model (1986)
DHB4	Δ(*ara-leu*)7697 *araD*139 Δ*lacX*74 *galE galK rpsL phoR* Δ(*phoA*)PvuII Δ*malF*3 *thi*	Boyd *et al*. (1987)
WP551	DHB4 + pAID135	Prinz *et al*. (1997)
Aegis256	WP551 *nadB*::Tn10	this work
Aegis257	WP551 Δ*trxC nadB*::Tn10	this work
Aegis258	WP551 *trxB*::kan *nadB*::Tn10	this work
Aegis259	WP551 *trxB*::kan Δ*trxC nadB*::Tn10	this work
Aegis260	WP551 Δ*trxA trxB*::kan *nadB*::Tn10	this work
Aegis261	WP551 Δ*trxA trxB*::kan Δ*trxC nadB*::Tn10	this work
Aegis262	WP551 Δ*trxA nadB*::Tn10	this work
Aegis263	WP551 Δ*trxA* Δ*trxC nadB*::Tn10	this work
Aegis269	WP551 Δ*trxA trxB*::kan *nadB*::Tn10 + pAM238	this work
Aegis270	WP551 Δ*trxA trxB*::kan *nadB*::Tn10 + pEJS80	this work
Aegis271	WP551 Δ*trxA trxB*::kan Δ*trxC nadB*::Tn10 + pAM238	this work
Aegis272	WP551 Δ*trxA trxB*::kan Δ*trxC nadB*::Tn10 + pEJS80	this work
Aegis281	DHB4 Δ*trxA gshA*::kan *nadB*::Tn10	this work
Aegis282	DHB4 Δ*trxA* Δ*trxC gshA*::kan *nadB*::Tn10	this work
Aegis283	DHB4 Δ*trxA grxA*::Tn10kan *nadB*::Tn10 + pEJS33	this work
Aegis284	DHB4 Δ*trxA* Δ*trxC grxA*::Tn10kan *nadB*::Tn10 + pEJS33	this work
FÅ295	DHB4 Δ*trxA grxA*::Tn10kan Δ*trxC nadB*::Tn10 + pFÅ12	this work
FÅ47	DHB4 Δ*trxA grxA*::Tn10kan Δ*ara*714 *leu*::Tn10	this work
FÅ48	FÅ47 + pFÅ12	this work
FÅ50	FÅ47 + pWP619	this work
FÅ51	FÅ47 + pEJS33	this work
FÅ120	FÅ47 + pFÅ1	this work

37°C. For phosphatase assays and pulse-labelings the cells were subcultured from overnight NZ cultures into minimal M63 medium [M63 salts with 0.2% glucose, 1 μg/ml vitamin B1, 1 mM $MgSO_4$, 50 μg/ml 18 amino acids (excluding methionine and cysteine) and supplemented with 2 μg/ml nicotinic acid] at a 100-fold dilution, and then incubated at 37°C. Antibiotic selection was maintained for all markers on plasmids, at the following concentrations: ampicillin, 200 μg/ml; spectinomycin, 100 μg/ml; and chloramphenicol, 10 μg/ml. Induction of alkaline phosphatase was accomplished by addition of IPTG to a final concentration of 5 mM at the time of subculturing. For testing growth on a minimal medium, M63 glucose (0.2%) plates were supplemented with leucine and isoleucine (50 μg/ml each) and incubated for 2 days at 37°C. The ability to utilize methionine sulfoxide as the sole source of methionine was tested on M63 plates as above, with methionine sulfoxide added to 100 μg/ml. The strain used (generously provided by M.Russel) is A313, a *trxA metE* mutant which is unable to synthesize methionine *de novo*. Nicotinic acid (2 μg/ml) was added to the media when strains with an insertion in *nadB* were grown on M63. Constructs in pBAD plasmids were induced by addition of 0.1% L-arabinose.

Cloning of the disulfide-reducing proteins

A PCR fragment encoding the *trxC* gene was cloned into pBAD33. This resulted in the expression of *trxC* under the control of the araBAD operon promoter. A DNA fragment containing *trxC* and enough upstream DNA (214 bp) to include its putative promoter was also generated by PCR and cloned into the vector pAM238. This PCR fragment was ligated into the vector in the opposite orientation to the lac promoter on the plasmid, resulting in the expression of the *trxC* gene under its own promoter. All other plasmids created for this study were cloned using PCR. Plasmids pFÅ1, pEJS33, pEJS62 and pEJS80 use the Shine–Dalgarno ribosome binding site native to the gene cloned, while pFÅ12, pFÅ13, and pWP619 employed the optimized Shine–Dalgarno contained in the vector.

