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Selective *N*-Bromosuccinimide Oxidation of the Nonfluorescent Tryptophan-31 in the Active Center of Thioredoxin from *Escherichia coli*†

Arne Holmgren

ABSTRACT: The two tryptophan residues (Trp-28 and Trp-31) of thioredoxin-S_2 from *Escherichia coli* were selectively tritiated with trifluoroacetic [^{3}H]acid. The ^{3}H label was introduced to permit quantitative amino acid sequence analyses of the result of *N*-bromosuccinimide oxidation of tryptophan to oxindolylalanine. Addition of 3-fold molar excess of *N*-bromosuccinimide at pH 4 modifies a tryptophan in thioredoxin-S_2 that is nonessential for enzyme activity with thioredoxin reductase and has a strongly quenched fluorescence in both oxidized and reduced thioredoxin. This residue was shown to be Trp-31 by amino acid sequence analyses of ^{3}H-labeled chymotryptic peptides from the modified protein. The results demonstrate that the second tryptophan residue, Trp-28, signals a conformational change on reduction of the active-center disulfide to a dithiol by increasing its fluorescence quantum yield about 6-fold at pH 7. The differential reactivity of the tryptophan residues agrees with the known three-dimensional structure of thioredoxin-S_2.

A 3-fold increase in the quantum yield of tryptophan fluorescence at pH 7 accompanies the reduction of *Escherichia coli* thioredoxin-S_2[1] to thioredoxin-$(SH)_2$ (Stryer et al., 1967; Holmgren, 1972a). This useful change is evidence for a localized protein conformational change involved in the reaction mechanism of thioredoxin-$(SH)_2$ as a disulfide reductase (Holmgren, 1979a,b). Both tryptophan residues of thioredoxin (Trp-28 and Trp-31) are located close to the functional di-

† From the Department of Chemistry, Karolinska Institutet, S-104 01 Stockholm, Sweden. *Received November 24, 1980.* This investigation was supported by grants from the Swedish Cancer Society (Project 961) and the Swedish Medical Research Council (Projects 13X-3529 and 13P-4292).

[1] Abbreviations used: dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; thioredoxin-S_2 and thioredoxin-$(SH)_2$, the oxidized and reduced forms of thioredoxin, respectively; 1-NBS-thioredoxin, thioredoxin with one tryptophan residue modified to oxindole with *N*-bromosuccinimide.

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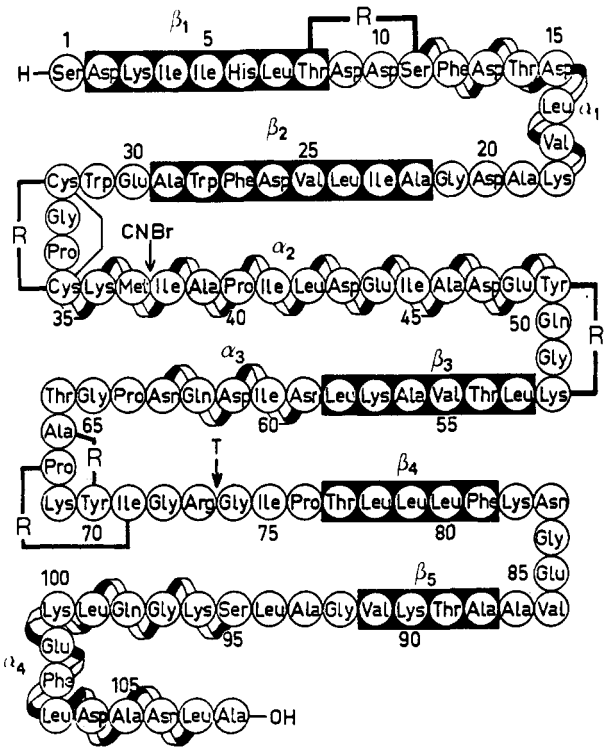

FIGURE 1: Amino acid sequence and secondary structure of *E. coli* thioredoxin-S_2 from X-ray crystallography at 2.8-Å resolution (Holmgren et al., 1975).

sulfide bridge, Cys-32 to Cys-35 (Holmgren, 1968), that occurs on a unique protrusion in the three-dimensional structure of the molecule (Holmgren et al., 1975, see Figure 1).

