The Sequence of Amino Acid Residues Around the Oxidation-Reduction Active Disulfide in Yeast Glutathione Reductase

HOMOLOGY WITH THE ANALOGOUS REGION IN LIPOAMIDE DEHYDROGENASE*

ERIC T. JONES AND CHARLES H. WILLIAMS, JR.

From the Veterans Administration Hospital and the Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48105

SUMMARY

A 14-residue peptide containing the oxidation-reduction active cystine residue from yeast glutathione reductase has been isolated from proteolytic digests of the enzyme in which the free sulfhydryl groups had been reacted with N-ethylmaleimide. The sequence of this disulfide-containing peptide was found to be:

$$\text{S} \quad \text{Ala-Leu-Gly-Gly-Thr-Cys-Val-Asn-Val-Gly-Cys-Val-Pro-Lys} \quad \text{S}$$

The sequence was highly homologous with the active cystine regions in Escherichia coli and pig heart lipoamide dehydrogenase.

The sequences of three of the postulated four thiol-containing regions of the enzyme are also presented, as well as evidence supporting the view that the enzyme is composed of two identical subunits.

EXPERIMENTAL PROCEDURES

Materials—Yeast glutathione reductase was obtained from Sigma Company and further purified by chromatography on calcium phosphate as described by Massey and Williams (3). Pepsin, twice crystallized, and TPCK-treated trypsin were obtained from Sigma. The abbreviations used are: TPCK, 1-chloro-4-phenyl-3-tosyl-amidobutan-2-one; SE-Sephadex, sulfoethyl-Sephadex;...
from Worthington. Thrombolyins and pronase were products of Calbiochem. N-ethyl[3,4-C]maleimide and iodo[2-14]Clacetic acid were purchased from Amersham/Searle, Trifluoroacetic acid, phenylisothiocyanate, and benzene were obtained from Pierce Chemical Co. Fluram (fluorescamine) was purchased from Roche Diagnostics. Pyridine was redistilled over ninhydrin prior to use. All of the other reagents were the purest commercially available grade.

Reaction of Enzyme Thiol Groups with Iodoacetic Acid and N-ethylmaleimide—The alkylation of guanidine-denatured enzyme with iodoacetic acid was carried out as described by Matthews et al. (21).

[14C]N-ethylmaleimide was added to yeast glutathione reductase from the side arm of an anaerobic cuvette (22). The enzyme was anaerobically denatured in 7.0 M GuHCl for 24 hours prior to being transferred to the cuvette. The transfer was accomplished inside an anaerobic "glove box." After reaction for 24 hours at 25° and dialysis against three changes of 5% formic acid for 24 hours, the incorporation of N-ethylmaleimide was determined by amino acid analysis as S-succinylcysteine and by taking aliquots for radioactive counting from the hydrolysate prior to application to the amino acid analyzer column.

Proteolytic Digestion—Pepsin digestion was carried out as described by Piskiewicz et al. (23). Three additions of 2% w/w pepsin were made at 0, 2, and 4 hours to the protein solution in 5% formic acid (1 mg/ml). After 24 hours at 40°, the digestion was halted by lyophilizing. Peptides were digested with TPCK-treated trypsin or pronase (1% w/w) for 8 hours at 40° in 0.5% ammonium bicarbonate. Peptide maps were prepared by Piskiewicz et al. (23). Three additions of 20/, w/w pepsin to the amino acid analyzer column.

Separation and Characterization of Peptides—Peptides were purified by ion-exchange chromatography on SE-Sephadex (Pharmacia), DEAE-cellulose DE52 (Whatman), PA35 (Beckman), and by gel filtration chromatography on Sephadex G-10. Peptides were detected by sampling a portion of the column effluent utilizing automatic amino acid hydrolysis and reaction with ninhydrin as described by Hill and Delaney (24). DE52 and Sephadex G-10 fractions were analyzed by reading the A280 nm. Paper chromatography and electrophoresis were carried out as described by Burleigh and Williams (15). Peptide maps were prepared as described by Matthews et al. (21). Peptides were detected on paper by spraying with 1% fluorescamine in acetone followed by 0.5% pyridine in acetone. After drying, the chromatograms were visualized under ultraviolet illumination (365 nm). Peptides were eluted with 0.1 M HCl, 0.5% ammonium bicarbonate, or water. Amide assignments were made at pH 6.5 relative to aspartic acid (m = -1) based on the plots of Offerd (25).