Deletion of trxC

As the sequence downstream of *trxC* indicates that it might be the first gene in an operon, we created a null mutation that is unlikely to affect expression of these downstream genes. DNA flanking the gene was amplified by PCR, and fragments consisting of 1.8 kb of upstream DNA and 1.4 kb of downstream DNA were generated using the primers (all are written 5′ to 3′, and were supplied by Genosys): upstream, F1-left-B, GGCCAGGATCCTTATCACGGACC; F1-right-P, ACAATGCTGC-AGAACGGTATTCATAACTAACCT; downstream, F2-left-P, GATA-GCCTGCAGAACGAATCTCTTTAATCTTAC; F2-right-S(Xho), GCG-CTCTCGAGACTGTCCCGGGCCAGATAGTCAAG. These fragments included the first four amino acids (upstream) and the last four amino acids (downstream) of *trxC*, but no other part of the coding sequence. They were cloned into pKO3, and following the published protocol (Link *et al.*, 1997), this vector was used to sequentially select and then screen for replacement of the wild-type *trxC* allele with the deletion allele. Presence of the deletion allele was verified by a PCR screen across the chromosomal *trxC* gene. The final result removes the central 131 codons of the *trxC* gene, and replaces them with the six bases of a *Pst*I site.

Transduction of trxC with a linked transposon

A P1 lysate of strain CAG18480, containing a Tn10 insertion in the *nadB* gene, was used to generate a marker linked to *trxC*. The tetracycline

resistance marker co-transduces *trxC* with a frequency of ~30%. The original deletion of *trxC* was moved by P1 transduction, using this marker to create the strains used in this study. The strain used to generate this lysate was PCR sequenced across the *trxC* region to verify the deletion.

Construction of multiple-mutant strains

Construction of the potentially lethal strains completely blocked for the disulfide-reducing pathways was accomplished as follows: a *trxA, gshA* strain was transduced with the lysate from the *trxC* strain described above, selecting for tetracycline resistance, on a plate containing a disk saturated with DTT. DTT-dependent colonies were then screened by PCR for the presence of the *trxC* deletion: a *trxA, grxA* mutant carrying a plasmid expressing *trxC* or another disulfide-reducing protein was also transduced with the *trxC* lysate, and selected for tetracycline resistance on plates containing arabinose, as described above. The resultant colonies were then screened for the *trxC* deletion.

Alkaline phosphatase assays

Cells were grown and induced as described above. At an optical density at 600 nm (OD_{600}) of 0.4–0.6, an aliquot of 1 ml was immediately added to 100 μl of room-temperature 1 M iodoacetamide. The sample was then incubated on ice for at least 20 min. AP activity was then determined as described previously (Derman *et al.*, 1993a) . All cultures were grown, subcultured, and assayed in duplicate.

Pulse-labelings and immunoprecipitations

Cells were grown and induced as described above. At an OD_{600} of 0.25–0.30, a 1 ml aliquot of culture was transferred to a Falcon 2059 culture tube in a shaking water bath at 37°C. After a 30 min incubation, radiolabel ([^{35}S]methionine, ICN) was added to a concentration of 40 μCi/ml. After a 1 min incubation, 100 μl of unlabeled 1% methionine was added and 700 μl of the culture was immediately removed to a tube containing 300 μl of ice-cold, unlabeled 0.5% methionine.

These cells were then lysed and immunoprecipitations performed using antibody to AP (1 μl/ml of culture; antibody purchased from Rockland) and antibody to OmpA protein (1 μl/ml of culture; antibody from laboratory-generated stock). Immunoprecipitations were performed as described previously (Pogliano and Beckwith, 1993). A 12% SDS–polyacrylamide gel (Mini-ProteanII, Bio-Rad) was then loaded with 20% of each of the total sample volumes, and electrophoresed at a constant voltage of 120 V until the dye front reached, but did not leave, the bottom of the gel. The gel was dried under vacuum at 80°C for 1.5 h, then exposed to film (Kodak X-OMAT) for 14 h. The gel was then immediately used to expose a Molecular Imager cassette for 3 h. The cassette was scanned and quantified at a resolution of 100 μm in a Bio-Rad GS-525 Molecular Imager System. The number of counts from the band corresponding to phosphatase was normalized to the number of counts from OmpA in each lane, to provide a control for the amount of protein loaded. All cultures were grown, labeled and quantitated in duplicate.

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