Conformational changes in redox pairs of protein structures

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Abstract: Disulfides are conventionally viewed as structurally stabilizing elements in proteins but emerging evidence suggests two disulfide subproteomes exist. One group mediates the well-known role of structural stabilization. A second redox-active group are best known for their catalytic functions but are increasingly being recognized for their roles in regulation of protein function. Redox-active disulfides are, by their very nature, more susceptible to reduction than structural disulfides; and conversely, the Cys pairs that form them are more susceptible to oxidation. In this study, we searched for potentially redox-active Cys Pairs by scanning the Protein Data Bank for structures of proteins in alternate redox states. The PDB contains over 1134 unique redox pairs of proteins, many of which exhibit conformational differences between alternate redox states. Several classes of structural changes were observed, proteins that exhibit: disulfide oxidation following expulsion of metals such as zinc; major reorganisation of the polypeptide backbone in association with disulfide redox-activity; order/disorder transitions; and changes in quaternary structure. Based on evidence gathered supporting disulfide redox activity, we propose disulfides present in alternate redox states are likely to have physiologically relevant redox activity.

Keywords: disulfide redox activity; thiol-based redox signaling; OxyR; oxidative stress; CLIC1; zinc signaling; disordered proteins; molten globule

Introduction
Emerging evidence supports the concept of two distinct types of disulfides in protein structures which have different functional roles.1 The best known group act as structural stabilizers in proteins, significantly lowering the Gibb’s free energy of the protein chain by crosslinking it, resulting in proteins that are more resistant to denaturation. The second group are redox-active and best known for their catalytic role in proteins such as thiol-disulfide oxidoreductases. An increasing role for the redox-active group in protein redox regulation is emerging.1,2 These disulfides act as redox-sensitive switches and are best viewed as post-translational modifications akin to phosphorylation. It has now been established that oxidative regulation: important in processes such as the cell cycle, apoptosis, and response to oxidative stress; is an important control mechanism in the cytosol. Transient oxidation mediates control of enzymes, signaling pathways, as well as transcriptional and translational control. However, these characterized cytosolic proteins likely only represent the tip of the iceberg. Increasingly, reduction of disulfides is also emerging as a key regulator of protein function in oxidizing environments.3–5 Importantly, ageing and several major diseases including...
neurodegenerative diseases such as Alzheimer’s disease, type II diabetes, cancer, and cardiovascular disease have been associated with abnormal redox conditions.

Structural and redox-active disulfides can be distinguished by their redox potentials. Disulfide redox potentials measured in thiol-disulfide oxidoreductases range from $-120$ to $-270$ mV.\textsuperscript{5–9} For disulfides serving structural purposes, the redox potential can be as low as $-470$ mV.\textsuperscript{10} Computational methods for distinguishing the two groups would be a boon. Reactivity of a redox-active Cys is modulated by its immediate protein environment. In addition to the effects of the electrostatic environment, physical stress applied to a disulfide bond both through stretching and twisting enhances its reactivity. These forces can be applied to a protein dynamically\textsuperscript{11} or may be captured during the enhanced reactivity. These forces can be applied to the redox-active disulfides are more susceptible to cleavage/oxidation under nonphysiologic conditions. For instance, redox-active disulfides are prone to cleavage by synchrotron radiation during the process of X-ray structure determination.\textsuperscript{13–15} Other factors such as the pH of crystallization or the presence of mutations that change the electrostatic environment near a disulfide may also influence reactivity. Here, we mined the Protein Data Bank (PDB)\textsuperscript{16} for highly similar proteins that have been solved in multiple redox states, that is, disulfide-bonded in one structure and reduced in another. We present evidence that most of the disulfides present in our Redox Pairs dataset are likely to be physiologically redox-active. Many of the proteins have known thiol-based redox activity, while others have a high likelihood of thiol-based redox activity based on their involvement with redox processes. We also observed and classified types of conformational changes which occur during redox transitions.

Results

**Redox processes are enriched in the redox pair dataset**

The Redox Pairs dataset contains 18,003 pairs of protein structures consisting of 4333 protein chains, of which 1238 (28.57%) are high-resolution structures ($<2.2$ Å). Many structures are of the same protein solved in different redox environments. A filtered dataset consists of 322 unique protein groups clustered at 95% sequence identity.

Further evidence of disulfide redox activity was sought based on experimental data in the literature, association of protein function with known redox activity, and implication of the disulfide in redox activity by independent computational prediction methods. For 41 proteins, direct experimental evidence of redox activity for the particular disulfide was available (designated by “K” in Table S1, Supporting Information). The proteins belong to several major groups known to have redox activity. 11 unique Redox Pairs are associated with aerobic metabolism in eukaryotes or carbon fixation in plants (in groups D and E of Table S1); a further five are associated with maturation of proteins of these pathways (in groups E and G). Four Redox Pairs are associated with ion channel activity (group A): two of these, the chloride channel CLC\textsubscript{1} and Cdc25B regulate their resident protein’s function.\textsuperscript{21,22} 41 Redox Pairs (group d) are involved in transcriptional control, replication, and DNA synthesis including the oxidative stress response transcription factor OxyR\textsuperscript{23} and ribonucleotide reductases from several species in multiple redox states.\textsuperscript{24} 12 Redox Pairs generated or destroyed reactive oxygen species (Groups F and Y). The latter comprised three different peroxiredoxins\textsuperscript{25} as well as superoxide dismutases from several species.\textsuperscript{26} Four different amine oxidases generate peroxide while inducible nitric oxide synthase generates NO. Other large groups consist of proteins devoted to cell entry\textsuperscript{27} (Group b, 15 examples), cell adhesion (group c, nine examples), immunity (group f, 43 examples), apoptosis and protein fate (groups Z and a, 10 examples), and redox signaling (group X, 13 examples). A schematic of superfamilies is shown in Figure 1.

To further assess the likelihood that the presence of proteins in the PDB with disulfides in both redox states is an indicator of physiological disulfide redox activity, we looked for Gene Ontology (GO) terms overrepresented in the Redox Pairs dataset compared to the entire PDB. GO is a dynamic controlled vocabulary of over 16,000 terms used to describe molecular function, process and location of action of a protein in a generic cell.\textsuperscript{28} The analysis revealed many GO terms consistent with redox function, including cell redox homeostasis and multiple pathways of cysteine biosynthesis (Supporting Information Table 2). Interestingly, several of the enriched GO terms are linked to proteins mediating cell entry, including bacterial toxins and viral entry proteins. Reduction of disulfides, often accompanied by irreversible conformational changes, have previously been implicated as a general mechanism in cell entry proteins of diverse types.\textsuperscript{27} Experimental evidence directly supporting such a general process exists for HIV gp120, Newcastle disease virus, SARS coronavirus and Chlamydia.\textsuperscript{29–34} Thus the GO terms support redox roles for proteins in the Redox Pair dataset.
Disulfides were tested computationally for likely redox activity by screening the dataset for oxidized structures where the disulfide had particular properties previously associated with redox activity. Flagged disulfides were those (a) with high torsional energies; (b) found in known redox-active sequence motifs; or (c) which disobeyed known rules of protein stereochemistry. Studies of disulfide-bonds in model proteins have shown that those bonds with high torsional energies are more easily cleaved than disulfide-bonds with lower stored energy. 148 disulfides in 139 proteins have torsional energies $>$15 kJ mol$^{-1}$: a conservative threshold associated with redox activity. These are designated by “H” in Supporting Information Table 1. Redox-pair disulfides were also found in sequence motifs previously shown to be associated with redox activity. 67 proteins, designated by “M” in Table S1, contain known redox-active motifs such as the CXXC motif: a redox-active disulfide motif commonly found in the thioredoxin fold, but which has also arisen convergently in other folds. A further 65 proteins, designated by “F” in Table S1, contain disulfides in protein structures that disobey known rules of protein stereochemistry. These disulfides, which
we refer to as “forbidden” disulfides, conform to canonical structural motifs. Deformation of the resident structure enhances disulfide reactivity by inducing stress in the protein chain.\textsuperscript{37,38} Forbidden disulfides are proposed to have a functional role through redox activity.\textsuperscript{12} In summary, a total of 226 proteins in the Redox Pair dataset contain disulfides predicted to be redox-active by a second independent bioinformatic analysis method.

