Alkyl Hydroperoxide Reductase from *Salmonella typhimurium*

SEQUENCE AND HOMOLOGY TO THIOREDOXIN REDUCTASE AND OTHER FLAVOPROTEIN DISULFIDE OXIDOREDUCTASES*

(Received for publication, November 21, 1989)

Louis A. Tartaglia†, Gisela Storz§, Michael H. Brodsky, Andrew Lai, and Bruce N. Ames‡

From the Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720

The DNA sequence of the *Salmonella typhimurium ahp* locus was determined. The locus was found to contain two genes that encode the two proteins (C22 and F52a) that comprise the *S. typhimurium* alkyl hydroperoxide reductase activity. The predicted sequence of the F52a protein component of the alkyl hydroperoxide reductase was found to be highly homologous to the *Escherichia coli* thioredoxin reductase protein (34% identity with many conservative substitutions). The homology was found to be particularly striking in the region containing the redox-active cysteines of the thioredoxin reductase molecule, and among the identities were the redox-active cysteines themselves. Aside from the strong similarity to thioredoxin reductase, overall homology between the F52a protein and other flavoprotein disulfide oxidoreductases such as glutathione reductase, dihydrolipoamide dehydrogenase, and mercuric reductase was found to be rather limited, and the conserved active site segment common to the three proteins was not observed within the F52a protein. However, three short segments that have been implicated in FAD and NAD binding were found to be conserved between the F52a protein and the other disulfide reductases. These results suggest that the alkyl hydroperoxide reductase is the second known member of a class of disulfide oxidoreductases which was represented previously by thioredoxin reductase alone; they also allow the putative assignment of several functional domains.

---

* This work was supported by National Institutes of Health Grant GM19993 and National Cancer Institute Outstanding Investigator Grant CA39910 (to B. N. A.) and by National Institute of Environmental Health Sciences Center Grant ES01896. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Div. of Biochemistry and Molecular Biology, 401 Barker Hall, University of California, Berkeley, CA 94720. Tel.: 415-642-5165; Fax: 415-643-7935.

‡ Supported by the GenBank/EMBL Data Bank accession number(s) J05479.

§ Supported by National Institutes of Health Training Grant ES07075.

§ Supported by National Institutes of Health Training Grant GM07232. Present address: Dept. of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114.

† Supported by National Institutes of Health Training Grant ES07075.

‡ Supported by National Institutes of Health Training Grant GM07232. Present address: Dept. of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114.

1 Supported by National Institutes of Health Training Grant ES07075.

† To whom correspondence should be addressed: Div. of Biochemistry and Molecular Biology, 401 Barker Hall, University of California, Berkeley, CA 94720. Tel.: 415-642-5165; Fax: 415-643-7935.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) J05479.

The alkyl hydroperoxide reductase activity of *Salmonella typhimurium* and *Escherichia coli* was first recognized through the isolation of mutant cells that were resistant to mutagenesis by alkyl hydroperoxides (1). By using as an assay the conversion of a model alkyl hydroperoxide to its corresponding alcohol, the activity was purified to homogeneity from a regulatory mutant strain that produced it at high levels (1). The purified activity was found to be composed of two proteins that had been identified previously by two-dimensional gel electrophoresis as hydrogen peroxide-inducible proteins F52a and C22, both of which were known to be positively regulated by the *S. typhimurium* oxyR locus (2). The F52a protein was found to be composed of two identical chains, each containing a bound FAD cofactor and having a subunit molecular mass of ~57 kDa. It can use either NADH or NADPH as electron donors for the direct reduction of redox dyes or of alkyl hydroperoxides when combined with the C22 protein. Mutant *S. typhimurium* strains that lack the alkyl hydroperoxide reductase are extremely sensitive to killing by organic hydroperoxides, and therefore a likely in vivo function for this activity would be the detoxification of lipid and other hydroperoxides that are produced during an oxidative stress (3).

Four other well studied flavoprotein oxidoreductases are glutathione reductase, mercuric reductase, dihydrolipoamide dehydrogenase, and thioredoxin reductase (4). Although each of these four enzymes is specific for a different substrate, each contains a pair of redox-active cysteines involved in the transfer of reducing equivalents from the FAD cofactor to the substrate. Although the mechanism and active site segment of the alkyl hydroperoxide reductase have not yet been determined, the activity has been shown to be inhibited by thiol-reactive reagents, suggesting that its mechanism may also involve catalytic cysteine residues (1).

