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20 June 2001; accepted 7 August 2001
Published online 16 August 2001;
10.1126/science.1063630
Include this information when citing this paper.

Conversion of a Peroxiredoxin into a Disulfide Reductase by a Triplet Repeat Expansion

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Pathways for the reduction of protein disulfide bonds are found in all organisms and are required for the reductive recycling of certain enzymes including the essential protein ribonucleotide reductase. An *Escherichia coli* strain that lacks both thioredoxin reductase and glutathione reductase grows extremely poorly. Here, we show that a mutation occurring at high frequencies in the gene *ahpC*, encoding a peroxiredoxin, restores normal growth to this strain. This mutation is the result of a reversible expansion of a triplet nucleotide repeat sequence, leading to the addition of one amino acid that converts the AhpC protein from a peroxidase to a disulfide reductase. The ready mutational interconversion between the two activities could provide an evolutionary advantage to *E. coli*.

The cytoplasms of all living cells are highly reducing environments compared with their external milieu. A host of cytosolic proteins are responsible for maintaining a redox balance. In *E. coli*, two pathways in the cytoplasm function in overlapping ways to maintain cysteine residues in the reduced state (*1*). The thioredoxin system includes thioredoxin reductase (*trxB*) and two thioredoxins; the glutaredoxin system includes glutathione reductase (*gor*), glutathione, and three glutaredoxins (*2, 3*). These two systems recycle certain reductive enzymes by reducing disulfide bonds formed at their active sites (*4–7*).

A *trxB,gor* double mutant, lacking both cytosolic disulfide reductive pathways, grows poorly under aerobic conditions unless a reducing agent such as dithiothreitol (DTT) is present. The poor growth is likely due to the inability of these cells to synthesize deoxyribonucleotides, because ribonucleotide reductase is not maintained in the reduced state.

We have previously described a derivative of the *trxB,gor* mutant strain, FÅ113, in which a suppressor mutation has restored a normal growth rate (*8*). Despite the likelihood that a source of disulfide-reducing power has been restored by the suppressor mutation, FÅ113 is able to efficiently catalyze disulfide bond formation in cytoplasmically retained secreted proteins. FÅ113 and its derivatives also enhance the expression in the cytoplasm of active tissue plasminogen activator (vtPA), a complex eukaryotic protein with multiple disulfide bonds (*8*).

We wished to determine the nature of the suppressor mutation in FÅ113. However, even though the cells were grown in the presence of DTT, suppressors arose in this strain at such a high frequency that we could not map them using transposon insertions that were cotransduced by bacteriophage P1 with the suppressor mutation. To overcome this problem, we introduced into the *trxB,gor* mutant a plasmid that contained the *trxB* gene under the control of the pBAD *ara* promoter (*9*). The frequency of suppressor mutations that occurred under restrictive growth conditions was still 0.5×10^{-3}. Transposon insertions linked to the suppressor mutation clustered to minute 14 of the *E. coli* chromosome, and the linkage data suggested that a mutation in the *ahpCF* locus might be responsible for the suppression (Fig. 1A).

The *ahpCF* locus of *E. coli* encodes the alkyl hydroperoxidase system, which reduces both hydrogen peroxide and alkyl hydroperoxides, thus protecting the cells from oxidative damage. Peroxides oxidize the active site Cys^{46} of the AhpC dimer to a sulfenic acid intermediate that is converted to a disulfide bond when it is attached by Cys^{165} in the other subunit of the homodimer (*10*). The mechanism of reductive electron transfer in the AhpCF system involves a cascade of disulfide bond reduction steps. First, electrons are transferred from NADH via a flavin moiety in the COOH-terminus of AhpF to reduce a disulfide bond in that portion of the protein. The electrons from these reduced cysteines are then donated to a disulfide bond in the NH$_2$-terminal domain of AhpF (*11*), from which they are transferred to the disulfide bond in AhpC, regenerating the reduced active form of the protein (*12, 13*).