Nonetheless, it is possible that some proteins with alternate Cys-Pair redox states are non-native, that is, not encountered under normal, or abnormal, physiological conditions. We reasoned that proteins demonstrated selective reduction of disulfides were more likely to represent physiological states. Accordingly, for proteins with more than one disulfide, the dataset was partitioned into pairs where all the disulfides were reduced and those where some disulfides were selectively reduced while others remained intact, to further test the likelihood of involvement in physiological redox processes. The enriched GO terms for both the partially and fully reduced sets contained terms previously associated with thiol-based redox regulation indicating both the fully and partially reduced sets have likely redox activity (Supporting Information Table 2).

**Conformational changes associated with redox activity**

Having established the Redox-Pair dataset is enriched in proteins involved in redox-regulated processes, we studied conformational changes between redox pairs of proteins. For many proteins in the data set there were negligible conformational differences between the SG atoms of each Cys residue of the reduced and oxidized states (see Fig. 2). For this group, other factors may be required for, or preclude, conformational changes in these proteins. Crystal packing forces, for example, may prevent a subsequent conformational change after cleavage of the disulfide by synchrotron radiation. For another group, the small distance changes of 2–4 Å observed are most likely due to rotation of one or both Cys sidechains toward each other to form the S=S bond. Some of the proteins exhibiting these small conformational changes are oxidoreductases with catalytic thiols such as thioredoxin, thioredoxin reductase, and protein disulfide isomerase.

For proteins which exhibit major conformational changes, four classes were identified based on the nature of the change. These groups were: proteins that oxidize disulfides following expulsion of metals such as Zn; proteins that exhibited major reorganization or “morphing” of portions of the polypeptide backbone in association with disulfide redox-activity; proteins that exhibited order/disorder transitions; and proteins that exhibited changes in quaternary structure.

**Redox sites associated with zinc and other metals**

Redox regulation of proteins that form disulfides following expulsion of Zn is an emerging area of signaling.\textsuperscript{40} For example, reversible redox regulation of the Zn-binding site has recently been proposed for small Tim proteins of the mitochondrial intermembrane space (IMS).\textsuperscript{41} Small Tim proteins have an essential role in the transport of proteins into the IMS. These proteins can exist in both a reduced Zn-bound state.
and an oxidized disulfide-bridged state. Both states likely represent physiological forms of the proteins which are adopted in response to changing redox conditions in the IMS.41 In addition, expelled Zn acts as a second messenger to effect further cellular responses.

Proteins that form disulfides following expulsion of metals such as Zn were identified by searching for ligation of metals by Cys in reduced structures. A total of 73 redox pairs in 53 proteins were found (Table I, z in S2). Proteins identified include transcriptional regulators such as SMAD and anti-TRAP; transcriptional silencers such as SIRT2; and the enzymes of sulfur metabolism, BHMT, MetE, and MetH. Also notable were a number of RNA-interacting proteins including amino-acid-tRNA synthetases, RNA/DNA polymerases, ribonucleotide reductase and the ribosome. In addition to Zn, a few other bound metal atoms were represented including Fe, Ni, Cu. There were several examples of inorganic iron-sulfur (Fe-S) clusters associated with redox-active disulfides including: xanthine dehydrogenase, a major source of ROS in the failing heart; and components of respiratory complexes (Table I). Other proteins containing Fe-S clusters contained other redox-active disulfides that were not associated directly with the Fe-S cluster. All proteins containing Fe-S clusters (designated with "C" for cofactor) are listed in Table I regardless of whether the Fe-S cluster is directly associated with a labile dithiol pair or not.

Crystallographers typically differentiate two types of Zn sites: catalytic Zn sites, which in their simplest, mononuclear form have three amino-acid sidechains and one water molecule as ligands; and structural Zn sites, which have four amino acid ligands. From a redox point of view, a second variable - lability, is important. Some structural Zn sites expel Zn upon variation of physiological redox conditions, whereas others bind Zn tightly, and would not be expected to release Zn under physiological conditions. We refer to this first redox-regulated group as "labile" Zn sites; and the second, purely structural group, as "inert."

We wished to determine whether Zn sites associated with Redox Pair disulfides detected in the study were labile or inert. The number of Zn ligands and their identity are key indicators of Zn site lability. For catalytic mononuclear Zn sites, three amino acids, which are often His, ligate the Zn. In binuclear Zn sites there are usually six ligands, and the carboxylated residues Asp and Glu predominate over His.56-57 Cys is more commonly found as a ligand in structural Zn sites which generally have four ligands. On consideration of ligand type alone, S₄ sites - those with four Cys ligands, are the most labile type of structural Zn site, and are almost as reactive as a free thiol.58 Most of the structural Zn sites identified in this study were of the S₄ type (Table I), suggesting they are labile. Four catalytic Zn sites were also found in the dataset. These catalytic Zn sites are highly unusual because of their utilization of Cys as a ligand instead of the more usual His, Glu, or Asp. Two of the catalytic sites are within BHMT and MetH: enzymes of sulfur metabolism, which is redox-regulated in plants.6 Selection of sulfur ligands for Zn in these enzymes may confer an additional level of redox control on sulfur metabolism.

As an additional control, we checked whether metal atoms were expelled from oxidized structures. A metal atom is not present in over a third of the oxidized structures. For another structure, aspartate transcarbamylase (1tug), the metal site was only partially occupied in the oxidized chain. For the remaining structures where the metal atom is retained in the oxidized structure, the shortened SG-SG distance may be caused by a heterogeneous mix of oxidized and reduced, metal-bound structures in the ensemble. We reasoned that if the metal was expelled from some members of the ensemble, or partially expelled (still in the site but held, for example, by only two of four potential ligating Cys), then the B-factor should be higher than for chains where no shortened SG-SG distances are observed. Although B-factors cannot be compared between different structures, for some of the redox pairs, different chains of the same structure in different disulfide redox states could be compared. Our analysis confirmed that the B-factor of the metal was higher in the oxidized chains for 80% of structures (see Fig. 3). A similar result was obtained for proteins with Fe-S clusters. There were no instances of expulsion of the Fe-S cluster from the oxidized structure but oxidation of Fe-ligating Cys residues to form disulfides was generally associated with a higher B-factor of the relevant Fe atom than Fe atoms where the ligating Cys remained reduced. In contrast, in reduced structures all Fe atoms of the Fe-S cluster had similar B-factors.