Radioactivity Measurements—Aliquots for counting were added to 6 ml of Aquasol (New England Nuclear) and counted in a Packard scintillation counter.

Amino Acid Analysis—Peptides and proteins were hydrolyzed for 18 to 24 hours in constant boiling HCl in evacuated, sealed tubes. (Thiodiglycol (0.5%) was added to all samples containing carboxymethylcyesteine [Cys(Cm)] or S-succinylcysteine.) The amino acids were analyzed on a modified Beckman 120B amino acid analyzer using the single column system of Durrum with DC-1A resin and Pic system II buffers. Half-cysteine was determined as cysteic acid after hydrolysis of the peptide or protein in the presence of MeSO as described by Spencer and Wold (26) or in hydrolysates of performic acid-oxidized peptides (27). All values are uncorrected for destruction of threonine and serine.

Sequence Studies—Dansyl NH2-terminal determinations were carried out as described by Gray (28), on 5 × 5-cm polyamide sheets (29). The "semi-micro" Edman method of Peterson et al. (30) was used with the substitution of 75% pyridine water (for dimethylallylamine) as the coupling buffer. Aliquots of 2 and 15 nmol were taken, respectively, for dansyl NH2-terminal determination and amino acid analysis following each step of the Edman degradation.

RESULTS

Characterization of Yeast Glutathione Reductase—From timed hydrolyses of yeast glutathione reductase, amino acid composition of MeSO, dimethylsulfoxide; Cys(Suc), S-succinylcysteine; Cys (Cm), carboxymethylcyesteine; dansyl or Dns, S-dimethylaminonaphthalene-1-sulfonlyl; GuHCl, guanidinium chloride.

Fig. 1. Peptide map of a tryptic digest of yeast glutathione reductase reacted with IAA. Fifty nanomoles of carboxymethylated-yeast glutathione reductase digested with trypsin were run in descending chromatography for 18 hours and followed by high voltage electrophoresis at 3,000 volts for 65 min. The map was sprayed with fluorescamine.

Fig. 2. Chromatography of a peptic digest of yeast glutathione reductase reacted with N-ethylmaleimide, on SE-Sephadex (C-25). N-ethyl[14C]maleimide-yeast glutathione reductase, after peptic digestion (6% w/w pepsin for 24 hours at 40°), was chromatographed on a SE-Sephadex column (1.5 × 90 cm). The column was developed with a concave gradient from 0.02 M pyridine acetate, pH 2.5, to 2.0 M pyridine acetate, pH 5.0, over 48 hours. The flow rate was maintained at 18.6 ml per hour and 10-min fractions were collected. Radioactivity was determined on 50-ml aliquots from each fraction. (Fig. 5 is a "map" of this same digest.)

Isolation and Sequence of Active Cystine Region—Yeast glutathione reductase (3.88 μmol) was reacted with a 100 fold excess of N-ethyl[14C]maleimide over FAD in 0.1 M phosphate, 7.0 μM GuHCl, pH 6.9, after anaerobic denaturation for 24 hours. After dialysis, the enzyme was exhaustively digested with pepsin (6% w/w) for 24 hours at 40° in 5% formic acid. The resulting peptides were fractioned on a SE-Sephadex (C-25) column (1.5 × 90 cm) (Fig. 2).

