Protein disulfide design

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Introduction

• Disulfides are one of the most prevalent long range covalent interactions in proteins

• Naturally occurring disulfides impart stability to the native folded form but the design of non-native disulfides to stabilize proteins has met with limited success

• Disulfides are also known to sense redox environment and act as conformational switches. Such switches having recently been implicated in the entry of HIV into target cells.
Fig: 1 (a) Definition of the various dihedral angles in a cystine disulfide bridge.
(b) Definition of interatomic distances used in geometrical fixing of S atoms.
Analysis of disulfide stereochemistry:
730 disulfides from 172 proteins
(a) $\chi^{ss}$

(b) $\chi^i$

(c) $\chi^2$
Flowchart for disulfide design
Structural correlates of disulfide induced stabilization

(a) Accessibility (%)
(b) Residue Depth (Å)
(c) Number of occurrences
(d) B-factor (Å²)
(e) Loop length (no. of residues)
(f) Δ Volume* (Å³)
Design of disulfide linked dimers and multimers of proteins based on analysis of crystal contacts

Possible intra-molecular disulfide bridges are predicted based on crystal structure of the protein.

Modeled disulfides are graded from A to C based on the stereochemistry of the disulfide bond.

**INTERMODIP:** modification of MODIP to predict intermolecular disulfide bridge formation was developed in the present studies.

Symmetry-related chains are generated based on the crystallographic symmetries present in the given space group.

Residue pairs from these chains in which at least two atoms are separated by 5Å or less are considered for intermolecular disulfide formation.

If the two new Cysteine residues are on the same face of the molecule, a dimer will be formed. If situated on opposite faces, a linear polymer will result.
Database Analysis

Database analysis carried out on 46 non homologous proteins from the PDB.

37 of these had one chain in the asymmetric unit while 9 had two.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th></th>
<th>B</th>
<th></th>
<th>C</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimer</td>
<td>18</td>
<td>Dimer</td>
<td>28</td>
<td>Dimer</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>Polymer</td>
<td>16</td>
<td>Polymer</td>
<td>36</td>
<td>Polymer</td>
<td>77</td>
<td></td>
</tr>
</tbody>
</table>

INTERMODIP predicted dimers in almost all the proteins studied.

It failed to find suitable sites for intermolecular disulfides only in two of the 46 proteins examined.
THIOREDOXIN

E. Coli thioredoxin consists of 108 amino acid residues and is a monomer in solution.

It is a protein disulfide reductase

Has an active site conserved sequence: Trp-Cys-Gly-Pro-Cys with a disulfide between Cys32 and Cys35.

Crystal structure has been solved. Two chains in the asym. unit.

Difficult to crystallize….requires almost a month for diffraction quality crystals to form.

INTERMODIP predicted the following intermolecular disulfides for E.coli thioredoxin using 2Trx as the PDB coordinates:

- T89C-T89C (Dimer)
- E101C-E101C (Dimer)
- E101C-A105C (Dimer)
- F102C-A105C (Dimer)
- D20C-R73C (Polymer)
- 98C-A105C (Dimer)
- N83C-E85C (Dimer)
Predicted sites for mutation in thioredoxin

- D20
- R73
- T89
- E101
- A105
- F102
Dimer and polymer formation of the thioredoxin mutants

Monomer and dimer were separated by gel filtration chromatography through Sephadex G-50

SDS-PAGE (15%)
Lane 1: E101C/A105C
Lane 2: E101C

Multimers of D20C/R73C
Thermodynamic parameters from isothermal GdmCl melt and DSC

These experiments were done at pH 7.0
Crystal structures of mutant dimers

Thioredoxin is difficult to crystallize – takes over a month

Mutant dimers crystallized in 4-7 days

All crystals obtained by hanging drop vapor diffusion method

E101C dimer crystallized under new conditions, lacking copper

Crystal structures compared with predicted dimer structures obtained by running INTERMODIPAROUND

