Homology between Egg White Sulfhydryl Oxidase and Quiescin Q6 Defines a New Class of Flavin-linked Sulfhydryl Oxidases*

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Karen L. Hoober‡, Nicole M. Glynn‡, Joan Burnside§, Donald L. Coppock¶, and Colin Thorpe**

From the Departments of Chemistry and Biochemistry and Animal and Food Sciences, University of Delaware, Newark, Delaware 19716, the Oncology Research Laboratory, Winthrop University Hospital, Mineola, New York 11501 and the Department of Medicine, State University of New York Stony Brook Medical Center, Stony Brook, New York 11790

The flavin-dependent sulfhydryl oxidase from chicken egg white catalyzes the oxidation of sulfhydryl groups to disulfides with the reduction of oxygen to hydrogen peroxide. Reduced proteins are the preferred thiol substrates of this secreted enzyme. The egg white oxidase shows an average 64% identity (from randomly distributed peptides comprising more than 30% of the protein sequence) to a human protein, Quiescin Q6, involved in growth regulation. Q6 is strongly expressed when fibroblasts enter reversible quiescence (Coppeck, D. L., Cina-Poppe, D., Gilleran, S. (1998) Genomics 54, 460–468). A peptide antibody against Q6 cross-reacts with both the egg white enzyme and a flavin-linked sulfhydryl oxidase isolated from bovine semen. Sequence analyses show that the egg white oxidase joins human Q6, bone-derived growth factor, GEC-3 from guinea pig, and homologs found in a range of multicellular organisms as a member of a new protein family. These proteins are formed from the fusion of thioredoxin and ERV motifs. In contrast, the flavin-linked sulfhydryl oxidase from Aspergillus niger is related to the pyridine nucleotide-disulfide oxidoreductases, and shows no detectable sequence similarity to this newly recognized protein family.

Both metalloprotein and FAD1-linked sulfhydryl oxidases catalyze the generation of disulfides with the reduction of molecular oxygen to hydrogen peroxide.

$$2 \text{R--SH} + \text{O}_2 \rightarrow 2 \text{R--S--S} + \text{H}_2\text{O}_2$$  \hspace{1cm} (Eq. 1)

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** To whom correspondence should be addressed. Tel.: 302-831-2689; Fax: 302-831-6335; E-mail: cthorpe@udel.edu.

† The abbreviations used are: FAD, flavin adenine dinucleotide; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; PDI, protein disulfide isomerase; PTH, phenylthiohydantoin; LysC, endoproteinase LysC; HPLC, high pressure liquid chromatography.
FIG. 1. Comparison of the sequence of egg white sulfhydryl oxidase peptides with sequence and domain structure of Quiescin Q6. Peptides 1–13 were obtained as described under “Experimental Procedures” and were located in the sequence of Q6 (U97276) as described. Peptide 10 contains the redox-active disulfide bridge in this flavoprotein. It was obtained by first converting the oxidase to the 4-electron reduced state by dithionite titration (9). The dithiol was then alkylated under anaerobic conditions with monobromobimane (18) (see “Experimental Procedures”). Under these conditions, both active site cysteine residues are labeled, as observed with iodoacetamide (9), yielding a strongly fluorescent peptide modified at Cys-450 and Cys-453 (Q6 numbering).

The egg white oxidase contains a total of about 595 amino acids (9), similar to the 582 residues of Q6 (14). In aggregate, the oxidase peptides shown in Fig. 1 represent 32% of the total sequence with an average identity of 64% (81% similarity). The probability that this degree of sequence identity would arise by chance is negligible. Thus, a sulfhydryl oxidase secreted into the egg white of the laying hen has clear homology to a protein involved in growth regulation in human fibroblast cells (14, 15).

This conclusion is strengthened by the demonstration that the egg white oxidase strongly cross-reacts (Fig. 2) with a peptide antibody raised against a peptide comprising residues 494–507 of Q6 (underlined in Fig. 1) show high levels of identity (from 45 to 100%) with Quiescin Q6 (Ref. 14 and see later). The 13 of 14 peptides are numbered in Fig. 1. Fortuitously, 3 peptides (11–13) are found to comprise a contiguous 51-residue segment toward the C terminus of Q6.