1 L. D. Arscott and C. H. Williams Jr., unpublished observations.
FIG. 3. Chromatography of the peptic disulfide peptide on DE-52. The peptic disulfide peptide (Peak D, from SE-Sephadex, Fig. 2) was fractionated on a 1.5 X 89-cm DE-52 column. The column was equilibrated with 20 mM ammonium bicarbonate. The flow rate was maintained at 13.5 ml per hour and 12 min fractions were collected. The column was developed by successive linear gradients from 20 mM to 0.2 M ammonium bicarbonate and from 0.2 M to 2.0 M ammonium bicarbonate (each over 24 hours). The disulfide location was determined by amino acid analysis.

FIG. 4. Tryptic digestion of the peptic disulfide peptide fractionated on Sephadex G-10. The peptide from Peak I of Fig. 3 was digested with 2% w/w TPCK-treated trypsin for 8 hours. The resulting fragments were separated by molecular-sieve chromatography on a Sephadex G-10 column (1.5 X 90 cm) equilibrated with 20 mM ammonium bicarbonate. The flow rate was 13.5 ml per hour. The disulfide-containing peak was located by amino acid analysis.

The disulfide-containing peak was further purified by chromatography on a DEAE-cellulose column (1.5 X 90 cm) (Fig. 3). Amino acid analysis of the cystine-containing peak showed the following composition: Asp(1.1), Thr(1.0), Pro(1.1), Gly(3.7), Ala(1.7), 1/2 Cys(1.5), Val(5.1), Met(1.1), Leu(1.3), Lys(3.4). The single NH₂-terminal residue of this peptide was found to be lysine. The yield was 34% of the starting material (based on amino acid analysis).

Following tryptic digestion of this 21-residue peptide, the resulting peptides were fractionated by molecular-sieve chromatography on Sephadex G-10. The elution profile is shown in Fig. 4. The disulfide-containing fraction (B) had the following composition: Asp(1.0), Thr(1.0), Pro(0.9), Gly(2.9), Ala(0.9), 1/2 Cys(1.6), Val(2.7), Leu(1.0), Lys(1.4). Dansylation showed NH₂-terminal alanine.

This peptide was performic acid oxidized and the sequence was determined by Edman degradation to be:

S---S

Ala-Leu-Gly-Gly-Thr-Cys-Val-Asp-Val-Gly-Cys-Val-Pro-Lys

Peaks C and D from the Sephadex G-10 column gave the following composition: C, Gly(1.0), Ala(1.0), Lys(1.7); D, Val(2.0), Met(0.9), Lys(1.0). Peak C contained the NH₂-terminal region of the 21-residue peptic peptide. This tetra-peptide with NH₂-terminal lysine was shown to have the sequence: Lys-Ala-Gly-Lys, by subtractive Edman degradation through two steps and then NH₂-terminal determination using dansyl chloride.

Peak D was shown to contain a peptide of the following composition: Val(2.0), Met(0.9), and the amino acid lysine. The sequence of the tri-peptide was shown to be Val-Val-Met by subtractive Edman degradation through two steps.

Isolation and Sequences of Three Unique Thiol-containing Regions—After reacting the enzyme with N-ethyl[¹⁴C]maleimide as described above, amino acid analysis indicated 1.9 S-succinylcysteine residues per FAD and 2.9 mol of N-ethyl[¹⁴C]maleimide incorporated per mole of FAD based on radioactivity. This is compared with 4 sulfhydryl residues per FAD demonstrated by amino acid analysis of oxidized enzyme.²

Amino acid analysis of native enzyme, after hydrolysis in the presence of Me₂S₀, yields 5.9 to 6.2 residues of cysteic acid per FAD, indicating 4 thiol residues in addition to the cystine residue. Radioautograms of N-ethyl[¹⁴C]maleimide labeled yeast glutathione reductase show four radioactive peptides (Fig. 5). SE-Sephadex elution profiles of pepsin-digested enzyme show four separate peaks of radioactivity (Fig. 2).