Sequence analysis of the entire *ahpCF* locus from the wild-type, FÅ113, and nine independently isolated suppressor strains revealed that all of the mutants contained the same mutation, an insertion of three bases (TCT) after base 103 of the *ahpC* coding region (Fig. 1B). The wild-type gene contains four direct repeats of TCT, whereas the mutants contain five. This change results in the addition of a single amino acid (Phe^{38}), located only nine amino acids away from the active site Cys^{46} (Fig. 1B). We refer to the mutant protein as AhpC*.

Expansion of a repeated sequence, as is found in the *ahpC** mutation, is usually a very frequent event (*14*) and would explain the finding that all the suppressors represent

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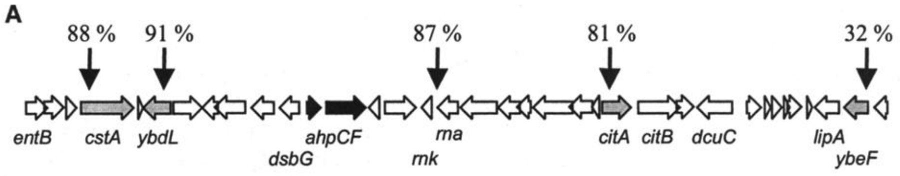
the same mutational event. The conserved triplet repeat sequence in which the *ahpC** mutation occurs is present in the *ahpC* gene of several facultative anaerobic enteric bacteria [*Salmonella*, *Shigella*, and *Klebsiella* (Fig. 1C) (*15*)].

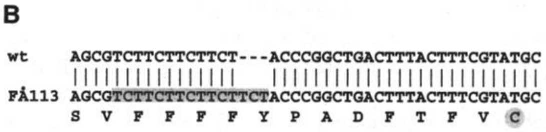
To examine whether the *ahpC** triplet repeat expansion is responsible for the restoration of growth in the *trxB,gor* double mutant, we generated plasmid constructs containing the genes from the *ahpCF* or *ahpC*F* operon (*16*). These were transformed into either the *trxB,gor* strain (JL10), or into a derivative that has a deletion of both *ahpC* and *ahpF* (DR456). Only the *ahpC** gene restored normal growth to the *trxB,gor* strain, whereas expression of both *ahpC** and *ahpF* was necessary for suppression in the *ahpCF* null background (Table 1). These results show that the effect of AhpC* is dominant over the wild-type allele and that AhpF is necessary for the phenotype of this mutation. Further, they suggest that the mutation in *ahpC** is a gain-of-function mutation, altering this peroxiredoxin so that it has a new function.

If the AhpC protein has a new function in the *ahpC** mutant, does it still retain its function as a peroxidase? To address this question, we determined whether the mutation had altered the resistance of *E. coli* to alkyl hydroperoxides. Comparison of strains expressing the plasmid-borne wild-type *ahpCF* and mutant *ahpC*F* operons revealed that the mutant operon conferred little or no increased resistance to cumene hydroperoxide (*17*). We also looked at the oxidative stress response mediated by the transcription factor OxyR in vivo. OxyR is activated by high intracellular concentrations of hydrogen peroxide to turn on genes involved in peroxide defense (*ahpCF*, *katG*) and disulfide bond reduction (*gor*, *grxA*, *trxC*) (*18, 19*). If the AhpC* mutant eliminates peroxidase activity, the concentration of cytoplasmic hydrogen peroxide should increase, inducing the OxyR response (*20*). Indeed, strains containing *ahpC** showed the same elevated OxyR response as a strain deleted for the *ahpC* locus (*21*). Combination of *ahpC** with a mutation inactivating the *katG* gene should then result in a maximal OxyR response. This is true of a *katG* strain that is deleted for the *ahpCF* locus (*20*). However, the double mutants were highly unstable; 75% of the mutants that had initially the desired *ahpC**, *katG*$^-$ genotype segregated to give colonies with an *ahpC*$^+$, *katG*$^-$ genotype (*22*). The instability of individual colonies suggests that there is a strong selective advantage to restore peroxidase activity in the *ahpC**, *katG*$^-$ strain background.