Peptide 10 contains the reactive disulfide bridge in this flavoprotein. It was obtained by first converting the oxidase to the 4-electron reduced state by dithionite titration (9). The dithiol was then alkylated under anaerobic conditions with monobromobimane (18) (see “Experimental Procedures”). Under these conditions, both active site cysteine residues are labeled, as observed with iodoacetamide (9), yielding a strongly fluorescent peptide modified at Cys-450 and Cys-453 (Q6 numbering).

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This conclusion is strengthened by the demonstration that the egg white oxidase strongly cross-reacts (Fig. 2) with a peptide antibody raised against a peptide comprising residues 494–507 of Q6 (underlined in Fig. 1). This reaction is completely blocked by the addition of competing peptide (Fig. 2). We have also partially purified sulfhydryl oxidase from bovine semen (see “Experimental Procedures”), based on the early work of Ostrowski et al. (17) on the rat seminal vesicle enzyme. This oxidase also contains FAD (6) and appears mechanistically similar to the egg white enzyme (9). Fig. 2 shows a strong specific response to the bovine enzyme. Thus, both vertebrate sulfhydryl oxidases appear related to Quiescin Q6. The differing apparent molecular weights in Fig. 2 may reflect varying carbohydrate contents (9).

The sequence of a third sulfhydryl oxidase, that from A. niger (8), is available from the patent literature (12). Despite markedly similar properties to the egg white protein mentioned earlier, there is undetectable sequence homology between them (not shown). However, data base searches with the A. niger sequence show 30% identity (46% similarity) with alkyl hydroperoxide reductase from Bacillus halodurans, 23% identity (40% similarity) with thioredoxin reductase (TRX B) from My-
The sequence of the egg white oxidase reflects a distinctly different lineage. Using the Q6 sequence, no members of the pyridine nucleotide-disulfide oxidoreductase family appear in any search protocols (not shown). However, these searches reveal that the egg white oxidase, like Q6 (14), was probably formed via fusion of at least two domains. First, a single thioredoxin motif is evident from residues 69–125 in Q6. Thioredoxins are small proteins of approximately 95–110 amino acids with a range of redox and nonredox roles (21, 22). A conspicuous member of the thioredoxin superfamily is mammalian protein-disulfide isomerase (21, 23, 24). PDI contains two functional thioredoxin domains with the active site sequence WCGHC (21, 24). Our peptide sequencing of sulfhydryl oxidase has yet to encounter a stringency of at least one in 10⁷. Cytochrome P450 reductase and nitric-oxide synthase contain an FNR domain. It appears to be secreted from hepatocytes in an active form in extracellular matrix formation as outlined above. Finally, the A. niger sulfhydryl oxidase and this new sulfhydryl oxidase-like protein family appear to have evolved convergently. Our current work is aimed at the cloning, overexpression, and characterization of Quiescin Q6, and a comparison of its properties with those of sulfhydryl oxidases from egg white and seminal vesicle. We are also exploring the role of the thioredoxin motif in these flavin-dependent oxidases.

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REFERENCES

1. Janolino, V. G., and Swaisgood, H. E. (1975) J. Biol. Chem. 250, 2532–2538
2. Schmelzer, C. H., Swaisgood, H. E., and Horton, H. R. (1982) Biochem. Biophys. Res. Commun. 107, 196–201
3. Lash, L. H., Jones, D. P., and Orrenius, S. (1984) Biochim. Biophys. Acta 779, 191–200
4. Goldsmith, L. A. (1987) Methods Enzymol. 143, 510–515
5. Clare, D. A., Pinnix, I. B., Lecce, J. G., and Horton, H. R. (1988) Arch. Biochem. Biophys. 265, 351–361
6. Ostrowski, M. C., and Kistler, W. S. (1988) Biochemistry 19, 2639–2645
7. Kusakabe, H., Kuninaka, A., and Yoshino, H. (1982) Agric. Biol. Chem. 46, 2057–2067
8. de la Motte, R. S., and Wagner, F. W. (1987) Biochemistry 26, 7363–7371
9. Hoober, K. L., Joneja, B., White, H. B., III, and Thorpe, C. (1996) J. Biol. Chem. 271, 30510–30516
10. Hoober, K. L., and Thorpe, C. (1999) Biochemistry 38, 3211–3217
11. Hoober, K. L., Sheasley, S. S., Gilbert, H. F., and Thorpe, C. (1999) J. Biol. Chem. 274, 22147–22150
12. Hoober, K. L., and Thorpe, C. (1999) Biochemistry 38, 3211–3217
13. Hoober, K. L., Sheasley, S. S., Gilbert, H. F., and Thorpe, C. (1999) J. Biol. Chem. 274, 22147–22150
14. Cockcroft, D. L., Cina-Poppe, D., and Gilberman, S. (1998) Genomics 54, 460–468
15. Cockcroft, D. L., Kopman, C., Scandalis, S., and Gilberman, S. (1999) Cell

