

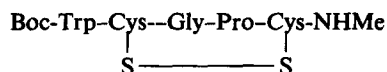
A fluorescent peptide model for the thioredoxin active site

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A synthetic model for the active site of the protein thioredoxin has been synthesized:



corresponding to residues 31–35 of the protein and possessing the 14-membered disulfide loop and a fluorescent chromophore. Dithiothreitol reduction of the disulfide bond results in a 50–60% enhancement of Trp fluorescence. The rate of reduction is solvent dependent, following the order 8 M urea >> methanol > water. The spectral changes observed in the model peptide are compared with those reported for the native protein. Circular dichroism studies suggest a substantial change in peptide backbone conformation, on disulfide reduction.

Thioredoxin active site

Peptide disulfide

Fluorescent peptide

Tryptophan fluorescence

1. INTRODUCTION

Thioredoxin is a redox protein which functions via a reversible disulfide–dithiol reaction [1,2]. Its active site consists of a 14-membered disulfide loop between residues Cys-32 and Cys-35 (fig. 1), which forms a protrusion on the surface of the molecule [3]. In the *Escherichia coli* enzyme, 2 Trp residues are present at positions 28 and 31 [4], while in the yeast enzyme there is only a single Trp at position 31 [5]. The fluorescence of these Trp residues has been used to probe the structural changes which

occur on reduction of the disulfide bridge [6]. Extensive studies of the fluorescence of the native protein and *N*-bromosuccinimide modified derivatives have established that the fluorescence of both Trp residues in *E. coli* thioredoxin is strongly quenched by the S–S bridge [7]. Reduction results in a large increase in the fluorescence of Trp-28 but has little effect on Trp-31 [7]. As part of a program to examine the effect of disulfide bonds on Trp fluorescence and to provide a model for the thioredoxin active site, we describe in this report spectroscopic studies on the synthetic peptide:

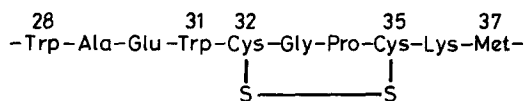
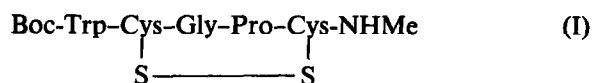


Fig. 1. Partial sequence in the vicinity of the active site of *E. coli* thioredoxin [4].



Fluorescence and CD spectral changes accompany reduction of the disulfide bridge.

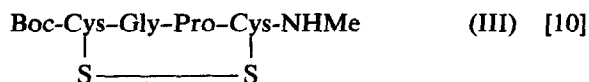
Abbreviations: Bzl, benzyl; DTT, dithiothreitol; NATA, *N*-acetyl-L-tryptophanamide; MeOH, methanol

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2. MATERIALS AND METHODS

All peptides were synthesized by solution phase procedures: (I) was prepared by a 2 + 3 coupling of

Boc-Trp-Cys(SBzl)-OH⁺ to Gly-Pro-Cys(SBzl)-NHMe, with dicyclohexylcarbodiimide-1-hydroxy benzotriazole to yield Boc-Trp-Cys(SBzl)-Gly-Pro-Cys(SBzl)-NHMe (II). Removal of the *S*-benzyl groups by reductive cleavage using Na/liquid NH₃ followed by oxidative cyclization under high dilution conditions in aqueous K₃Fe(CN)₆ yielded crude (I). Silica gel chromatography afforded (I) as a homogeneous solid in ~5% yield. Detailed synthetic protocol for the preparation of model 14-membered cyclic peptide disulfides [8,9] and:



have been reported. All peptides used in this study were purified by silica gel column chromatography and fully characterized by 270 MHz ¹H-NMR. Full procedures for the preparation of peptides I and II will be presented elsewhere.

N-Acetyltryptophanamide (NATA), L-Trp and dithiothreitol (DTT) were obtained from Sigma Chemical Co. USA. Fluorescence spectra were recorded on a Perkin-Elmer Model MPF-44A Fluorescence Spectrometer, with 5 nm excitation and emission band pass and an excitation wavelength of 290 nm. Absorbance at 290 nm of all solutions was adjusted to 0.1 corresponding to 22.1 μM Trp (peptide) concentration. Reductions were carried out using a final concentration of 500 μM DTT in MeOH, H₂O and 8 M urea. Relative quantum yields were estimated with respect to L-Trp for which a value of 0.14 is reported [11].