The isolation of three unique cysteine-containing peptides is summarized in Table I. The thiol peptides were isolated by combination of column and paper chromatography and paper electrophoresis. Both N-ethylmaleimide-labeled enzyme, described above, and iodoacetic acid-labeled enzyme were utilized, as well as both tryptic and peptic digests of labeled enzyme. The sequences are as follows:

Cys-Asn-Asp

Lys-Ile-Ala-Cys-Pro-Gly-Asn-Val-Gln-Lys

Asp-Thr-Ile-Tyr-(His, Glx)-Val-Cys-Lys-(Thr, Gly, Ala, Leu)
**Amino acid composition and sequence of yeast glutathione reductase sulfhydryl peptides**

The abbreviations used are: NEM, N-ethylmaleimide; IAA, iodoacetic acid.

| Sulfhydryl Peptide A: | Cys(Suc)(1.00);Asx,1.87 |
|----------------------|-------------------------|
| NEM-P-S-A*           |                         |
| Purification method* |                         |
| Dansylation          |                         |
| Edman                |                         |
| Purification method  |                         |
| Dansylation          |                         |
| Edman                |                         |
| Purification method  |                         |
| Dansylation          |                         |
| Sequence             |                         |

| Sulfhydryl Peptide C1: |                         |
|-----------------------|-------------------------|
| IAA-P-S-C1            |                         |
| Purification method   |                         |
| Dansylation           |                         |
| Sequence              |                         |

| Sulfhydryl Peptide C2: |                         |
|-----------------------|-------------------------|
| IAA-T-S-C2            |                         |
| Purification method   |                         |
| Dansylation           |                         |

| Symbols used to label peptides and to describe the method of purification are as follows: NEM, yeast glutathione reductase labeled with [14C]NEM prior to digestion. Conditions are described in the text. IAA, yeast glutathione reductase labeled with [14C]IAA prior to digestion. P, digestion of whole protein with pepsin. T, digestion of whole protein with trypsin. Th, digestion of a previously isolated peptide with thermolysin. Pr, digestion of a previously isolated peptide with pronase. S, purification on SE-Sephadex cation exchange resin. D, purification on DE52 anion exchange resin. PA, purification of a thermolysin or a pronase digested peptide on PA35 cation exchange resin. 6, purification by electrophoresis at pH 6.5. 3, purification by electrophoresis at pH 3.5. A, C1, and C2 are symbols given to specific sulfhydryl-containing peptides, based on their position of elution from SE-Sephadex resin. (See text.) |
| Numbers in parentheses indicate the residue assumed to equal one for the composition calculation. Radioactivity was used in some cases involving Cys(Suc) and Cys(Cm) where values were less than unity for these residues in amino acid analysis. |
| Dns-Cys(Suc) is above and between Dns-Ala and Dns-amide after the second solvent on the polyamide sheets. |
The fourth major radioactive fraction (Peak D, in Fig. 2) was further purified by chromatography on DEAE-cellulose. The radioactivity was found to be contained in a highly cationic peptide with the composition: Lys, His, Arg, Asx, Glx, Gly, Met, Ile, Tyr, Phe. This peptide corresponds to the cationic peptide on a radioautogram; it contained no S-succinylcysteine and the location of the radioactivity was not determined.

**DISCUSSION**

The sequence of amino acid residues around the active site disulfide in yeast glutathione reductase shows a very high degree of homology with the analogous region in pig heart lipoamide dehydrogenase. This represents yet another similarity between these closely related enzymes. There is a growing list of enzyme families in which a high degree of homology has been demonstrated around a common active site residue (31). These include the serine proteases, the cysteine proteases, the carboxypeptidases, the ATP-guanidine phosphotransferases (creatinine, arginine, and lombricine kinases), and the aldolases. The pyridine nucleotide-disulfide oxidoreductases can now be added to this list. A parallel can be drawn between this group and the aldolases, where the closely related aldolases A and B show extensive homology but do not show homology with transaldolase. Such is the case with glutathione reductase and lipoamide dehydrogenase where there is no homology with thioredoxin reductase around the active center cystine residue. Fig. 6 compares these structures. The two substitutions in the immediate disulfide region between glutathione reductase and pig heart lipoamide dehydrogenase are conservative both chemically and genetically. It is of interest that the prokaryote *Escherichia coli* lipoamide dehydrogenase has a valine preceding the first half-cystine, whereas the eukaryotic enzymes have a threonine. The possible genetic significance of this change may become clearer when the sequences of *E. coli* glutathione reductase and yeast lipoamide dehydrogenase are known. Chemically this is a relatively conservative change since the side chains of these amino acids are virtually identical in volume.