Further inspection of proteins with likely redox-active Zn sites reveals that two forbidden disulfide motifs, the Jump Strand Disulfide (JSD) and a type of β-diagonal disulfide (BDD),12 are repetitively associated with redox-active Zn-binding sites in the dataset. Oxidized structures that contain a BDD include iso-loculeyl tRNA synthetase (1ile 180–184, 502–504), the apoptosis-related proteins birc8 and survivin (1xb1, 300–303;1f3h, 57–60), aspartate transcarbamylase (1rob, 138–141) and the DNA repair enzyme RecR (1vdd, 57–60). Oxidized structures that contain a JSD include the amino-acyl tRNA synthetases for Leu and Ile (1obh, 439–484; 1jzs, 461–502), the enzymes of sulfur metabolism MetH and BHMT (1q7m, 207–273; 1tug, 217–300) and GTP hydrolase GTPφl (1gtp, 110–181).

Morphing transitions in the dataset
A large group of proteins in the dataset were observed to undergo plastic deformations involving large scale rearrangements of the polypeptide backbone. We refer to these conformational changes as morphing transitions. These transitions, which typically involve...
| Group                  | Proteins         | Sitea                  | ox  | re | M  | Ref. |
|-----------------------|------------------|------------------------|-----|----|----|------|
| Sulfur metabolism     | Hs/ReBHMT        | ZnS        | i17 | i18 | Y  | 42  |
|                       | TnMetH           | ZnS       | i17 | i18 | Y  | 43  |
|                       | TnMetE           | ZnS        | i17 | i18 | Y  | 43  |
|                       | HsNedd8          | ZnS        | i17 | i18 | Y  | 43  |
|                       | HsSurvivin        | ZnS        | i17 | i18 | Y  | 43  |
|                       | HsBirc8          | ZnS        | i17 | i18 | Y  | 43  |
| Nucleotide metabolism | Bs-G-Deaminase   | ZnS        | i17 | i18 | Y  | 43  |
|                       | EcATCase         | ZnS        | i17 | i18 | Y  | 43  |
|                       | EcGTPPhl         | ZnS        | i17 | i18 | Y  | 43  |
|                       | AChE             | ZnS        | i17 | i18 | Y  | 43  |

Table I. Proteins with Likely Redox-Active Metal Sites

| Group                      | Proteins          | Sitea                  | ox  | re | M  | Ref. |
|----------------------------|-------------------|------------------------|-----|----|----|------|
| NAD+-dependent oxidoreductases | TnSir2         | ZnS        | i17 | i18 | Y  | 43  |
| RNA/DNA                    | Other viral proteins | HsSMAD        | ZnS        | i17 | i18 | Y  | 43  |
|                            | Transcriptional regulators | HsRc         | ZnS        | i17 | i18 | Y  | 43  |
|                            | DNA repair        | DrRecR       | ZnS        | i17 | i18 | Y  | 43  |
|                            | Metallo-chaperones | ScATx         | CuS  | i17 | i18 | Y  | 43  |
|                            | Other viral proteins | MjHypB        | ZnS         | i17 | i18 | Y  | 43  |
|                            | Oxidoreductases   | SARSpol10      | ZnS         | i17 | i18 | Y  | 43  |
|                            | aa-tRNA synthetases | TtVal-tRNAs   | ZnS        | i17 | i18 | Y  | 43  |
|                            | RNA/DNA polymerases | TtRNApolB1B1  | ZnS        | i17 | i18 | Y  | 43  |
|                            | Ribosomal proteins | Tt30SribosomS4 | ZnS        | i17 | i18 | Y  | 43  |
|                            | Replication, DNA synthesis | EcRNApolIiIIc | ZnS        | i17 | i18 | Y  | 43  |
|                            | Fe-S containing  | Rsxanholde hydrogenase | ZnS        | i17 | i18 | Y  | 43  |
|                            | Other            | Hslnk2       | ZnS        | i17 | i18 | Y  | 43  |

Ligands in metal sites are Cys unless indicated by a one-letter-code amino acid prefix, B indicates ligation by the backbone of the indicated residue.

ox, oxidized structure; re, reduced structure; M, metal expelled; N, metal present in oxidized structure; Metal present in oxidized structure with partial occupancy; ADH, alcohol dehydrogenase; Anti-TRAP, tryptophan RNA-binding attenuator protein-inhibitory protein; ATCase, aspartate carbamoylase; ATx, alcohol dehydrogenase; B4, Bacteriophage 14; Bs, Bacillus subtilis; Ds, Drosophila; Dv, Drosophila vulgaris; Ec, Escherichia coli; Gg, Gallus gallus; HepC, Hepatitis C; Hs, Homo sapiens; Mm, Mouse; Mt, Mollicutes; Ns3, Ns3 Protease; Pa, Pau; Pp, Pseudomonas; Pt, Pseudocarcinoma thermophila; Rp, Rhodococcus sp R312 plastid; SARS, severe acute respiratory syndrome coronavirus; Sc, Saccharomyces cerevisiae; Ss, Sus scrofa; SV40, Simian Virus 40; Tf, Thermus thermophilus.

* Zn sites in italics are catalytic.

b Cys residue is in another chain (interchain disulfide).
unraveling portions of secondary structure, are illustrated in Figure 4 and in animations in the Supporting Information. The well known redox-sensitive protein OxyR belongs to this group. In OxyR, a protein of 305 residues, significant refolding of the C-terminus occurs involving residues 196–221. In the reduced structure, residues 200–204 form a short \(3_1\) helix flanked by regions of coil. Upon oxidation, residues 196–221 adopt a different fold with residues 189–193 and 213–218 forming two parallel \(\beta\)-strands joined by a loop with a disulfide-linkage between Cys199 and Cys208. In the reduced OxyR structure, the two Cys that form the disulfide are separated by almost 13 Å. Cys 199 in OxyR is known to undergo alternate modifications such as glutathionylation. Because of the spectacular nature of the transition between the reduced and oxidized structures, and the existence of an alternate mode of redox regulation, the physiological relevance of the disulfide-linked structure has been called into question.

To determine whether the oxidized structure of OxyR is indeed an oddity, or if other proteins exhibit similar conformational changes, we systematically searched for morphing proteins in the dataset by scanning for protein structure pairs that exhibited large differences of the backbone torsional angles over at least seven contiguous residues. Changes in pseudo-dihedral angles calculated using four consecutive C\(\alpha\) atoms exceeding a summed threshold of 1000 degrees over seven consecutive residues were used to detect changes in secondary structure. Twenty-two parents were found in the fully reduced set and four in the partially reduced set (designated \(m\) in Supporting Information Table 1). Proteins showing the largest conformational changes are in Table II. These proteins include the \(\mathrm{Cl}^-\) channel (CLIC1), a protein that forms ion channels by inserting itself into membranes in response to oxidation; the detoxification enzyme arsenate reductase, which undergoes multiple conformational changes associated with a disulfide relay as part of its catalytic mechanism.