We also purified the wild-type and mutant AhpC and assayed their peroxidase activity in vitro in the presence of AhpF, using stopped flow spectrophotometry with hydrogen peroxide and cumene hydroperoxide as substrates (*23*). A sustained high rate of loss of absorbance at 340 nm (A_{340}) due to NADH consumption was observed only when AhpC and peroxide were included. In contrast, with AhpC*, a sustained A_{340} loss was not observed with either cumene hydroperoxide or hydrogen peroxide. In a separate assay for disulfide reductase activity, AhpC* was able to reduce the substrate DTNB [5,5′-dithio-bis(2-nitrobenzoic acid)]; AhpC showed a similar activity (*24*). Thus, AhpC* has disulfide reductase activity in the presence of AhpF, but no peroxidase activity. Although the wild-type AhpC is capable of reducing the generic disulfide bond–containing substrate DTNB in vitro, unlike AhpC* it does not exhibit any activity in vivo contributing to the reduction of ribonucleotide reductase, even when overexpressed.

These results suggest that the mutant AhpC* protein, although it has acquired a new reductive function, has effectively lost its original ability to reduce peroxides. Be-





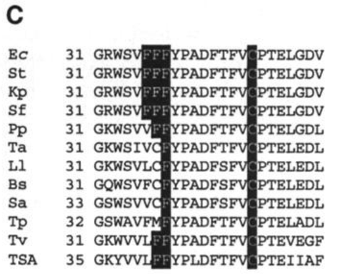
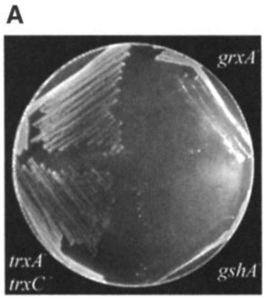


Fig. 1. The *ahpC* locus is responsible for the restoration of disulfide bond reduction in a *trxB,gor* strain. (**A**) Identification of the FÅ113 suppressor locus by analysis of randomly inserted λNK1324 transposons. The open reading frames that were disrupted by the transposon insertions are shown in gray and the *ahpCF* locus is marked in black. (**B**) Sequence comparison of the wild-type and mutant loci. The mutation is a triplet insertion (TCT) leading to the expansion of a triplet repeat sequence in the *ahpC* open reading frame. The insertion leads to the addition of a single amino acid (Phe) near Cys^{46}, which is essential for the peroxidase activity of the peroxiredoxin AhpC. (**C**) Peroxiredoxins are present in all organisms, and the region containing the active site cysteine is well conserved. Ec, *Escherichia coli*; St, *Salmonella typhi*; Kp, *Klebsiella pneumoniae*; Sf, *Shigella flexneri*; Pp, *Pseudomonas putida*; Ta, *Thermus aquaticus*; Ll, *Lactococcus lactis*; Bs, *Bacillus subtilis*; Sa, *Staphylococcus aureus*; Tp, *Treponema pallidum*; Tv, *Thermoplasma volcanium*; TSA, thiol-specific antioxidant protein from *Rattus norvegicus*. Sequences were retrieved from GenBank and aligned using ClustalW.