The lack of homology between lipoamide dehydrogenase and thioredoxin reductase, and the apolar nature of the residues around the active center disulfide in lipoamide dehydrogenase, an enzyme with an apolar substrate, led to the postulation that the disulfide region contained important determinants for interaction with the respective substrates (10, 17, 19). The very high degree of homology between lipoamide dehydrogenase and glutathione reductase suggests that some modification of that postulation is in order; clearly glutathione and lipoamide are very different molecules. It is possible that the apolar region is important in the binding of lipoamide whereas the determinants for binding the larger glutathione molecule are elsewhere. One would predict that ionic interactions would play a role in the binding of anionic glutathione and the nearby lysine residues may function in this way.

Glutathione reductase (3) and lipoamide dehydrogenase (1, 2) cycle in catalysis between the oxidized state and a 2-electron reduced state. The spectral characteristics of this intermediate make it likely that it is a charge transfer complex in which the donor is a thiolate anion and the acceptor is FAD. Thus the reduced disulfide and the flavin share 2 electrons. It seems reasonable then to suggest that the structure of the disulfide region in these enzymes confers a special reactivity on that disulfide.

The resolved spectrum of FAD when bound to glutathione reductase and lipoamide dehydrogenase indicates that the flavin is bound in a hydrophobic region. It was suggested for lipoamide dehydrogenase (15) that the tight loop in the polypeptide chain imposed by the proximity of the half-cystines in the sequence formed part of this region. The tight loop would favor a flat conformation accommodating the planar isoalloxazine ring and al...
lowing multiple van der Waals contacts. Electron sharing between the 
flavin and the disulfide demands that they be close.

Previous studies have indicated that glutathione reductase is 
composed of two electrophoretically similar subunits and 2 mole-
cules of FAD (3, 7). The subunit molecular weight, based on 
amino acid analysis of samples of known FAD content, is 50,600.2
Each subunit contains 6 half-cystines; 2 are present as the active 
center disulfide and 4 as cysteine residues. Only 1 of the cysteine 
residues reacts readily with iodoacetate or N-ethylmaleimide in 
the presence of 7 M GSHCl; two others react partially; the fourth 
reacts very slowly with phenyl mercuric acetate (3). The data 
presented here would indicate that the subunits of glutathione re-
ductase are identical or very nearly so. Thus, only a single 
NH2-terminal residue, glycine, was found; only one disulfide-
containing peptide was isolated; the expected number of peptides 
were found in maps of tryptic digests based on the number of 
arginine plus lysine residues per FAD; and finally peptides con-
taining three of the expected four thiols were isolated and shown 
 to have unique sequences. None of the sequences around the 
thiols of glutathione reductase shows obvious homology with any 
of the seven sequences of thiol-containing peptides in pig heart 
lipoamide dehydrogenase (20, 21).

The sequences reported here constitute about 10% of the poly-
peptide chain. Speculation on the relatedness of glutathione re-
ductase and lipoamide dehydrogenase is not possible with so small 
a proportion of the total sequence. Preliminary work indicates 
that the disulfide region is located in a 57-residue peptide isolated 
from glutathione reductase reacted with cyanogen bromide; its 
sequence, shown in Fig. 6, is identical with the COOH-terminal 
portion of the cyanogen bromide peptide. A common location for 
the active cystine residue in the primary structure of glutathione reductase and lipoamide dehydrogenase would provide strong 
evidence for any evolutionary relationship between these two en-
zymes.