Figure 3. Comparison of B-factors between multiple chains of structures where oxidation of the metal site is heterogeneous. Bars represent the difference of the average B-factor of the metal between oxidized and reduced chains. In structures where there is partial oxidation of the metal site, the B-factor of the metal should be higher because it has fewer ligands and hence more conformational freedom. This is true for most structures (bars above the axis). Standard errors are depicted where they could be calculated. Key to structures: Clostridium acetobutylicum thymidine kinase 1-1x6; E. coli aspartate transcarbamylase 2-1r0b, 3-1tug; Thermotoga maritima Sir2 4-2h59; Homo Sapiens Sir2 5-1j8f; Equus caballus alcohol dehydrogenase 6-1axg; Bacillus subtilis Tryptophan RNA-binding attenuator protein- inhibitory protein (antiTRAP) 7-2bx9; severe acute respiratory syndrome coronavirus nonstructural protein 10 8-2g9t, 9-2ga6; Homo sapiens estrogen receptor 10-1hcq; Deinococcus radiodurans recombinational repair protein 11-1vdd; norovirus 3c-like protease 12-1wqs; Moorella thermoacetica bifunctional carbon monoxide dehydrogenase/acetyl-coa synthase 13-1mjg; Rhodococcus sp R312 nitrile hydratase 14-1ahj; Desulfovibrio s. NiFe hydrogenase 15-1fr; Pseudomonas aeruginosa formate dehydrogenase (fdhE) 16-2fly; Thermus thermophilus valyl-\(\text{tRNA}\) synthetase 17-1gax; Thermus thermophilus RNA polymerase 18-2cw0; E. coli ribosomal RNA large subunit methyltransferase A (rmmA) 19-1p91; E. coli DNA polymerase III \(\beta\) 20-1xxh; Rhodobacter capsulatus xanthine dehydrogenase 21-1jrp. Metal in all structures is Zn unless specified otherwise. Metal atoms in structure 21 are Fe atoms in Fe-S cluster.
of its reaction cycle\textsuperscript{17,61} and cyclic phosphodiesterase, a tRNA-splicing enzyme where the disulfide modulates access to the active site.\textsuperscript{59} Like OxyR, several of these proteins exhibit large changes in $\text{C}_\alpha$ separations of the two Cys between alternate redox states (Table II). Thus large physical separation of the reduced Cys pair seems to be a common feature of many of these proteins and does not preclude disulfide formation.

**Order/disorder transitions**

Another recognizable group of conformational changes involves order/disorder transitions. In these transitions, alternate redox states of the protein correlated with differing amounts of disorder in the protein structure, as evidenced by missing electron density. A total of 282 redox pair structures in 27 parents exhibited order/disorder transitions correlated with the

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**Figure 4. Morphing transitions in protein Redox Pairs.** A: A large morphing transition in the chloride channel CLIC1: a protein that forms ion channels by inserting itself into membranes in response to oxidation. B: A smaller morphing transition in cyclic phosphodiesterase (CPDase) a protein involved in the tRNA splicing pathway of yeast, plants and vertebrates. Redox activity of the disulfide modulates access to the active site.\textsuperscript{59} Oxidized structures are shown on the left. Reduced structures are on the right. The region of the polypeptide chain between the two redox-active Cys is shown in green in the oxidized structure and red in the reduced structure. Other morphing regions are shown in blue. C: Flocco-Mowbray diagram for CLIC1 (left) and CPDase (right). Y-axis SFMI-Smoothed Flocco-Mowbray Index. The polypeptide chain is coloured according to the corresponding oxidized structure. Redox-active thiols are indicated with open squares. Animations of the conformational changes in CLIC1 and CPDase are included in the Supporting Information.
Proteins were classified into four groups based on the location of disulfide-forming Cys residues with respect to the disordered region in the protein chain. Proteins in Group A had a single Cys residue in a disordered region, with the other Cys residing on a more stable region of structure. In Group B, both Cys residues reside on a single disordered region. In Group C, both residues lie on disordered regions which are not contiguous in the sequence. In Group D, disulfide reduction is associated with multiple regions of disorder in the reduced protein chain.

Disorder-to-order transitions have previously been observed upon binding of ligands. Acquisition of order upon binding of ligands concomitant with disulfide formation was apparent for the group B oxidoreductase gdhB, where the disulfide straddled part of the PQQ binding site, and the group A RNA sulfuration enzyme EcTrmU, where the disulfide straddles the tRNA-binding site. However, the reverse was true for

| Name                  | Disulfide | Structures | Morphing region | ΔSG Å | Transition Reduced → Oxidized |
|-----------------------|-----------|------------|-----------------|-------|------------------------------|
| E. coli OxyR          | 199–208   | 1i6a       | 1i69            | 281–298 | 15.9  Helix, coil to sheet    |
| H. sapiens Actin      | 24–59     | 1rK4       | 1k0m            | 27–56  | 13.5  Sheet to helix, coil    |
| Vibrio ChpP           | 424–493   | 1v7v       | 1v7w            | 483–499 | 10.8  Strand to coil          |
| Staphylococcus Aureus | 82–89     | 1j1u       | 1j18            | 81–95  | 10.7  Helix to coil           |
| Allophosphodehydrogenase | 104–110 | 1f5i       | 1j6h            | 99–119 | 9.0   C-term of helix to coil  |
| Ec TrmU               | 49–54     | 2ec3       | 2ec4            | 44–60  | 7.6   Helix to β-hairpin      |
| H. sapiens RNPh        | 203–334   | 1tdw       | 1phz            | 131–149| 7.2   Helix to coil           |
| H. sapiens AKT         | 60–77     | 1unr       | 1unq            | 39–53  | 6.8   Helix to coil           |
| Staphylococcus Aureus | 10–82     | 1lko       | 1j18            | 80–94  | 5.7   Helix to coil           |
| H. sapiens SSAT        | 120–122   | 2bg5       | 2g3t            | 145–155,155–170 | 5.2  Coil to strand transition |
| H. sapiens IL2         | 58–105    | 1m48       | 1m4a            | 29–36,71–87 | 5.1  Coil to N-term of helix  |
| T. foetus IMPDH        | 26–459    | 1meh       | 1pvn            | 317–342,412–435 | 5.0  Helical phase shift, helix to coil |
| R. norvegus PR-1 Ppase | 49–104    | 1x24       | 1x2l            | 20–30,46,56,103–110 | 4.9  R1 helix to coil, R3 coil to helix |
| E. coli OxyR          | 208–227   | 1uag       | 1eeh            | 345–354 | 4.6   Helix to coil transition |
| HepCRNA polymerase 1a | 303–311   | 1inhu      | 1c2p            | 303–318 | 3.8   β-hairpin straddled by disulfide (motif C) curls in oxidized structure |
| T. foetus tRNAS       | 439–484   | 1obh       | 1h3n            | 150–193,436–447,539–546 | 3.8  Sheet melts, helical phase shift |
| E. coli ATCase        | 114–141   | 1tug       | 1d09            | 45–58  | 3.7   Helix to coil transition |
| HepCRNA polymerase 2b | 316–366   | 1yvx       | 1uyy            | 19–38,445–451,539–546 | 3.6   Helix to coil |
| H. sapiens TSP1       | 153–214   | 2es3       | 1zaa            | 17–29  | 3.1   Helix to coil transition |
| M. musculus Activin receptor | 86–91 | 1bte       | 2g00            | 58–73,87–94 | 3.0  R1 helix to coil, R2 coil to helix |
| Staphylococcus Aureus | 393–193   | 1txn       | 1tkl            | 40–60,187–202 | NA  R1 coil to strand, R2 coil to helix/strand |
| H. sapiens DNA pol III λ | 543–543   | 1xsl       | 1xsn            | 460–474 | NA  Strand to coil |
| H. sapiens Activin    | 80–80     | 2arv       | 2arp            | 20–27,65–79 | NA  R1 helix to coil transition |
| H. sapiens RXR        | 269–269   | 1i55y      | 1f9m            | 242–264,434–451 | NA  C-term to helix |