CD spectra were recorded on a JASCO J-20 Spectropolarimeter using cells of 1 mm path-length. Intensities are expressed as molar ellipticities [θ]_M deg.cm².dmol⁻¹.

3. RESULTS AND DISCUSSION

Fig. 2a shows the fluorescence spectrum of I in MeOH and the effect of addition of dithiothreitol (DTT). A clear *increase* in the Trp fluorescence is noted on reduction of the S-S bond. In the appropriate control experiments (fig. 2) addition of DTT to *N*-acetyl-tryptophanamide (Ac-Trp-NH)₂ or the acyclic precursor peptide Boc-Trp-Cys(SBzl)-Gly-Pro-Cys(SBzl)-NHMe (II) resulted only in a small *decrease* in emission intensity. This is due to a dilu-

tion effect and presumably also a consequence of quenching by the excess thiol. DTT reduction of the disulfide was also monitored in water (fig. 2b) and 8 M urea. The time course of the reactions in these solvents is shown in the inset to fig. 2. The rate of disulfide reduction follows the order 8 M urea >> MeOH > water. The quantum yields relative to L-Trp measured for peptides I, II and Ac-Trp-NH₂, under different conditions are summarized in table 1. The intrinsic quantum yield for the Trp chromophore is significantly higher in MeOH and 8 M urea as compared to water. Further, thiol quenching on addition of DTT is larger in MeOH and 8 M urea. Reduction of the disulfide bridge results in limiting intensity enhancements of 67% and 60% for MeOH and 8 M urea, respectively, whereas the corresponding increase in water is only 45%.

To establish structural changes on reduction of the disulfide loop, the CD spectra of the related peptide (III) were monitored in MeOH after addition of DTT. Peptide (III) was chosen to avoid interference from the aromatic sidechain of Trp, thereby permitting direct observation of the CD band due to the -S-Sθ *n*-σ* transition. Fig. 3 shows the CD spectra of III recorded at intervals after addition of DTT. The S-S band at ~280 nm is abolished on reduction. The peptide *n*-π* band at ~225 nm is observed on reduction. ¹H NMR studies suggest that III adopts a folded conformation with a Gly-Pro β-turn, stabilized by a transannular 4→1 hydrogen bond, accommodated within the 14-membered disulfide loop [10]. DTT reduction of the disulfide should destabilize this β-turn, resulting in a disordered conformation in the reduced product. This conclusion is supported by the CD spectral changes in fig. 3.

Disulfide quenching of the fluorescence of aromatic chromophores like Tyr and Trp in peptides and proteins is well established [12,13]. In *E. coli* thioredoxin, DTT reduction of the lone S-S bond between Cys-32 and Cys-35 results in a 3-fold enhancement of protein Trp fluorescence [6]. An average quantum yield of 0.02 was determined for the 2 Trp residues in the native protein. Using an *N*-bromosuccinimide modified derivative in which Trp 31 was selectively converted to non-fluorescent oxindolylalanine, Holmgren has established that Trp-31 is essentially completely quenched in the native protein [7]. Further, the increase in fluores-