The amino acid sequences of glutathione reductase, mercuric reductase, and dihydrolipoamide dehydrogenase have each been determined from two or more organisms (6–12). These sequences show substantial sequence homology to one another, particularly around the 2 redox-active cysteine residues that are separated by 4 conserved amino acids. However, the sequence of *E. coli* thioredoxin reductase shows quite poor overall sequence homology to these other disulfide reductases, even around the active site, and contains 2 redox-active cysteines separated by only 2 amino acids (13, 14).

We report here the DNA sequence of both genes encoding the alkyl hydroperoxide reductase activity located near 13 min on the *S. typhimurium* chromosome. The predicted amino acid sequence of the F52a protein is compared with the known sequences of other flavoprotein disulfide oxidoreductases and is shown to be highly homologous to the thioredoxin reductase molecule although exhibiting similarities to other reductases that are limited to short segments probably involved in FAD and NAD binding.

**EXPERIMENTAL PROCEDURES**

*DNA Manipulations*—DNA isolation, analysis, and cloning were generally carried out as described by Maniatis *et al.* (15).
S. typhimurium Alkyl Hydroperoxide Reductase Sequence

Strains and Plasmids—Strains TA4190 (ahp::TnlO), TA4485 (ahp2::TnlO), TA4486 (oxyR1 ahp::TnlO), and TA4487 (oxyR1 ahp2::TnlO), along with otherwise isogenic ahp+ control strains, were used to examine the effects of single TnlO insertions within the ahp locus on expression of the two encoded gene products (F52a and C22). The construction of TA4190 has been described previously (3). TA4485 was isolated by a separate but otherwise identical localized P22 mediated TnlO mutagenesis procedure. Both ahp+ and ahp2::TnlO mutant alleles were moved into TA4110 (oxyR1) (2) by P22-mediated transduction to give strains TA4486 and TA4487. The starting plasmid for this study was pAQ27, which contains the ahp locus on a 4.1-kilobase fragment cloned into the polylinker sites of pUC18 (16). pAQ28 is a smaller derivative of pAQ27 in which sequences between the ScaI polylinker site and the B&I site just upstream of the insert within pAQ38. The arrows show the locus on an 8.6-kilobase fragment (3). Subcloning experiments in Luria broth or Luria broth with the appropriate antibiotic at 37 °C were performed. Oligonucleotide primers complementary to ahp insert sequences were used to amplify the ahpC and ahpF genes as shown below the restriction site map. bp, base pairs.

Results

Organization and Nucleotide Sequence of the ahp Locus—In a previous study we reported the isolation of a plasmid (pAQ9) from a S. typhimurium library that contained the ahp locus on an 8.6-kilobase fragment (3). Subcloning experiments showed that a smaller DNA fragment, a 3.0-kilobase BglII-BamHI fragment from pAQ92 (see Fig. 1), contained all sequences sufficient for both complementation of the extreme sensitivity to organic hydroperoxides characteristic of ahp deletion mutants and restoration of expression of the two proteins that comprise the alkyl hydroperoxide reductase activity (data not shown). The 3.0-kilobase BglII-BamHI fragment was sequenced using the strategy shown in Fig. 1. The DNA and predicted amino acid sequences of the two long open reading frames contained on this clone are shown in Fig. 2. The two open reading frames are separated by 250 base pairs and would be transcribed in the same direction. To investigate the possibility that the two open reading frames are cotranscribed, we looked for the expression of their gene products in strains containing single TnlO insertions within the ahp locus. Immunoblot analysis of strains containing the ahp::TnlO loci and ahp2::TnlO mutations (see “Experimental Procedures”) showed that both of the independently isolated single TnlO insertions eliminated the basal level and oxyR-stimulated synthesis of both the C22 and F52a proteins (data not shown). It is therefore likely that both of these proteins are synthesized from a single transcript.