<table>
<thead>
<tr>
<th>Parameter</th>
<th>T89C</th>
<th>E101C</th>
<th>E101C/A105C</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray source</td>
<td>PF-AR NW-12A</td>
<td>PF-AR NW-12A</td>
<td>FR-D</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.5418</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cryo protectant</td>
<td>none</td>
<td>40% PEG400</td>
<td>none</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>54.6 – 1.90 (1.97 – 1.90)</td>
<td>38.6 – 2.60 (2.69 – 2.60)</td>
<td>33.5 – 2.50 (2.59 – 2.50)</td>
</tr>
<tr>
<td>Space group</td>
<td>C2</td>
<td>P2_12_1</td>
<td>P2_1</td>
</tr>
<tr>
<td>a (Å)</td>
<td>89.2</td>
<td>101.7</td>
<td>50.1</td>
</tr>
<tr>
<td>b (Å)</td>
<td>49.6</td>
<td>59.2</td>
<td>51.0</td>
</tr>
<tr>
<td>c (Å)</td>
<td>59.4</td>
<td>75.1</td>
<td>89.0</td>
</tr>
<tr>
<td>α (°)</td>
<td>90.0</td>
<td>90.0</td>
<td>90.0</td>
</tr>
<tr>
<td>β (°)</td>
<td>113.3</td>
<td>90.0</td>
<td>90.4</td>
</tr>
<tr>
<td>No. of observed reflections</td>
<td>69983 (6981)</td>
<td>104396 (10373)</td>
<td>49214 (1829)</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>18976 (1887)</td>
<td>14514 (1421)</td>
<td>15784 (1076)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.7 (99.9)</td>
<td>99.9 (100.0)</td>
<td>95.4 (70.0)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>8.3 (30.4)</td>
<td>8.1 (25.8)</td>
<td>9.3 (35.7)</td>
</tr>
<tr>
<td>Rwork (%)</td>
<td>16.1 (3.2)</td>
<td>24.3 (6.7)</td>
<td>9.0 (1.2)</td>
</tr>
<tr>
<td>No. of monomer in asymmetric unit</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>122</td>
<td>57</td>
<td>41</td>
</tr>
<tr>
<td>B (Å²)</td>
<td>18.4</td>
<td>23.1</td>
<td>23.2</td>
</tr>
<tr>
<td>RMSD (%)</td>
<td>21.8</td>
<td>29.2</td>
<td>31.1</td>
</tr>
<tr>
<td>RMSD bonds (Å)</td>
<td>0.017</td>
<td>0.006</td>
<td>0.011</td>
</tr>
<tr>
<td>RMSD angles (°)</td>
<td>1.58</td>
<td>1.30</td>
<td>1.45</td>
</tr>
</tbody>
</table>
Clear electron density maps for the intermolecular disulfide bonds confirm the formation of the engineered dimers.

All the Cys residues in the dimers (active site) are fully oxidized as evident from mass spectrometry and iodoacetamide and MMTS labeling.
E101C/A105C — model
— expt
E101C — model
— expt
Summary

- It is possible to engineer specific intermolecular disulfide bonds to create dimers in proteins that are normally monomeric in solution.

- For *E. coli* thioredoxin, 5 mutants were constructed, expressed, purified and characterized. Four formed dimers and one formed polymers as predicted.

- Three of these had similar stability to WT and their structures were solved by X-ray crystallography.

- In all three cases dimerization greatly facilitated crystallization.

- The E101C/A105C and T89C dimers had structures very similar to those predicted. E101C had a change in the relative orientation of the monomers compared to that predicted.

- If the mutant Cys residues are on the same face of the molecule, dimers result, whereas if they lie on opposite faces, multimers result.

- For virtually any protein there are multiple sites where intermolecular disulfide bonds can be engineered.