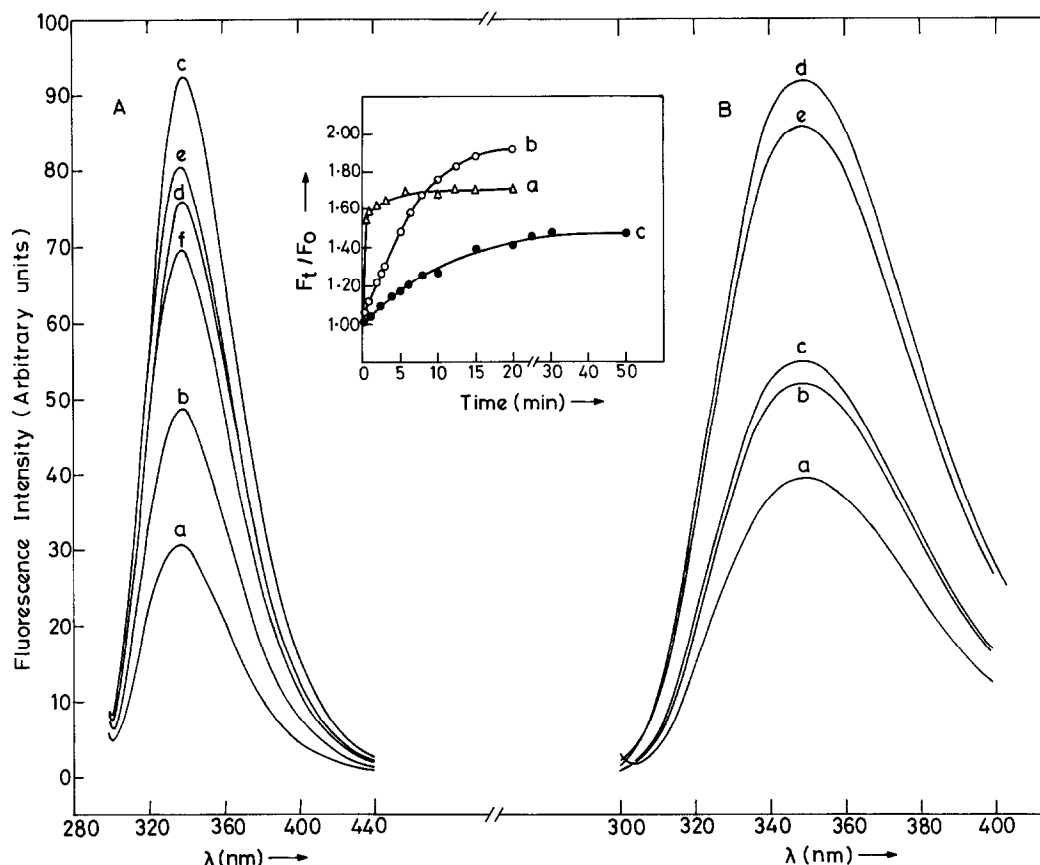


Fig. 2. Fluorescence emission spectra of (I) excited at 290 nm. (A) In methanol: (a) I; (b) I + DTT after 20 min; (c) NATA; (d) NATA + DTT; (e) II; (f) II + DTT. (B) In water: (a) I; (b) I + DTT after 20 min; (c) I + DTT after 50 min; (d) L-Trp; (e) L-Trp + DTT. Inset: time course of fluorescence increase normalised to initial fluorescence intensity: (a) in urea; (b) in methanol; (c) in water.

cence on disulfide reduction may be almost exclusively attributed to Trp-28, suggesting that even in the reduced form Trp-31 is quenched by other proximate groups or the vicinal dithiol. In a more recent study the contributions from the Tyr residues, 49 and 70, to the fluorescence of native, oxidized *E. coli* thioredoxin have been evaluated. While Tyr residues appear to contribute significantly to the protein emission in the oxidized form, the fluorescence of the reduced form is dominated by Trp [14].

In yeast thioredoxin which has only a single Trp at position 31, the fluorescence quantum yield is 0.03 and there is a 1.2-fold increase on reduction [6]. In an early study it was observed that in the CNBr fragment of *E. coli* thioredoxin containing

residues 1-37, including both Trp residues, a small increase in Trp quantum yield was observed on disulfide reduction [15]. In the active site model peptide [I], the fluorescence quantum yield of the Trp residue in water is higher than in the thioredoxins. An enhancement of 1.5-1.6-fold is observed on S-S reduction. This difference is undoubtedly because in the native protein the Trp-31 emission is quenched by neighbouring groups other than the S-S bond. The present report suggests that pentapeptide (I) can be used to model fluorescence changes that occur on reduction of the disulfide loop in thioredoxin. (I) may prove useful in evaluating solvent effects on disulfide reduction and in examining the stereochemical requirement for quenching of Trp emission by proximate S-S