ahpC Gene—Comparison of the DNA sequence near the beginning of the more upstream open reading frame with the known N-terminal amino acid sequence of the S. typhimurium C22 protein (1) confirmed that this gene encodes the C22 protein component of the alkyl hydroperoxide reductase and identified the probable position of the translation start point. We are designating this gene as ahpC. The C22 protein is predicted to be 183 amino acids long with a molecular mass of 20,699 daltons (minus the initiating methionine). This predicted mass is in reasonable agreement with a subunit molecular weight of 22,000 extrapolated from its mobility in denaturing polyacrylamide gels (1). Shortly upstream of the ahpC coding region is a sequence that closely matches the bacterial Shine-Dalgarno consensus sequence. The start point of the ahp transcript (indicated on Fig. 2) and the regulation of its promoter are described in a separate report (16). A comparison of the predicted amino acid sequence of the C22 protein with two protein data bases (NBRF/Dayhoff and translated GenBank) showed no significant sequence similarities to other known proteins.

ahpF Gene and Homology of the Encoded F52a Protein to Thioredoxin Reductase—Comparison of the DNA sequence near the beginning of the more downstream open reading frame with the known N-terminal amino acid sequence of the purified F52a protein (1) confirmed that this gene encodes the F52a protein component of the alkyl hydroperoxide reductase. We are designating this gene as ahpF. The N-terminal protein sequence was also used to identify the likely translation start point on the ahpF transcript, and inspection of this region revealed a Shine-Dalgarno consensus sequence just upstream of the initiating methionine codon. The F52a protein is predicted to be 521 amino acids long with a molecular mass of 55,959 daltons (without the FAD), in good agreement with a molecular weight of 57,000 extrapolated from its mobility in denaturing polyacrylamide gels (1). The NBRF/Dayhoff and translated GenBank data bases were searched for proteins with sequences similar to that of the F52a protein using the FASTP and TFASTN computer programs (21). The protein giving both the highest initial and optimized scores observed in any search was E. coli thioredoxin reductase (see Table 1). Computer alignment of the entire E. coli thioredoxin reductase protein with residues 207–521 of the F52a protein revealed 34% identity and many conservative changes (Fig. 3). A particularly striking align-
ment requiring no deletions or insertions occurs in the 65 showed that the overall sequence similarity to these enzymes was rather poor and limited to three short regions of strong local sequence similarity. The redox-active segment, which is among the identities are the highly conserved glutathione reductase, mercuric reductase, and dihydrolipoamide dehydrogenase, is not observed in the F52a protein. The three regions of local sequence similarity which are shared by the F52a protein and other FAD-containing disulfide reductases (13). The first region above the mean score (Table I). Further analysis, however, showed that the overall sequence similarity to these enzymes was rather poor and limited to three short regions of strong local sequence similarity. The redox-active segment, which is highly conserved among glutathione reductase, mercuric reductase, and dihydrolipoamide dehydrogenase, is not observed in the F52a protein. The three regions of local sequence similarity which are shared by the F52a protein and other disulfide reductases (13). The first region of sequence similarity (the most N-terminal of the three

FIG. 2. Nucleotide sequence of the ahp locus and the predicted amino acid sequences of the encoded C22 and F52a proteins. The nucleotide sequence of the two genes encoding the alkyl hydroperoxide reductase and their flanking regions is shown in the 5' to 3' direction. The predicted amino acid sequences of the C22 protein (encoded by the more upstream ahpC gene) and the F52a protein (encoded by the more downstream ahpF gene) are indicated above the relevant nucleotides. Likely Shine-Dalgarno (SO) sequences upstream of each gene are underlined. The transcription start (indicated by an arrow) and important promoter elements of the ahp locus (16) are also indicated.

Comparison of the F52a Protein Sequence with That of Other Disulfide Oxidoreductases—Computer searches of the NBRF/Dayhoff and GenBank data bases using FASTP and TFASTN showed that the functionally related proteins glutathione reductase, mercuric reductase, and dihydrolipoamide dehydrogenase also showed sequence similarity scores significantly above the mean score (Table I). Further analysis, however,
regions in each of the proteins) includes amino acids 214–229 of the F52a protein. The corresponding region in human erythrocyte glutathione reductase has been implicated in the binding of FAD based on its position in the crystal structure (22). The second region of sequence similarity includes residues 357–371 of the F52a protein and shows strong homology to the known NADP-binding domain of glutathione reductase (9). The third region (residues 470–488 in the F52a protein), which is near the carboxyl end of each of the proteins, aligns well with the central domain of glutathione reductase, which has also been shown to make contacts with the FAD molecule (22). More recently, the analogous region in a crystal of the F52a protein. The corresponding region in human thioredoxin reductase molecule and their counterparts in the F52a protein are highlighted with shaded rectangles.