The role of the tryptophan residues of thioredoxin has been studied with chemical modification (Holmgren, 1972a, 1973). Modification of both tryptophans destroyed the biological activity of the molecule. Tritration experiments with *N*-bromosuccinimide at pH 4.0 showed a proportional destruction of the two tryptophan residues; complete oxidation required a 6-fold molar excess of reagent (Holmgren, 1973). A differential reactivity of the two residues was, however, suggested from the biphasic loss of enzymatic activity and fluorescence of thioredoxin-$(SH)_2$. Thus, treatment of thioredoxin with a 3-fold molar excess of *N*-bromosuccinimide resulted in oxidation of half of the tryptophans to oxindolylalanine (1-NBS-thioredoxin) (Holmgren, 1973). The fluorescence quantum yield of 1-NBS-thioredoxin-$(SH)_2$ was almost the same as that of the native thioredoxin-$(SH)_2$, strongly suggesting that a nonfluorescent tryptophan residue had been converted to oxidolylalanine.

This paper describes the identification of the modified residue in 1-NBS-thioredoxin. The position of the tryptophan residues in the amino acid sequence of thioredoxin makes it difficult to obtain peptides for reliable sequence analyses, particularly since the character of the tryptophan indole is lost after oxidation with *N*-bromosuccinimide. For quantitative analyses, thioredoxin-S_2 was selectively tritiated in its tryptophan residues by the method of Holt et al. (1971) in order to permit identification of all indole- or oxindole-containing peptides.

Experimental Procedures

Materials. Thioredoxin-S_2 and thioredoxin reductase from *E. coli* B were homogeneous preparations obtained as described (Holmgren & Reichard, 1967; Thelander, 1967). Tritiated water (5 Ci/mL) was obtained from the Radiochemical Centre (Amersham, England). *N*-Bromosuccinimide was a product of Fluka AG and was recrystallized before use. Trifluoroacetic anhydride was from Pierce, Eurochemie, Rotterdam. Other materials were as described (Holmgren, 1973; Hall et al., 1971).

Tritiation of Thioredoxin. The method of Holt et al. (1971) was used. Tritiated trifluoroacetic acid was prepared by addition of an equimolar amount of tritiated water (5 mCi/mL) to trifluoroacetic anhydride. Thioredoxin (12 mg) was dissolved in 0.75 mL of tritiated trifluoroacetic acid and incubated for 15 min at 25 °C in a stoppered tube. After dilution with 4 mL of water, the mixture was lyophilized. The dry protein was lyophilized once more from H_2O before it was dissolved in 0.06 M NH_4HCO_3 at 6 mg/mL. The radioactivity was determined with a Packard Tricarb liquid scintillation spectrometer.

Measurement of Enzymatic Activity of [^{3}H]Thioredoxin. The activity of thioredoxin with thioredoxin reductase was determined by coupling the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (Slabý & Holmgren, 1975). The K_m for native and tritiated thioredoxin with thioredoxin reductase was determined by the method of Moore et al. (1964).

Spectrophotometric Determinations. Protein was determined by measuring the absorbance at 280 nm in a Zeiss PMQII spectrophotometer or by amino acid analysis (Holmgren, 1973).

N-Bromosuccinimide Oxidation. [^{3}H]Thioredoxin-S_2 (1.35 μmol) was dissolved in 15 mL of 0.10 M sodium acetate, pH 4.0, at 25 °C and rapidly mixed with 450 μL of freshly prepared 10 mM *N*-bromosuccinimide (Holmgren, 1973). This resulted in oxidation of 1.05 residues of tryptophan as calculated from the ΔA_{280nm} (Holmgren, 1973). The 1-NBS-thioredoxin had a A_{260}/A_{280} ratio of 1.06 as compared to 0.58 in thioredoxin-S_2. The modified protein was reduced with 100 μL of 2-mercaptoethanol for 2 h at 37 °C and aminoethylated by addition of 220 μL of ethylenimine. The mixture was incubated for 30 min at room temperature and the protein then freed from excess reagent on a column of Sephadex G-25 (150 mL) in 0.6 M NH_4HCO_3 and lyophilized. The *N*-bromosuccinimide oxidation and reduction plus aminoethylation resulted in a loss of 36% of the ^{3}H from thioredoxin.