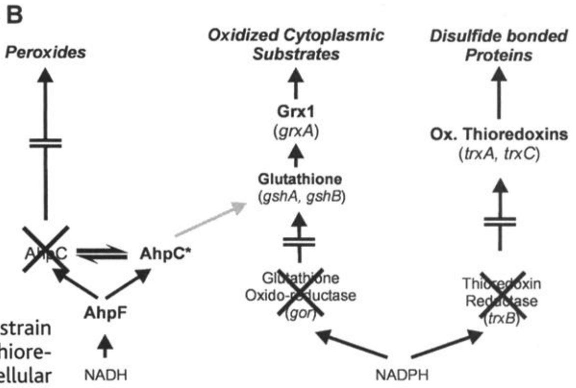


Fig. 2. Thiol redox pathways in a strain lacking glutathione reductase and thioredoxin reductase. (**A**) Additional cellular factors are required for AhpC* to function as a disulfide reductase in vivo. Strains were constructed by generalized transduction of the mutant alleles into a *trxB*$^-$,*gor*$^-$,*ahpC** strain expressing *trxB* from plasmid under the control of the *araBAD* promoter. Growth on rich medium with 0.2% glucose was scored after 1 day as the ability to form single colonies. (**B**) Model of the electron flow in a *trxB,gor* double mutant. The mutant form of the peroxiredoxin AhpC* is required to restore growth, because it can donate electrons to oxidized cytoplasmic substrates, such as ribonucleotide reductase. The effect depends on the presence of glutathione and glutaredoxin 1 (Grx1) as well as AhpF. The channeling of electrons into the glutathione pathway has not been shown biochemically (gray arrow). Oxidized thioredoxins are presumably involved in disulfide bond formation in the cytoplasm.

cause the mutant protein still functions as an electron acceptor from AhpF, the electrons necessary to restore growth to the *trxB,gor* strain must be derived from NADH.

The *ahpC** mutation must have restored essential disulfide-reducing capacities to the cytoplasm. To determine whether AhpC* acts by reducing any of the known components of the cytoplasmic thiol-redox pathways, we introduced additional mutations into the *trxB,gor* suppressor strain and determined their effect on the suppression. Both glutathione and glutaredoxin 1 were required for *ahpC** to restore growth to the *trxB,gor* strain; the thioredoxins are not required (Fig. 2A).

So far, we have been unable to detect significant glutathione or glutaredoxin reductase activities using the AhpC*F enzyme system in vitro. Nevertheless, the in vivo data suggest that electrons are being channeled into the glutathione pathway from the AhpC*F complex, thus replacing NADPH with NADH as the source of the electrons. The most likely explanation for these findings is that the AhpC* protein has been altered so that it now uses its disulfide reductase activity to partially replace glutathione reductase in the double mutant (Fig. 2B).

Even though the AhpC*F system sustains sufficient disulfide-reducing capacity to allow cells to grow, the cytoplasm remains oxidizing and favors the formation of disulfide bonds in proteins. The reducing ability of the thioredoxin branch is not restored in the suppressor strains, and it is therefore likely that the oxidized forms of the thioredoxins actively catalyze disulfide bond formation in the cytoplasm, as they do in a *trxB*⁻ mutant strain (*25*).

The conservation of the triplet repeat sequence across species and the high frequency of genetic interconversion between the peroxidase and the apparent glutathione-glutaredoxin reductase forms of alkyl hydroperoxidase raise the possibility that this change represents an evolutionarily significant advantage for these bacteria. The ability to switch back and forth between the peroxidase activity and the activity that channels electrons into the glutathione pathway may allow a fraction of the bacterial population to survive either oxidative stress or disulfide stress, respectively. Disulfide stress would occur when bacteria encounter oxidative agents, other than peroxides, that promote disulfide bond formation (*1, 26*). Alternatively, the switch may be important for growth conditions in which bacteria become depleted of NADPH. The switch would allow the cells to use NADH via the AhpC*F system as the normal source of electrons for glutathione. The frequency of the *ahpC** mutation is comparable to other cases of evolutionarily significant genetic switches, including the activation of cryptic β-glucosidases (*27*) and the alternation between flagella types in phase variation (*28–30*). The AhpC example differs from these in that the genetic event is a reversible interconversion of a single enzyme between two forms that have substantially different activities.

In humans, triplet repeat expansions are deleterious and have been causally linked to certain neurodegenerative disorders (*31, 32*). In contrast, the repeat expansion in AhpC represents a genetic switch leading to a protein with a new and advantageous enzymatic function.