Transitions are expressed from reduced to oxidized but are likely to be reversible. Structures where change in SG separation is caused by quaternary structure changes are indicated as NA. ox, oxidized structure; re, reduced structure; AKT, Rac-alpha serine/threonine kinase; ArsC, arsenate reductase; ATCase, aspartate carbamoyltransferase; bcp, bacterioferritin comigratory protein; ChpP, chitobiose phosphorylase; CLIC1, chloride intracellular channel protein 1; CPDase, cyclic nucleotide phosphodiesterase; DNA pol III λ, DNA polymerase III λ; hem13, coproporphyrinogen III oxidase; IL2, interleukin 2; IMPDH, inosine monophosphate dehydrogenase; Leu-tRNAs, leucyl-tRNA synthetase; MurD, UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase; OxyR, hydrogen peroxide-inducible genes activator; PR-1 Ppase, protein tyrosine phosphatase 4a1; RXR, retinoic acid receptor; SSAT, spermidine/spermine N1-acetyltransferase; TSP1, thrombospandin-1; Ae, Aeropyrum pernix; At, Arabidopsis thaliana; Ec, E. coli; HepC, Hepatitis C; Hs, Homo sapiens; Mm, Mus musculus; Rn, Rattus norvegus; Sa, Staphylococcus aureus; Sc, Saccharomyces cerevisiae; Tf, Tritrichomonas foetus; Tt, Thermus thermophilus; Vp, Vibrio proteolyticus.

* 2nd Cys residue is in another chain (interchain disulfide).
The Thermotoga maritima tRNA-processing enzyme, Psii55s, where significant disorder of the protein chain concomitant with disulfide reduction occurred upon binding of the tRNA substrate fragment. The introduction of disorder in this case may facilitate further co-operative binding of the RNA and protein after the initial docking step. The introduction of disorder in this case may facilitate further co-operative binding of the RNA and protein after the initial docking step.
are depleted in disordered sequences, whereas Lys is enriched.\textsuperscript{76,77} The disordered sequences in the Redox Pair dataset are also depleted in aromatics, in agreement with more general studies of disordered sequences, but are not depleted in Cys, or enriched in Lys; and thus do not conform entirely to previously detected sequence patterns. Redox Pair disordered sequences are instead enriched in oxygen-bearing side-chains such as Asp, Glu, Ser and Thr.

Some changes in secondary structure are also apparent during the order/disorder transitions. In the oxidized state, both Cys residues generally reside on coil in their proteins, but for a small number of pairs the oxidized form has a secondary structure that “melts” upon reduction of the disulfide. Examples include vitamin D-binding protein\textsuperscript{76,79} and the \textit{E. coli} oxidoreductase ghdB,\textsuperscript{74,80} where helices are lost upon reduction; and the bacteriophage cell entry protein PRD\textsuperscript{181} where a small $\beta$-sheet disappears upon reduction.

The amount of disorder in the chains is also of interest. For most reduced structures, the disorder introduced upon reduction of the disulfide affects less than 10% of the polypeptide chain. However, three of the four group D proteins exhibited large regions of disorder in excess of 20% of the involved protein chain. Interestingly, all were associated with a second more-ordered monomer that was oxidized. For example, the \textit{Thermotoga maritima} tRNA-processing enzyme, Ps55s undergoes a spectacular loss of secondary structure in one monomer upon binding of RNA.\textsuperscript{75} It is possible that the fully reduced state of these protein dimers is uncrystal-lisable and that only partially and fully oxidized oligomers are sufficiently ordered to enable structure solution.

\textbf{Changes in quaternary structure}

We also investigated whether formation of interchain disulfide bonds altered the quaternary state of proteins. Different redox states of the interchain disulfides are associated with two general types of quaternary interaction: those where the number of oligomers differs between the reduced and oxidized state; and those where the number of subunits involved in the oligomer stays constant, but the oligomerization interface changes. Some Redox Pairs exhibit a mixture of the two types of quaternary change. For example, disulfide-bonded dimers of the macrophage sialoadhesin

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\textbf{Figure 5.} Order/disorder transitions in protein Redox Pairs. A: A small order/disorder transition in the T-loop of the signaling protein Rac-beta serine/threonine-protein kinase (AKT2).\textsuperscript{67} In the more ordered oxidized structure: a disulfide is formed between Cys 297 and Cys 311 which straddles a key phosphorylation site at Thr 309. Formation of the disulfide is proposed to inhibit kinase activity by recruiting the cognate phosphatase.\textsuperscript{70} The T-loop was recently identified as a target for selective cancer drugs.\textsuperscript{56} B: A larger order/disorder transition associated with expulsion of Zn from the oxidized structure of leucyl-tRNA synthetase.\textsuperscript{71,72} In the more ordered reduced structure, several new secondary structures condense enabling rigid co-ordination of the Zn. Zn is represented by red spheres in the reduced structure. Oxidized structures are shown on the left, reduced structures on the right.
form an interface distinct from that found in trimers formed upon binding of sialosides, and may negatively regulate ligand binding.

Twenty-nine Redox Pair protein clusters with intermolecular disulfide bonds exhibit changes in quaternary structure upon oxidation/reduction. Redox regulation of the quaternary state has previously been associated with cooperative behavior in proteins. For example, the HoxB5 protein binds DNA in vitro when either oxidized or reduced but formation of an intramolecular disulfide between HoxB5 monomers is necessary for co-operative binding. Other well-known examples are OxyR and p53. Several other proteins in the dataset exhibit co-operative behavior. Cytoglobin is a member of the vertebrate haemoglobin family residing in the nucleus and cytosol that is upregulated under conditions of hypoxia and certain types of stress. Intermolecular disulfide bonds in cytoglobin modulate the quaternary structure and may regulate the ligand-binding properties. Both the reduced and oxidized states are dimers.
under conditions of hypoxia and certain types of stress (see Fig. 6). It has recently been implicated as a tumor suppressor. Intermolecular disulfide bonds in cytoglobin modulate the quaternary structure and may regulate the ligand-binding properties (Table IV). The homologous lamphrey haemoglobin is in an equilibrium