Digestion with Chymotrypsin. Aminoethylated 1-NBS-thioredoxin (1.1 μmol) was dissolved in 1.0 mL of 0.06 M NH_4HCO_3 and digested with 300 μg of chymotrypsin for 7 h at 37 °C. The digest was concentrated to 0.75 mL by lyophilization and applied to a column of Sephadex G-25 (0.9 × 140 cm) equilibrated with 0.06 M NH_4HCO_3. Four fractions were isolated on the basis of ^{3}H content and 280-nm absorbance.

Peptide Separations. High-voltage paper electrophoresis was carried out as described previously at pH 6.5 and 2.0 (Hall et al., 1971). Peptides were eluted from paper by 0.10 M NH_4OH.

Amino Acid Analysis. Peptides were hydrolyzed for 24 h at 110 °C in 1.0 mL of 6 M HCl in carefully evacuated and sealed tubes. Quantitative amino acid analyses were performed with a Spinco 120B amino acid analyzer equipped with high sensitivity cuvettes.

NH_2-Terminal Sequence Analysis. The NH_2-terminal sequence of peptides was determined by the dansyl–Edman technique as described in detail previously (Hall et al., 1971).

Results

Tritiation of Thioredoxin. Treatment of thioredoxin-S_2 for 15 min with trifluoroacetic [^{3}H] acid gave incorporation of 3.4 mol of nonexchangeable tritium per mol of protein. The tritiated thioredoxin-S_2 had an essentially unchanged ultra-

Table I: Specificity of Labeling of Tryptophan Residues in Thioredoxin by Tritiated Trifluoroacetic Acid

	sp act. (cpm/nmol)
thioredoxin	2600
thioredoxin-C(1–37)	2750
peptide P6 (Trp_{28}–Met_{37})[a]	2550

[a] Met_{37} is homoserine in the peptide.

Table II: Properties of Fractions from Sephadex G-25 Chromatography of Chymotryptic Digest of 1-NBS-thioredoxin

	fraction			
	I	II	III	IV
total cpm	57 000	193 000	46 800	104 000
A_{260}/A_{280}	1.12	1.03	1.96	0.79

violet spectrum but contained only 79% of disulfide reducible with NADPH and thioredoxin reductase as compared to thioredoxin-S_2. Chromatography of the [^{3}H]thioredoxin on Sephadex G-50 resulted in separation of two peaks. Only the main second peak, which was eluted in the same position as native thioredoxin (K_d = 0.5) (Holmgren & Reichard, 1967), was enzymatically active. The first peak appeared to be an inactive dimer of thioredoxin. This material was not used in the present experiments. The tritiated thioredoxin showed a K_m of 6.3 μM with thioredoxin reductase as compared to 5 μM for untreated thioredoxin. The V_{max} was identical.

Location of Tritium Label in Thioredoxin. Tritiated thioredoxin containing 2600 cpm/nmol was cleaved with CNBr, and the tryptophan-containing peptide thioredoxin-C-(1–37) (Holmgren & Slaby, 1979) was isolated by chromatography in 50% acetic acid (Holmgren & Reichard, 1967). After reduction and aminoethylation, peptide-C(1–37) was digested with pepsin, and a tryptophan-containing peptide, P6 (Holmgren et al., 1968), was isolated by paper electrophoresis. As shown in Table I, the total radioactivity per mole of peptide was the same for thioredoxin, thioredoxin-C(1–37), and peptide P6. This observation shows that all the radioactive label was confined to residues 28–37 (i.e., peptide P6), and it is reasonable to conclude that the two tryptophans in this sequence are the sites of selective labeling.

Location of Oxindolylalanine in 1-NBS-thioredoxin. The addition of 3 mol of *N*-bromosuccinimide to [^{3}H]thioredoxin-S_2 oxidized 1.05 mol of tryptophan. The calculated tryptophan fluorescence quantum yield of 1-NBS-thioredoxin-S_2 was 50% of that of native thioredoxin-S_2 (Holmgren, 1973) whereas the corresponding fluorescence for 1-NBS-thioredoxin-$(SH)_2$ was 92% of that of native thioredoxin-$(SH)_2$. The reduced and aminoethylated 1-NBS-thioredoxin was digested with chymotrypsin, and four main peaks were separated by chromatography on Sephadex G-25 (Figure 2). The characteristics of the four fractions are given in Table II. The material of fraction I was not studied further. Its A_{260}/A_{280} ratio and elution volume indicated that it contained undigested protein. Fractions II, III, and IV were further purified by paper electrophoresis (Table III).