### DISCUSSION

We have sequenced the *S. typhimurium* *ahp* locus and have shown that it contains the two genes that encode the two proteins comprising the alkyl hydroperoxide reductase activity. We have designated the more upstream of the two genes as *ahpC* (encoding the C22 protein) and the more downstream gene as *ahpF* (encoding the F52a protein). Earlier work has shown that the synthesis of both proteins is regulated by the oxyR promoter (1). However, little is known about the mechanism by which reducing equivalents are transferred to the FAD in each of the proteins. The corresponding region in human erythrocyte glutathione reductase has been implicated in the binding of FAD based on its position in the crystal structure (22). The second region of sequence similarity includes residues 357–371 of the F52a protein and shows strong homology to the known NADP-binding domain of glutathione reductase (9). The third region (residues 470–488 in the F52a protein), which is near the carboxyl end of each of the proteins, aligns well with the central domain of glutathione reductase, which has also been shown to make contacts with the FAD molecule (22).

#### Table 1

| Protein (organism)                  | Initial score* | Mean initial score (+S.D.)* | Optimized score* | Sequence ref. |
|-------------------------------------|----------------|-----------------------------|------------------|---------------|
| Thioredoxin reductase (*E. coli*)   | 188            | 29.2 (7.35)                 | 408 (13)         |               |
| Mercuroc reductase (plasmid R100)   | 60             | 29.1 (7.29)                 | 102 (15)         |               |
| Mercuroc reductase (*Tn501*)        | 60             | 29.1 (7.29)                 | 88 (10, 11)      |               |
| Fumarate reductase (*E. coli*)      | 91             | 29.1 (7.29)                 | 94 (26)          |               |
| Glutathione reductase (*E. coli*)   | 45             | 29.1 (7.29)                 | 86 (8)           |               |
| Glutathione reductase (human)       | 58             | 29.1 (7.29)                 | 63 (9)           |               |
| Dihydrolipoamide dehydrogenase (*E. coli*) | 40      | 29.1 (7.29)                 | 77 (5)           |               |
| Dihydrolipoamide dehydrogenase (Saccharomyces cerevisiae) | 69 | 29.1 (7.29) | 104 (7) |               |
| Dihydrolipoamide dehydrogenase (human) | 49          | 24.2 (6.37)                 | 74 (6)           |               |

* Initial and optimized scores were calculated using FASTP or TFASTN (21). The initial score ranks regions of local sequence similarity between the test sequence and all sequences in the database but does consider deletions or insertions. The mean initial score and its standard deviation reflect the average score of comparisons between the test sequence and each sequence in the database. The scores of high ranking similarities are then recalculated allowing for deletions and insertions to give an optimized score.
The role of the C22 subunit of the alkyl hydroperoxide reductase activity is still not well understood. No significant homologies were found between the C22 protein and other proteins in the available data bases, including the protein thioredoxin that, together with thioredoxin reductase, comprises a general protein disulfide-reducing system. The F52a protein is therefore likely to be the second member of a class of disulfide oxidoreductases which is distinct from the class of reductases represented by glutathione reductase, dihydrolipoamide dehydrogenase, and mercuric reductase. Mutagenesis of the likely active site region of the alkyl hydroperoxide reductase should complement the ongoing mutagenesis and x-ray crystallography studies of the thioredoxin reductase molecule (27, 28) and help elucidate the mechanistic details of this type of disulfide reductase.
ides (1). It is therefore likely that the C22 protein plays a role in substrate binding and targeting the activity for hydroperoxide substrates. The role of the N-terminal domain of the F52a protein is also unclear. Although the F52a protein and thioredoxin reductase align well throughout the length of the thioredoxin reductase molecule, the smaller size of thioredoxin reductase (322 amino acids as opposed to 521 for the F52a protein) results in a segment of approximately 200 amino acids at the N terminus of the F52a protein which has no counterpart in thioredoxin reductase. This N-terminal region showed no significant similarities to other proteins in the NBRF/Dayhoff or translated GenBank data bases.

Aside from the common subunit organization and sequence homology between the F52a protein and thioredoxin reductase, there is another striking similarity between these two proteins. Both interact with a smaller protein chain to fulfill their physiologically important function. It will therefore be interesting to see if other reductases that show homology to the F52a protein and thioredoxin reductase will be found whose function also requires an additional protein factor.