| Protein                      | Reduced       | Oxidized                  | Function                                      |
|------------------------------|---------------|---------------------------|-----------------------------------------------|
| Number of subunits differs between redox states                                           |
| CrRuBisCoL                   | 8ruC octamer  | 1uzd 16mer                | CO₂ fixation in plants                        |
| NtRuBisCoL                   | 1e7 monomer   | 4rub tetramer              | CO₂ fixation in plants                        |
| Sp chloroplast GAPDH         | 2hki monomer  | 1bo tetramer with NAD      |                                              |
| HsGrx                        | 1bwc monomer  | 2g85 dimer                 | Redox homeostasis                             |
| Ae bcp                       | 2cx3 tetramer | 2cx4 octamer               | Redox homeostasis                             |
| HsPNMT                       | 1y23 monomer  | 2an5 dimer                 | Catecholamine biosynthesis, dimer is active   |
| MeChiT                       | 1kx2 octamer  | 1l3j hexamer               | B12 synthesis, SAM-binding                    |
| Af/DsrC                      | 1sa monomer   | 2a5w trimer                | sulfite reductase, [4Fe-4S]                   |
| HsVEGF                       | 1j1 dimer     | 1mkg tetramer              | Angiogenesis                                   |
| Rp activin                   | 2arp monomer  | 1nys dimer                 | TGFβ ligand                                    |
| HsBMP2                       | 2g00 ternary complex/hexamer | 1rew binary complex/tetramer |                                           |
| Hsartemin                    | 2gay monomer  | 2gpy hexamer               | TGFβ ligand                                    |
| BPV1-E2                      | 2bop dimer    | 1ijh trimer                | Viral transcription factor                    |
| HPV6a-E2                     | 2ayb_immer, complex with DNA | 1rh8 hexamer | Viral transcription factor                    |
| FMDV leader protease         | 1qmy trimer   | 1qol octamer               | Viral protease                                 |
| HsAPE-1                      | 1de8 dimer, complex with DNA | 1nq1 dimer | Repairs oxidative DNA damage                |
| Mm Macrophage sialoadesin    | 1qfo trimer, complex with sialoadesin | 2hve dimer | Cell attachment, lectin                     |
| HsGGA1                       | 1py1 tetramer | 1jwg dimer                 | Sorting and trafficking                        |
| PsaO                         | 1i9i dimer    | 1w2z tetramer              | Oxidoreductase, Pro-eis/trans isomerization    |
| Spaquaporin                  | 1zp8 dimer/closed | 2h5f tetramer/open | Membrane water pore                           |
| HsDNA pol III λ             | 1xsn monomer  | 1xol tetramer              | DNA repair                                    |
| Mpmethylenetetrahydrofolate synthetase | 1ug3 monomer | 1sbq dimer | Folate biosynthesis                          |
| Hs granzyme A                | 1orf monomer  | 1op8 hexamer               | Immunity, apoptosis (111)                      |
| TEF-Ts                       | 1tfe monomer  | 1aip tetramer              | Translation, GEF for EF-Tu, dimer is active (112) |
| Dm ph sam domain             | 1kw4 monomer  | 1pk1 dimer                 | Chromatin protein, development                |
| Mm Grp1                      | 1fgy monomer  | 1fhw dimer                 | Signaling, binds phosphoinositides            |
| Oligomerization interface changes between redox states                                      |
| Tl hemerythrin               | 1hmd tetramer 1 | 1hmz tetramer 2 | Oxygen transport                              |
| Hs PCBP2                     | 2ax monomer   | 1ztg tetramer 2            | Translation regulation                        |
| Hs cytoglobin                | 1umo monomer  | 2dx3 dimer 2               | Oxygen carrier, upregulated in response to hypoxia |

Italicized prefixes are species abbreviations. Protein names in roman font.

AO, amino oxidase; bcp, bacterial comigratory protein peroxiredoxin; BMP2, bone morphogenetic protein 2; DNA pol III λ, DNA polymerase III λ; EF-Ts, elongation factor Ts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEF, guanine nucleotide exchange factor; GGA1, golgi-localized γ adaptin; Grp1, guanine nucleotide exchange factor and integrin binding protein homolog grp1; Grx, glutathione reductase; PCBP2, poly(C)-binding protein-2; phsam domain, polyhomeotic-proximal chromatin protein sterile alpha motif domain; PNMT, phenylethanolamine N-methyltransferase; RuBisCoL, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; SAM-S-adenosyl-L-methionine; VEGF, vascular endothelial growth factor; Ae, Aeropyrum pernix; Af, Archaeoglobus fulgidus; BPV, bovine papilloma virus; Cr, Cryptidia fasciculata, Dm, Drosophila melanogaster; FMDV, foot and mouth disease virus; HPV, human papilloma virus; Hs, Homo sapiens; Me, Methanobacterium thermautotrophicum; Mm, Mus musculus; Mp, Mycoplasma pneumoniae; Nt, Nicotiana tabacum; Ps, Pisum sativum (Pea); Rn, Rattus norvegus; Sp, Spinacia oleracea; Td, Themiste dyscritum; Tt, Thermus thermophilus.

a 2nd Cys in different monomer.
state between a low O\textsubscript{2} affinity oligomer and a high-affinity monomer which contributes to its co-operativity.

There was also a small group of transmembrane receptors which form covalent dimers in a neck region close to the membrane. These include the low-density oxidized LDL receptor, and the asialoglycoprotein receptor. Covalent dimer formation may promote further quaternary changes such as higher oligomer formation in response to bound ligands as has been recently demonstrated for the metabotropic glutamate receptors.\textsuperscript{96}

**Relevance to disease**

Importantly, ageing and several major diseases including neurodegenerative diseases such as Alzheimer’s disease, type II diabetes, cancer and cardiovascular disease have been associated with abnormal redox conditions. Inadvertent triggering of redox-active disulfide switches is a likely contributor to these phenotypes. This view is supported by the association of several Redox Pairs in the dataset with disease. SOD\textsubscript{1} (also known as CuZn-SOD) is an enzyme mutated in familial lateral sclerosis (FALS), an age-dependent degenerative disorder of motor neurons in the spinal cord, brain stem and brain. SOD\textsubscript{1} is largely localized to the cytosol but is also found in the intermembrane space of mitochondria. SOD\textsubscript{1} is activated by formation of the disulfide and insertion of Cu by the copper carrier CCS. Both of these proteins are represented in the Redox Pair dataset (Tables I and S2). The conserved disulfide in SOD\textsubscript{1} is essential for activity and unusual because it remains oxidized in the cytosol. Although the disulfide is intrachain, it is thought to play a critical role in stabilizing the SOD\textsubscript{1} homodimer. Formation of the disulfide is part of the SOD\textsubscript{1} maturation process which is regulated by physiological oxidative stress.

The three reduced proteins in the dataset are three distinct mutants associated with familial lateral sclerosis (FALS): G37R, H46R, and I113T. The exclusive association of the reduced proteins with the disease mutants suggests redox imbalance of this disulfide is implicated in the disease. This hypothesis is supported by the finding that 14 FALs mutants have the common property of being more susceptible to disulfide reduction than wild-type protein.\textsuperscript{97} Toxicity associated with disulfide-linked multimerization of SOD\textsubscript{1} mutants has recently been implicated in the disease mechanism.\textsuperscript{98}

**Discussion**

Several types of conformational change were observed in the Redox Pair dataset suggesting there may be common structural modes of redox regulation. Proteins exhibited disulfide oxidation following expulsion of metals such as Zn, morphing, order/disorder transitions, and changes in quaternary structure. In an individual protein more than one mode may be employed. For example in Leu-tRNA synthetase, part of the protein chain becomes disordered upon Zn expulsion but this is not the case for all proteins with labile Zn.

Redox regulation of proteins that form disulfides following expulsion of Zn is an emerging area of signaling. Well known and characterized examples including Hsp33 which is activated by Zn expulsion as part of the oxidative stress response\textsuperscript{99}, and the small Tim proteins which are kept in a transport-competent state in the cytosol by conjugation with Zn.\textsuperscript{43} Upon delivery to mitochondria, Zn is expelled from small Tim proteins which are activated by disulfide formation. Zn is not expelled from all protein Zn-binding sites with equal facility, and is probably not expelled from some sites at all under physiologic conditions. For structural Zn sites an analogous situation may exist to the bipartite distribution of redox-active and structural disulfides in proteins, with labile Zn sites being redox-active and inert Zn sites corresponding to the purely structural group. Key to Zn expulsion under oxidizing conditions is the presence of oxidation-prone Cys as a Zn ligand. Sites with more Cys are likely to be more facile. Based on the number and nature of ligands, the Zn sites detected in the Redox Pair dataset likely belong to the redox-active group.