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Flavin-linked Sulfhydryl Oxidases

SOX #10

| Quescin Q6 | I R G Y V H Y F F G C R D C A S H F E Q M L A |
| GEC-3 | I R N Y V F F G C R D C A S H F E Q M |
| CeQ6r1 | I Q G W V K S Y F G C E H C R N F H M H M T T |
| CeQ6r2 | I R D W V G S F F G C N H C R D H F L K M T T D |
| CeQ6r3 | F R A F I H F L S C E A C A N F T K E A K |
| DmQ6 | M H G Y I N K F G C T E C S E H F F Q V S Q A |

Fig. 3. The active site disulfide of egg white sulfhydryl oxidase is homologous to Quiescin Q6 homologs. The sequence of this active site peptide was aligned with representative members of the Quiescin Q6 family: the guinea pig homolog GEC-3 (U82982); three C. elegans homologs (CeQ6r1–3: Z69637, U80848, and U39646, respectively), and a D. melanogaster homolog (AC005655). Alignments were calculated using PileUp (Genetics Computer Group 9.0) and the consensus was calculated with the program Pretty (Genetics Computer Group 9.0) with a plurality of 3.

C. elegans homologs (CeQ6r1–3: Z69637, U80848, and U39646, respectively) and cell growth inhibitory factor (GenBank TM L42379) and secreted proteins.

Although its precise physiological role is not known, ALR appears to be secreted from hepatocytes in an active form in liver regeneration after chemical injury. This enzyme family could easily be envisaged with the loss of a redox-active disulfide bridge (residue 450–453; Q6 numbering; see Fig. 1) (14). Interestingly, the redox-active disulfide in this sequence, although it is observed between residues 69 and 73 in Q6 (Fig. 1) (14). Interestingly, the redox-active disulfide in the oxidase (9, 16) lies toward the C terminus within the second recognizable feature: the ERV/ALR motif (residues 406–503 of Q6; Fig. 1). ERV1 was the first identified member of the pyridine nucleotide-disulfide oxidoreductase family appear in any search protocols (not shown). However, these searches reveal that the egg white oxidase, like Q6 (14), was probably formed via fusion of at least two domains. First, a single thioredoxin motif is evident from residues 35–125 in Q6. Thioredoxins are small proteins of approximately 95–110 amino acids with a range of redox and nonredox roles (21, 22). A conspicuous member of the thioredoxin superfamily is mammalian protein-disulfide isomerase (21, 23, 24). The role of PDI in shuffling disulfide bonds during the maturation of proteins within the endoplasmic reticulum has been extensively documented (21, 23, 24). PDI contains two functional thioredoxin domains with the active site sequence WCGHC (21, 24). Our peptide sequencing of sulfhydryl oxidase has yet to encounter a stringency of at least one in 10⁷. Cytochrome P450 reductase and nitric-oxide synthase contain an FNR domain. It appears to be secreted from hepatocytes in an active form in response to liver damage (27).

Quiescin Q6 homologs containing both thioredoxin and ERV motifs are not found in any prokaryote genome sequence or in Saccharomyces cerevisiae. They are, however, widely distributed in metazoans (e.g., Caenorhabditis elegans, Drosophila melanogaster, guinea pig). In humans, sequences similar to Q6 have been named bone-derived growth factor (GenBank™ L42379) and cell growth inhibitory factor (GenBank™ E12644). All these homologs and the ERV proteins contain a region homologous to the active site disulfide bridge of the egg white sulfhydryl oxidase. Fig. 3 illustrates this homology with representative examples. These studies suggest that Quiescin Q6 is a flavoprotein sulfhydryl oxidase. Indeed, immunoprecipitates of fibroblast cell extracts show detectable sulfhydryl oxidase activity.² Quiescin Q6 does not apparently contain the ADP-binding motif (28) found in a number of FAD-linked flavoproteins including the A. niger sulfhydryl oxidase (not shown). However, searching the Q6 sequence using the program eMOTIF (29) identifies the region 442–451 (Fig. 1) as a pattern found in the ferredoxin-NADPH reductase (FNR) superfamily of flavoproteins (30) with a stringency of at least one in 10⁷. Cytochrome P450 reductase and nitric-oxide synthase contain an FNR domain. It is of interest that this 442–451 region is contiguous with the active site disulfide loop (residues 450–453; Q6 numbering; see earlier). Obviously, the ultimate significance of these observations must await further work. There are a number of observations suggesting a connection with the extracellular matrix (14, 15). Q6 lacks the endoplasmic reticulum retention signal and is secreted into the fibroblast culture medium.³ In addition, a number of the genes expressed at the onset of reversible quiescence, including 4 collagen and decorin, are well known components of the extracellular matrix. These secreted proteins typically contain multiple disulfide bridges essential for structural integrity. Taking one example, disulfide bond formation in many collagens is required to direct the assembly of trimeric proteins and, in some cases, is involved in the generation of extracellular collagen networks (31–33). If the specificity of Quiescin Q6 were as broad as the egg white protein (11), Q6 might contribute to disulfide bond formation in a variety of secreted proteins.