REFERENCES
1. Massey, V., Gibson, Q. H., and Veeger, C. (1960) Biochem. 
J. 77, 341–351
2. Massey, V., and Veeger, C. (1961) Biochim. Biophys. Acta 
48, 33–47
3. Massey, V., and Williams, C. H., Jr. (1965) J. Biol. Chem. 
240, 4470–4480
4. Zanetti, G., and Williams, C. H., Jr. (1967) J. Biol. Chem. 
242, 5232–5236
5. Thelander, L. (1968) Eur. J. Biochem. 4, 407–422
6. Williams, C. H., Jr., Burleigh, B. D., Jr., Ronchi, S., 
Abscott, L. D., and Jones, E. T. (1971) in Flavins and 
Flavoproteins (Kamin, H., ed) pp. 295–311, University Park 
Press, Baltimore
7. Mavis, R. D., and Stellwagen, E. (1968) J. Biol. Chem. 243, 
809–814
8. Coleman, R. F., and Black, S. (1965) J. Biol. Chem. 240, 1796– 
1803
9. Massey, V., Hofmann, T., and Palmer, G. (1962) J. Biol. 
Chem. 237, 3820–3828
10. Williams, C. H., Jr., Zanetti, G., Abscott, L. D., and 
McAllister, J. K. (1967) J. Biol. Chem. 242, 5226–5231
11. Williams, C. H., Jr. (1965) J. Biol. Chem. 240, 4793–4800
12. Thelander, L. (1967) J. Biol. Chem. 242, 852–859
13. Massey, V., Palmer, G., Williams, C. H., Jr., Swoboda, 
B. F. P., and Sands, R. H. (1966) in Flavins and Flavopro-
teins (Slater, E. C., ed) pp. 133–152, Elsevier Publishing 
Company, Amsterdam
14. Palmer, G., and Massey, V. (1968) in Biological Oxidations 
(Singer T. P., ed) p. 263, Interscience Publishers, New York
15. Burleigh, B. D., Jr., and Williams, C. H., Jr. (1972) J. 
Biol. Chem. 247, 2077–2082
16. Brown, J. P., and Perham, R. N. (1972) FEBS (Fed. Eur. 
Biochem. Soc.) Lett. 26, 221–224
17. Ronchi, S., and Williams, C. H., Jr. (1972) J. Biol. Chem. 
247, 2083–2086
18. Thelander, L. (1970) J. Biol. Chem. 245, 6026–6029
19. Williams, C. H., Jr., and Abscott, L. D. (1972) Z. Natur-
forsch 27b, 1078–1080
20. Brown, J. P., and Perham, R. N. (1974) Biochem. J. 138, 
505–512
21. Matthews, R. G., Abscott, L. D., and Williams, C. H., Jr. 
(1974) Biochim. Biophys. Acta 370, 26–38
22. Burleigh, B. D., Jr., Foust, G. P., and Williams, C. H., 
Jr. (1969) Anal. Biochem. 27, 538–544
23. Pereszkely, D., Landou, M., and Smith, E. L. (1973) J.
Biol. Chem. 248, 3067–3081
24. Hill, R. L., and Delaney, R. (1967) Methods Enzymol. 11, 
339–351
25. Offord, R. E. (1966) Nature 211, 591–593
26. Spencer, R. L., and Wold, F. (1969) Anal. Biochem. 32, 185– 
189
27. Moos, S. (1963) J. Biol. Chem. 238, 235–237
28. Gray, W. R. (1972) Methods Enzymol. 25, 121–138
29. Hartley, B. S. (1970) Biochem. J. 119, 805–822
30. Peterson, J. D., Nehrlich, S., Oyer, P. E., and Steiner, 
D. F. (1972) J. Biol. Chem. 247, 4860–4871
31. Dayhoff, M. O. (1972) Atlas of Protein Sequence and Structure 
Vol. 5, p. 56, National Biomedical Research Foundation, 
Washington, D.C.
The sequence of amino acid residues around the oxidation-reduction active disulfide in yeast glutathione reductase.
E T Jones and C H Williams, Jr

J. Biol. Chem. 1975, 250:3779-3784.