The repeated association of forbidden disulfides of the BDD type with labile Zn binding sites is particularly interesting because of the recent characterization of the oxidative process that leads to activation of the bacterial chaperone Hsp33.\textsuperscript{99} The presence of the Zn center keeps Hsp33 monomeric and functionally inactive. Upon exposure to elevated temperatures, the C-terminal domain swings away from the protein’s center of mass, exposing the Zn centre. The N-terminal thiols in the Zn site, Cys 232 and Cys 234, are preferentially oxidatively modified. The remaining two Cys, 269 and 272, form a disulfide of the BDD type, releasing Zn and unfolding the C-terminal domain. Unfolding the C-terminus removes a steric barrier to dimerization, activating the protein’s chaperone function.

The function of disulfide formation between Cys 269 and 272 in the Zn binding site has not been fully delineated. Potential benefits are protection from other oxidation modifications and restriction of the conformational freedom of the protein chain allowing easier refolding upon return of the protein’s milieu to normal conditions. However, these benefits apply to any disulfide and the repeated appearance of the BDD in labile Zn sites of proteins of the Redox Pair dataset suggests a more specific function, possibly recognition by a foldase or metallochaperone. We hypothesize the forbidden disulfide would become stressed upon condensation of the β-sheet in its vicinity,\textsuperscript{12} possibly allowing easier re-insertion of Zn. Without any detailed experiments as a guide, the significance of the other repeated forbidden disulfide motif, the JSD, is not clear, but the same arguments relating to its forbidden disulfide nature would apply. The JSD-containing proteins MetH and BHMT are known to be reversibly inactivated by disulfide formation.\textsuperscript{100} In vitro, BHMT can be reactivated by dialysis against DTT and Zn.\textsuperscript{101}
but the specific method of reactivation in vivo has not been characterized.

For most structures exhibiting order/disorder transitions in the dataset, the reduced structure is associated with more disorder than the oxidized structure. Energetically, formation of a disulfide bond between Cys residues would have a favorable entropic contribution to the stability of the chain. A notable exception is Leu-tRNA synthetase where the more ordered reduced structure binds Zn (Fig. 5(B)). This may be generally true for order/disorder transitions associated with labile Zn sites: the Zn-bound state is entropically favored over a single disulfide because the polypeptide chain is constrained at three or four points instead of two.\textsuperscript{102}

A subset of Redox Pair proteins exhibiting order/disorder transitions correlated with disulfide redox status contain regions of disorder in excess of 20% of the protein chain. All the proteins in this group exist as dimers with the other monomer being more ordered. The disordered monomers in these class D structures may correspond to the Molten Globule state. For these proteins, the Molten Globule state adopted may be physiologically relevant to their function. Molten Globules are collapsed forms of the protein chain which have some native-like secondary structure but a dynamic tertiary structure as seen by far and near circular dichroism spectroscopy, respectively. The Radius of Gyration of proteins in the Molten Globule state is \(\sim10\)% larger than the native state but precise tertiary structures at atomic resolution have not yet been obtained for commonly studied Molten Globule proteins such as lactoglobulin A. However, one of the proteins in group D: Platelet Factor 4, which like hepatocyte growth factor, binds heparin and regulates angiogenesis, has been shown to adopt the Molten Globule state upon reduction of its disulfides.\textsuperscript{102} It seems likely proteins in group D adopt a Molten Globule state in vivo but can also adopt more ordered states upon binding of appropriate ligands. Several of the proteins bind large polymers such as RNA and heparin and the order/disorder transitions may mediate co-operative behavior between monomers.

General studies of disordered sequences suggest there may be several different types of disordered sequence, three of which have been previously detected using an automatic classifier.\textsuperscript{103} The oxygen-rich sequences of the disordered regions of proteins of the Redox Pair dataset seem to represent a novel type of disordered sequence not previously recognized. Oxygen-rich sequences may confer bistable switch-like properties on the sequence: an oxygen-rich environment stabilizes the disulfide in the oxidized state by increasing its pKa\textsuperscript{104}; while the subset of negatively charged Asp and Glu residues in oxygen-rich sequences may contribute to disorder in the reduced state via like-charge repulsion. Disordered regions are known to undergo post-translational modifications including acetylation, glycosylation, methylation, and phosphorylation which may regulate the order/disorder transition.\textsuperscript{73} Redox-activity of resident Cys residues should be added to this list of post-translational modifications.

Perhaps the most astonishing conformational changes associated with disulfide reduction are the morphing transitions. The current paradigm, due to Anfinsen, is that the primary sequence of a protein encodes a unique three-dimensional structure. Various discoveries that have challenged this notion include the spectacular pH-induced conformational change in influenza A haemagglutinin (HA),\textsuperscript{105} molecular chaperones, and the previously discussed disordered sequences. All of these discoveries have been difficult to reconcile with the concept that protein sequences adopt a unique minimally frustrated fold. HA is believed to be kinetically trapped in a higher energy fold by an N-terminal domain which is subsequently cleaved, allowing it to cascade into a minimally frustrated fold. The conformations of disordered sequences appear to be principally governed by entropic factors: weak sequence preferences for a variety of conformational substates or modes appear to be co-selected by ligands. Morphing structures appear to be a new type of challenge to the concept of a unique minimally frustrated fold. For morphing sequences, the structure appears to be dependent on the environment. Unlike influenza A HA, where kinetic trapping during the folding process clearly plays a part in the structure adopting two different folds, for morphing sequences the two folds are energetic minima for the sequence in a particular environment. As such structural changes are reversible and dependent on environment.

We were previously aware of two instances where subdomain morphing of proteins has been associated with reversible disulfide reduction: a redox-controlled structural reorganization of the ion channel CLIC\textsubscript{1} proposed to regulate its insertion into membranes,\textsuperscript{18} and sequential oxidation of the transcription factor OxyR in response to oxidative stress which modulates its quaternary structure and DNA-binding properties.\textsuperscript{73} Here we showed that morphing is a common type of major structural change associated with disulfide reduction. The most spectacular morphing transition is that of CLIC\textsubscript{1}, where an entire subdomain of the protein rearranges in response to different redox conditions. Several Redox Pairs were found exhibiting large morphing conformational transitions of similar magnitude to OxyR, supporting the notion that these types of transitions may be reasonably common. Large separations of the Cys residues in the reduced state are a typical feature of these proteins, and do not preclude disulfide linkages forming. Possibly attack by a helper molecule, such as glutathione, may be required as an intermediate in disulfide formation to bridge the intervening distance. Large physical separation of partner Cys residues may represent an example of negative
design against inadvertent disulfide formation under mild oxidizing conditions.

Morphing transitions are not limited to changes in disulfide redox status. Other morphing transitions not associated with disulfide redox state have been noted in Mad2 and lymphotactin.\textsuperscript{106–108} pH and redox are probably only two of multiple environmental changes associated with structural morphing. The relative importance of these and other environmental changes in protein morphing remain to be determined.