Inter-strand disulfides, their stability and their role in protein stabilization
*E. coli* thioredoxin
Hydrogen bonded and non-hydrogen bonded pairs in adjacent strands of anti-parallel beta sheets
Analysis of disulfides occurring between adjacent antiparallel strands of beta sheets in proteins (bridging disulfides)

- Bridging disulfides are not strained in terms of conformation

- Only one type of rotamer is found in these disulfides. This is a right-handed rotamer with $\chi_{ss} \approx +90^0$. These disulfides have a preference to occur at edge strands

- They are formed only between the non-hydrogen bonded pairs of adjacent strands of anti-parallel beta sheet
Why are bridging disulfides not found at the hydrogen bonded pairs?

**Possible Reasons**

1. Disulfide bond formation is stereochemically disallowed at such positions.

2. These disulfides induce strain in the protein structure and hence destabilize the protein. Hence, they have been deselected during evolution.
Experimental Validation

Thioredoxin was chosen as a model system

- This protein is well characterized in terms of stability and folding thermodynamics and is expressed in high yields

- Has an antiparallel beta sheet in which one of the strands is an exposed edge strand.
Design of Disulfide mutants in Thioredoxin

Red: Hydrogen bonded pair
Cyan: Non-hydrogen bonded pair
Bridging disulfides can be designed at hydrogen bonded and non-hydrogen bonded pairs in adjacent beta strands.

Disulfides formed at hydrogen bonded positions destabilize the protein whereas the disulfides at non-hydrogen bonded positions stabilize the protein.
Disulfides at non-hydrogen bonded positions increase the thermal stability of Thioredoxin whereas the disulfide at a hydrogen bonded position decreases its thermal stability
Engineered bridging sheet disulfides in other proteins

<table>
<thead>
<tr>
<th>protein</th>
<th>N-term res</th>
<th>C-term res</th>
<th>registered pair type</th>
<th>%accessibility</th>
<th>∆C\textsubscript{m} (M)</th>
<th>∆T\textsubscript{m} (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIVBP247-331</td>
<td>247T</td>
<td>331V</td>
<td>NHB</td>
<td>0.4</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td>LBP245-335</td>
<td>245L</td>
<td>335Q</td>
<td>NHB</td>
<td>7.2</td>
<td>47.9</td>
<td>-0.5</td>
</tr>
<tr>
<td>LBP247-333</td>
<td>247T</td>
<td>333V</td>
<td>NHB</td>
<td>0.5</td>
<td>5.9</td>
<td>-0.7</td>
</tr>
<tr>
<td>MBP110-261</td>
<td>110V</td>
<td>261V</td>
<td>NHB</td>
<td>0.1</td>
<td>0</td>
<td>-0.2</td>
</tr>
<tr>
<td>LBP245-335\textsubscript{red}</td>
<td>245L</td>
<td>335Q</td>
<td>NHB</td>
<td>7.2</td>
<td>47.9</td>
<td>-0.5</td>
</tr>
<tr>
<td>LBP247-333\textsubscript{red}</td>
<td>247T</td>
<td>333V</td>
<td>NHB</td>
<td>0.5</td>
<td>5.9</td>
<td>Destabilised</td>
</tr>
<tr>
<td>MBP110-261\textsubscript{red}</td>
<td>110V</td>
<td>261V</td>
<td>NHB</td>
<td>0.1</td>
<td>0</td>
<td>-1.0</td>
</tr>
</tbody>
</table>

Green: NHB disulfide mutant with at least one residue exposed  
Black: NHB disulfide mutant with both residues buried  
Red: No disulfide formation observed.
Conclusions

- Bridging disulfides can be formed at both hydrogen bonded and non-hydrogen bonded positions.

- The designed bridging disulfides at hydrogen bonded positions destabilize the protein.

- Bridging disulfides at non-hydrogen bonded positions can potentially be used to improve protein stability, provided at least one of the WT residues (before mutation) is solvent accessible.
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