Table 1
Quantum yields (ϕ)^a of peptides and NATA under different conditions

	Methanol		Water		8 M Urea	
	ϕ	% enhancement	ϕ	% enhancement	ϕ	% enhancement
NATA	0.315		0.168		0.273	
NATA + DTT	0.273	-13.3	0.161	-5.2	0.252	-7.7
II	0.273		b		b	
II + DTT	0.252	-7.7	b		b	
I	0.105		0.060		0.111	
I + DTT	0.175	+66.6	0.087	+46.8	0.178	+60
$(\phi_I + \text{DTT} / \phi_{\text{NATA} + \text{DTT}}) \times 100$		64		54		70

^a Relative quantum yields were determined using a value of 0.14 for L-Trp [11]

^b Peptide was not soluble

bridges. A more appropriate model for the thioredoxin active site should incorporate a second Trp residue. Studies in this direction are underway.

ACKNOWLEDGEMENT

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REFERENCES

- [1] Holmgren, A. (1981) *Curr. Topic Cell. Reg.* 19, 47-76.
- [2] Holmgren, A. (1981) *Trends Biochem. Sci.* 6, 26-28.
- [3] Holmgren, A., Söderberg, B.-O. Eklund, H. and Brändén, C.-I. (1975) *Proc. Natl. Acad. Sci. USA* 72, 2305-2309.
- [4] Holmgren, A. (1968) *Eur. J. Biochem.* 6, 475-484.
- [5] Hall, D.E., Baldesten, A., Holmgren, A. and Reichard, P. (1971) *Eur. J. Biochem.* 23, 328-335.
- [6] Holmgren, A. (1972) *J. Biol. Chem.* 247, 1992-1998.
- [7] Holmgren, A. (1981) *Biochemistry* 20, 3204-3207.
- [8] Venkatachalapathi, Y.V., Prasad, B.V.V. and Balaram, P. (1982) *Biochemistry* 21, 5502-5509.
- [9] Ravi, A., Prasad, B.V.V. and Balaram, P. (1983) *J. Am. Chem. Soc.* 105, 105-109.
- [10] Ravi, A. and Balaram, P. (1983) *Biochim. Biophys. Acta*, in press.
- [11] Wiget, P. and Luisi, P.L. (1978) *Biopolymers* 17, 167-180.
- [12] Cowgill, R.W. (1967) *Biochim. Biophys. Acta* 140, 37-44.
- [13] Chen, R.F. (1973) in: *Practical Fluorescence. Theory, Methods and Techniques* (Guilbault, G.G., ed.) pp. 467-541, Marcel Dekker, New York.
- [14] Reutimann, H., Straub, B., Luisi, P.L. and Holmgren, A. (1981) *J. Biol. Chem.* 256, 6796-6803.
- [15] Holmgren, A. and Reichard, P. (1967) *Eur. J. Biochem.* 2, 187-196.

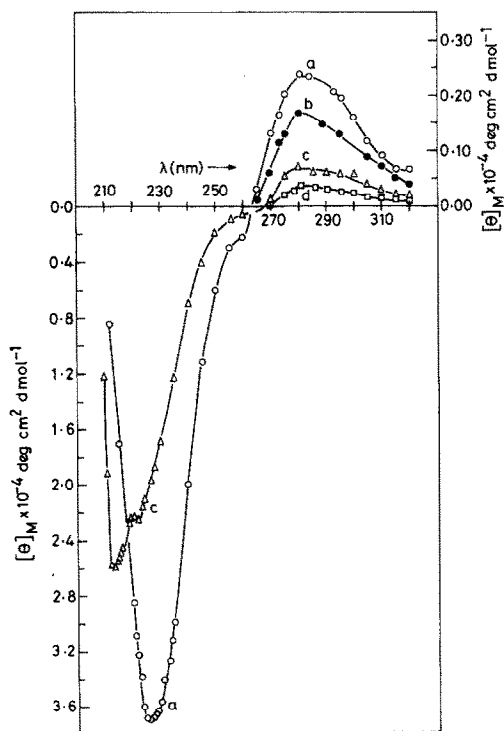


Fig. 3. CD spectra of (III) (2 mM) in methanol at different time intervals after adding DTT (2 mM). Left hand scale 210-260 nm; right-hand scale 260-320 nm: (a) without DTT addition; (b) 1 min after adding DTT; (c) 35 min after addition; (d) 1 h after addition.