In summary, the egg white oxidase has multiple homologs in multicellular organisms. Apart from the rat seminal vesicle enzyme, none of these proteins have been assigned an enzymatic function. It now appears likely that some will be involved in extracellular matrix formation as outlined above. Finally, the A. niger sulfhydryl oxidase and this new sulfhydryl oxidase-like protein family appear to have evolved convergently. Our current work is aimed at the cloning, overexpression, and characterization of Quiescin Q6, and a comparison of its properties with those of sulfhydryl oxidases from egg white and seminal vesicle. We are also exploring the role of the thioredoxin motif in these flavin-dependent oxidases.

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² K. L. Hoober, C. Thorpe, and D. L. Cockcroft, unpublished observations.
³ D. L. Cockcroft, unpublished observations.
Growth Differ. 4, 483–493
16. Williams, C. H., Jr. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., ed) Vol. III, pp. 121–211, CRC Press, Boca Raton, FL.
17. Ostrowski, M. C., Kistler, W. S., and Williams-Ashman, H. G. (1979) *Biochem. Biophys. Res. Commun.* 87, 171–176
18. Kosower, E. M., and Kosower, N. S. (1995) *Methods Enzymol.* 251, 133–148
19. Hu, L., Borleske, B. L., and Coleman, R. (1997) *Protein Sci.* 6, 42–52
20. Arita, Y., Buffolino, P., and Coppock, D. L. (1998) *Exp. Cell Res.* 242, 381–390
21. Ferrari, D. M., and Soling, H.-D. (1999) *Biochem. J.* 339, 1–10
22. Holmgren, A. (1989) *J. Biol. Chem.* 264, 13963–13966
23. Gilbert, H. F. (1997) *J. Biol. Chem.* 272, 29399–29402
24. Freedman, R. B., Hirst, T. R., and Tuite, M. F. (1994) *Trends Biochem. Sci.* 19, 331–336
25. Lisowsky, T., and Stein, G. (1998) *Yeast* 14, 171–180
26. Hagiya, M., Francavilla, A., Polimeni, L., Ihara, I., Sukai, H., Seki, T., Shimonishi, M., Porter, K. A., and Starzl, T. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 8142–8146
27. Gandhi, C. R., Kuddus, R., Subbotin, V. M., Prellich, J., Murase, N., Rao, A. S., Nalesnik, M. A., Watkins, S. C., DeLeo, A., Trucco, M., and Starzl, T. E. (1999) *Hepatology* 29, 1435–1445
28. Wierenga, R. K., Terpstra, P., and Hol, W. G. J. (1986) *J. Mol. Biol.* 187, 101–107
29. Nevill-Manning, C. G., Wu, T. D., and Brutlag, D. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 5865–5871
30. Correll, C. C., Ludwig, M. L., Bruns, C. M., and Karplus, P. A. (1993) *Protein Sci.* 2, 2112–2133
31. McLaughlin, S. H., and Balleiras, N. J. (1998) *Matrix Biol.* 16, 369–377
32. Gunwar, S., Ballester, F., Noe, C. E., Sado, Y., Ninomiya, Y., and Hudson, B. G. (1998) *J. Biol. Chem.* 273, 8767–8775
33. Zhu, Y., Oganessian, A., Keene, D. R., and Sandell, L. J. (1999) *J. Cell Biol.* 144, 1069–1080