Fraction IV. This contained only one ^{3}H-labeled peptide which also gave a positive Ehrlich stain. The amino acid composition of this peptide and its NH_2-terminal residue was consistent with the sequence of residues 25–28, Val-Asp-Phe-Trp.

Fraction III. This contained only one ^{3}H-labeled peptide which was not staining with Ehrlichs reagent, indicating that it contained a residue of oxindolylalanine. The amino acid

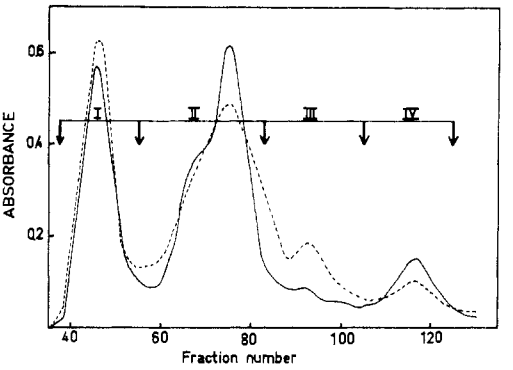

FIGURE 2: Separation of a chymotryptic digest of aminoethylated 1-NBS-thioredoxin by chromatography on Sephadex G-25 (140 × 0.9 cm) equilibrated with 0.06 M NH_4HCO_3. Fractions of 0.75 mL were collected every 20 min. Pools were taken as indicated by the arrows. A_{280} (—); A_{260} (- - -).

Table III: Amino Acid Composition of Peptides Isolated from Chymotryptic Digest of 1-NBS-thioredoxin

amino acid	peptide		
	IIa	III	IV
aspartic acid	1.22 (1)		1.08 (1)
glutamic acid		1.16 (1)	
alanine		1.00 (1)	
valine	0.94 (1)		0.96 (1)
phenylalanine	0.85 (1)		0.93 (1)
tryptophan	1[a]	*b*	1[a]
purification by electrophoresis at pH	6.5, 2.0	6.5, 2.0	6.5, 2.0
mobility	−0.43	−0.50	−0.39
yield (%)	20	16	20
NH_2 terminal	Val	Ala	Val

[a] Determined spectrophotometrically; also from positive Ehrlich stain. [b] Contains oxindolylalanine spectrophotometrically.

composition and NH_2-terminal residue showed that it contained residues 29–31, Ala-Glu-Trp, and that Trp-31 was in the form of oxindolylalanine.

Fraction II. On paper electrophoresis, this fraction yielded several labeled bands. All Ehrlich-positive material was recovered in a broad, weakly acidic band that was eluted and redigested with chymotrypsin. This generated an Ehrlich-positive peptide (m − 0.44) that was obtained pure by paper electrophoresis (Table III). Its amino acid composition and NH_2-terminal residue showed that it came from the sequence 28–31, Val-Asp-Phe-Trp, and thus was equivalent to the peptide in fraction II.

Discussion

The results of the sequence analysis of 1-NBS-thioredoxin-S_2 showed that the tryptophan-containing peptides were derived from the sequence Val-Asp-Phe-Trp, indicating that Trp-28 was not modified by *N*-bromosuccinimide. Furthermore, the peptide Ala-Glu-Trp containing Trp-31 was isolated and shown to have a residue of oxindolylalanine instead of tryptophan. These results demonstrate that *N*-bromosuccinimide at pH 4.0 specifically oxidizes Trp-31 in thioredoxin.

The selective tritiation of the tryptophan residues of thioredoxin was of major help in identifying the *N*-bromosuccinimide modified residue. Thus, isolation of all labeled peptides ensured that no oxindole-containing peptides escaped detection during the isolation procedure.