Acknowledgments—We wish to thank Fredric S. Jacobson, Marjorie Russel, Amy R. Axelrod, and Jeffrey W. Kitzler for critically reading this manuscript.

REFERENCES
1. Jacobson, F. S., Morgan, R. W., Christman, M. F., and Ames, B. N. (1989) J. Biol. Chem. 264, 1488-1496
2. Christman, M. F., Morgan, R. W., Jacobson, F. S., and Ames, B. N. (1985) Cell 41, 755-762
3. Storz, G., Jacobson, F. S., Tartaglia, L. A., Morgan, R. W., Silveira, L. A., and Ames, B. N. (1989) J. Bacterial. 171, 2049-2055
4. Schirmer, R. H., and Schulz, G. E. (1987) in Coenzymes and Cofactors, Vol. 2B: Pyridine Nucleotide Coenzymes: Chemical, Biochemical and Medical Aspects (Dolphin, D., Poulsen, R., and Avramovic, O., eds) pp. 333-379, John Wiley & Sons, New York
5. Stephens, P. E., Lewis, H. M., Darlinton, M. G., and Guest, J. R. (1983) Eur. J. Biochem. 135, 519-527
6. Otolakowski, G., and Robinson, B. H. (1987) J. Biol. Chem. 262, 17313-17318
7. Browning, K. S., Uhlinger, D. J., and Reed, L. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1831-1834
8. Greer, S., and Perham, R. N. (1986) Biochemistry 25, 2736-2742
9. Krauth-Siegel, R. L., Blatterspiel, R., Saleh, M., Schlitiz, E., Schirmer, R. H., and Untucht-Grau, R. (1982) Eur. J. Biochem. 121, 259-267
10. Brown, N. L., Ford, S. J., Pridmore, R. D., and Fritzsche, D. C. (1983) Biochemistry 22, 4089-4095
11. Fox, B. S., and Walsh, C. T. (1985) Biochemistry 24, 4082-4088
12. Mita, T. K., Brown, N. L., Haberstroh, L., Schmidt, A., Goddete, D., and Silver, S. (1985) Gene (Amst.) 34, 253-262
13. Russel, M., and Model, P. (1985) J. Biol. Chem. 263, 9015-9019
14. O’Donnell, M. E., and Williams, C. H., Jr. (1985) Biochemistry 24, 7617-7621
15. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Tartaglia, L. A., Storz, G., and Ames, B. N. (1988) J. Mol. Biol. 210, 709-719
17. Laemmli, U. K. (1970) Nature 227, 680-685
18. Blake, M. S., Johnston, K. H., Russell-Jones, G. J., and Gotschlich, E. C. (1984) Anal. Biochem. 136, 175-179
19. Kincirli, D. A., and Dinurd, R. L. (1984) Anal. Biochem. 136, 180-184
20. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
21. Lipman, D. J., and Pearson, W. R. (1985) Science 227, 1435-1441
22. Schulz, G. E., Schirmer, R. H., and Pai, E. F. (1982) J. Mol. Biol. 160, 287-308
23. Schierbeck, A. J., Swarte, M. B. A., Dijkstra, B. W., Vriend, G., Read, R. J., Hol, W. G. J., and Drenth, J. (1989) J. Mol. Biol. 206, 385-397
24. Westphal, A. H., and de Kok, A. (1988) Eur. J. Biochem. 172, 299-305
25. Perham, R. N., Harrison, R. A. S., and Brown, J. P. (1978) Biochem. Soc. Trans. 6, 47-50
26. Cole, S. T. (1982) Eur. J. Biochem. 122, 479-484
27. Prongay, A. J., Engelke, D. R., and Williams, C. H., Jr. (1989) J. Biol. Chem. 264, 2666-2664
28. Kuriyan, J., Wong, L., Russel, M., and Model, P. (1989) J. Biol. Chem. 264, 12752-12753
Alkyl hydroperoxide reductase from Salmonella typhimurium. Sequence and homology to thioredoxin reductase and other flavoprotein disulfide oxidoreductases.

L A Tartaglia, G Storz, M H Brodsky, A Lai and B N Ames

*J. Biol. Chem.* 1990, 265:10535-10540.

Access the most updated version of this article at [http://www.jbc.org/content/265/18/10535](http://www.jbc.org/content/265/18/10535)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/18/10535.full.html#ref-list-1](http://www.jbc.org/content/265/18/10535.full.html#ref-list-1)