Table 1. Ability of plasmid-borne *ahpCF* to restore the growth defect of *trxB,gor* and *trxB,gor,ahpCF* mutant strains. Strains were grown on plates containing 0.2% arabinose (to allow the expression of the *araBAD* controlled *trxB* gene), and the antibiotics to maintain the plasmids were restreaked onto plates containing only 0.2% glucose. Growth was scored after 24 hours at 37°C as the ability to form single colonies. The strain backgrounds used are JL10 (*trxB,gor*) and DR456, which was obtained by introducing *ahpCF::Km* (deletion of codons 62 to 187 of *ahpC* and codons 1 to 406 of *ahpF*) into JL10. NZ, rich medium containing NZ amine.

	Growth on NZ	
	trxB,gor	*trxB,gor,ahpCF::Km*
pLAC-*ahpCF*	–	–
pLAC-*ahpC*	–	–
pLAC-*ahpF*	–	–
pLAC-*ahpC*F*	+	+
pLAC-*ahpC**	+	–

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16. Plasmids with *ahpC*F* and *ahpCF* were made by amplifying *ahpF* and *ahpCF* from DHB4 and FÅ113 using the polymerase chain reaction, and these plasmids were cloned into the pACYC derived pLAC-YC under the control of the *lac*UV5 promoter to generate pJL32, 34, and 35, which correspond to *ahpF* from DHB4, *ahpCF* from DHB4, and *ahpC*F* from FÅ113. For the *ahpC* and *ahpC** constructs, *ahpF* was deleted from pJL34 and pJL35 with Sma I and Hind III, generating pJL77 and pJL78, respectively.
17. *Escherichia coli* DHB4 containing either wild-type *ahpCF* or *ahpC*F* was grown in 0.2% arabinose to an absorbance of A_{600} of 0.6. Cells (1 ml) were washed and resuspended in 0.9% NaCl solution and incubated for 20 min with a final concentration of 5 mM cumene hydroperoxide (Sigma) solution in dimethyl sulfoxide (DMSO). Appropriate dilutions were plated, and colony-forming units were counted after 24 hours at 37°C. In a control reaction, the cells were treated with 10 μl DMSO alone. Survival of cells expressing the wild-type was 10 times (3.2% of cells) those of a vector-only control and of the *ahpC**-expressing strains (0.3%). Both the wild-type and mutant AhpC proteins were expressed at similar concentrations as indicated by Coomassie-stained gels.
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21. The OxyR-dependent translational *trxC′-′lacZ* fusion was used as a reporter for the peroxide-induced oxidative stress response. β-Galactosidase activity was measured in strains with different mutations in the *ahpCF* locus. Strains were constructed by transduction of *ahpC* or *ahpC** into HP5 [DHB4 Δ(λ*att-lom*)::*bla trxC′-′lacZ*; (*33*)]. The fusion was expressed at a high concentration in an *ahpCF::Km* deletion mutant (393 U) and in an ahpC* strain (409 U), whereas it was found at basal concentration in wild-type backgrounds (61 U).
22. The *ahpC** allele was transduced into a strain containing the *katG* deletion and the *trxC′-′lacZ* reporter fusion. Colonies were analyzed after 24 hours on indicator plates as described (*33*).
23. Assays were carried out as described (*34*). At AhpC concentrations ranging from 2.5 to 50 μM, turnover of AhpF with AhpC had a k_{cat} of 190.5 ± 3.7 s^{-1}, and a K_m for AhpC of 19.3 ± 0.9 μM.
24. Disulfide reductase assays were carried out as described previously for AhpF (*34*), with 10 nM AhpF and 5 to 40 μM AhpC or AhpC*. In the absence of AhpC or AhpC*, DTNB was oxidized at 4.2 μM per minute 40 μM AhpC* or AhpC gave rates of 21.4 and 32.1 μM per minute, respectively. The reaction rate was linearly dependent on AhpC or AhpC* concentration.
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5 June 2001; accepted 6 August 2001