The tryptophan emission in 1-NBS-thioredoxin (Holmgren, 1973) must be attributed to Trp-28 since oxindole lacks fluorescence. Both tryptophan residues of native and active

thioredoxin-S_2 have a strongly quenched fluorescence; the active center disulfide has been strongly implicated as the quencher (Holmgren, 1972b). Upon reduction of the disulfide to the dithiol in thioredoxin-$(SH)_2$, a 3-fold increase in fluorescence per tryptophan takes place. The similar tryptophan emission of 1-NBS-thioredoxin-$(SH)_2$ and thioredoxin-$(SH)_2$ (Holmgren, 1973) is consistent with a 6-fold increase in quantum yield for Trp-28. Thus, Trp-28 signals the conformational change in thioredoxin accompanying reduction of the active-center disulfide.

The environments of Trp-28 and Trp-31 are quite different, as seen from the X-ray structure of thioredoxin-S_2, although both are located on the surface of the protein (Holmgren et al., 1975). As shown in Figure 1, Trp-31 is located in the active-site protrusion; its side chain appears free and has no intramolecular interactions. In contrast, Trp-28 is part of a β-pleated sheet (β_2) and is partly shielded. These locations agree with the initial modification of Trp-31 and the apparently small effects on the structure of thioredoxin-S_2 given by modification of Trp-31 to oxindolylalanine. Proton nuclear magnetic resonance spectra of thioredoxin in H_2O led to the identification of two resonance peaks from the indole NH proton of the two tryptophan residues of the molecule (Holmgren & Roberts, 1976). Upon reduction of oxidized thioredoxin, only one of these two peaks showed a major change in chemical shift while both peaks shifted as a function of pH.

The fluorescence quantum yield of *E. coli* thioredoxin-$(SH)_2$ is strongly pH dependent with a maximum at pH 5 (Holmgren, 1972b). The increase follows an apparent titration curve for a group with a pK of ~6.75 (Holmgren, 1972b), suggested to be a cysteinyl residue. The two cysteines in thioredoxin-$(SH)_2$, Cys-32 and Cys-35, have different reactivities toward iodoacetic acid (Kallis & Holmgren, 1980); only Cys-32 is modified at pH 7, and its pK value has been determined to be 6.7 (Kallis & Holmgren, 1980). Thus, an interaction of Cys-32 and Trp-28 is suggested as an explanation of the fluorescence behavior of thioredoxin-$(SH)_2$.

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Selective Inactivation of Lactate Dehydrogenase Isoenzymes with Ionic Surfactants†

Karl J. Sanford,* Donna J. Meyer, Michael J. Mathison, and John Figueras

ABSTRACT: Ionic surfactants selectively inactivate porcine lactate dehydrogenase (LDH) isoenzymes in 30 mM phosphate buffer, pH 7.4. The cationic surfactants hexadecylpyridinium bromide and hexadecyltrimethylammonium bromide rapidly inactivate LDH isoenzymes containing the B subunit; inactivation of LDH-A_4 is slower and also retarded by the cofactor reduced nicotinamide adenine dinucleotide. The anionic surfactants sodium decyl sulfate and sodium dodecyl sulfate rapidly inactivate LDH isoenzymes containing the A subunit; inactivation of LDH-B_4 is much slower and retarded by the cofactor. The selectivity of the inactivation process correlates with electrostatic interactions: positively charged surfactants preferentially inactivate isoenzymes containing a subunit of net negative charge, and negatively charged surfactants preferentially inactivate isoenzymes containing a subunit of net positive charge. Inactivation takes place near the critical micelle concentration for the cationic surfactants. Inactivation with anionic surfactants occurs above the critical micelle concentration. The cationic surfactants show little discrimination among LDH-B_4 and the hybrid isoenzymes, AB_3, A_2B_2, and A_3B, inactivating all at approximately the same surfactant concentration. The anionic surfactants, however, show a more graded inactivation–concentration profile with discrete differences in threshold surfactant concentrations required for complete inactivation of the four A subunit containing isoenzymes. At a particular surfactant concentration, loss in activity can be correlated with the percent A- or B-subunit composition of the isoenzyme.

The interaction of surfactants with proteins has been the subject of numerous investigations over the past several decades, and the topic has been reviewed by Steinhardt & Reynolds (1969), Tanford (1973), Waehneldt (1975), and Steinhardt (1975). Much of the interest in this area was generated because protein–surfactant systems can serve as models for the study of interactions between membrane proteins and

† From the Research Laboratories, Eastman Kodak Company, Rochester, New York 14650. *Received May 14, 1980.*

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