Morphing transitions may also have some relevance to evolutionary bridging states. In 1995, based on structures of Arc repressor, Sauer proposed the existence of evolutionary bridging states. A substructure in wild-type Arc repressor adopts a β-strand conformation which forms a two-stranded β-sheet with the adjacent monomer. In the N11L mutant of Arc repressor, the substructure was shown to fluctuate between the native state and a helical state on a millisecond time scale. A double mutant known as “switch Arc” (N11L, L12N) was shown to be stabilized in the alternate helical conformation. Sauer proposed the fluctuating N11L mutant represented an evolutionary bridge between two stable low energy folds. Proteins that can adopt two different folds from the same sequence could evolve two different functions upon gene duplication and mutation hence acting as an evolutionary bridge between two different folds.\textsuperscript{109} It is clear morphing proteins could also act as bridge states: deselection of one state over the other by point mutation may lead to loss of the ability to morph in the daughters resulting in proteins with different but related folds.

Although we have concentrated solely on disulfide reduction in this study, several anecdotal examples of disulfide isomerization should be mentioned in the context of the aforementioned discussion. A second example of an evolutionary bridging state was proposed based on a study of cnidarian collagen. The N-terminal domain of minicollagen 1 has 44% sequence identity with the C-terminal domain, but the structures show a different disulfide bonding pattern and fold.\textsuperscript{110} At this stage it is not clear if the two forms are interconvertible or represent true one-state endpoints evolved from an evolutionary bridging state. A different example where major structural organization is regulated by disulfide isomerization is the activation of P1 lysozyme.\textsuperscript{111} This conformational change is irreversible and associated with release of a hydrophobic transmembrane sequence by membrane depolarization, suggesting the latent state may be a kinetically trapped state rather than a true morphing protein.

**Conclusions**

Our study mined pairs of protein structures which contain disulfides in both reduced and oxidized states and represents the first systematic compilation of such a dataset. Quaternary changes, morphing and expulsion of transition metals, particularly Zn, have previously been associated with redox-related changes in proteins and here we showed these conformational changes are more common than anecdotal examples suggest. To our knowledge order/disorder transitions have not previously been linked with redox changes but also seem to be a common type of conformational change. Like morphing transitions, changing redox conditions appear to be only one of many mechanisms regulating order/disorder transitions. The oxygen-rich sequences of the disordered regions of proteins of the Redox Pair dataset seem to be a novel type of disordered sequence not previously recognized. Finally, two forbidden disulfide motifs are associated with redox-regulated Zn binding here for the first time.

The physiological significance of redox-controlled structural changes is only beginning to be appreciated. Previously, all oxidative changes were thought to be irreversible and damaging. It is now known that redox signaling pathways can modulate homeostatic and adaptive responses. However, continued stimulation of adaptive responses may lead to maladaptive responses. The Redox Pair dataset is a valuable resource for the investigation of physiological and pathological processes associated with disulfide redox activity.

**Materials and Methods**

**Identifying redox protein-pairs in the PDB**

The PDB\textsuperscript{116} was mined for pairs of structures with Cys in different redox states. First, all disulfide-containing structures were identified by querying their DSSP records,\textsuperscript{112} as SSBOND records in the PDB header were found to be unreliable. For each disulfide the atomic residue positions were mapped to the sequence position. The connectivity of the disulfides in the sequence was then used to assign a unique identifier, $d_{MN}$ score [Eq. (1)], to each protein.\textsuperscript{113} The $d_{MN}$ score allows direct comparison of the disulfide connectivity between structures as structures with the same disulfide bonding will have the same $d_{MN}$ score.

$$d_{MN} = \sqrt{\sum (j - i)^2}$$ (1)

where $i$ and $j$ are the sequence positions of disulfide-bonded Cys.

All protein sequences in the PDB were clustered at 95% sequence identity using BlastClust.\textsuperscript{114} The 95% threshold was chose to capture the same protein with some natural and engineered mutations allowed. Some highly conserved orthologs from closely related species were also captured in the same cluster, for example, the NF-κB orthologs in human and mouse. Proteins within each group were compared to detect subgroups with different $d_{MN}$ scores indicating alternative disulfide connectivity. For fully oxidized proteins, a set of “pseudo-reduced” $d_{MNS}$ scores were calculated by iteratively...
ignoring one or more disulfides. The set of pseudo-reduced $d_{\text{MSMS}}$ for the oxidized protein were matched against $d_{\text{MSMS}}$ for the reduced proteins in the dataset to select Redox Pairs. As an additional control, proteins were split into two groups based on whether some or all disulfide bonds in the protein were reduced. Proteins in which Cys residues were engineered into proteins were filtered from the list.

**Analysis of gene ontology term enrichment**

GO terms that are over and under-represented in the oxidized proteins in the Redox-Pairs dataset compared to the PDB as a whole were identified using Fisher's Exact Test, in the same way as applied in tools that analyze gene-expression data. GO annotations for the entire PDB were taken from the GOA resource, downloaded from the EBI.

To ensure a fair comparison, the GO annotations of a nonredundant set of the oxidized structures (95% sequence identity) were compared to GO annotations of a nonredundant PDB data set (95% sequence identity). To improve overall annotation coverage, a representative PDB structure is assigned all the GO terms belonging to the PDBs within its cluster group.

Because GO is a hierarchical data structure, GO terms are set to a single level in the hierarchy to enable a fair comparison. Here we used GO level three. At this level there is a compromise between information quality and the number of annotations available.

Where a GO term is below this level we move up the hierarchy until we reach the third level GO term(s). GO terms above level three cannot be used.

A $P$-value was calculated representing the probability that the observed number of each GO term ($n$) could have resulted from randomly selecting this GO term in the PDB as a whole ($N$). Fisher's Exact test was used in order to approximate this $P$-value. GO terms that are most specific to the Redox Pairs set will have $P$-values near zero, and those that are under-represented will be near one.

**Analysis of protein conformational change**

Protein conformational changes were examined using a variety of complementary approaches. We used ProFit (www.bioinf.org.uk/software/profit) to search for large conformational changes such as hinge movements. ProFit superimposes two structures using Cα atoms and was used to calculate RMSD and distances between residues. Large movements were indicated by the change in distance between the sulfur groups of the Cys residues in a disulfide.

Because RMSD is averaged over the entire molecule, large changes confined to a subdomain of protein may not be obvious using RMSD. Large conformational changes between structure-pairs corresponding to rearrangements of the polypeptide backbone were also identified using an implementation of a method by Flocco and Mowbray. For pairs of structures, the method compares the change in a pseudo-torsional angles defined by four consecutive Cα-atoms. Conformational changes were identified by running a smoothing window (five residues long) over the torsional-difference plot. Stretches of contiguous residues with seven or more pseudo-dihedral angles that exhibited individual movements in excess of $20^\circ$ were initially filtered from the dataset. These conformational changes were further classified by comparing DSSP annotations, solvent accessibility and secondary structure, between the protein pairs. Proteins with conformational changes where the summed changes in the pseudo-torsional angles exceeded a threshold of 1000 degrees with accompanying secondary structure changes were classified as morphing. The presence or absence of multiple chains in the PDB structure could cause errors when calculating changes in solvent accessibility; therefore, DSSP was run on individual chains only. All solvent accessibility measures were normalized using values derived by Chothia. Each amino acid was assigned to one of two states; core or exposed. Core residues are those with a solvent accessibility of less than 10% and exposed residues are those with a solvent accessibility equal to 10% or more. Secondary structures were assigned to one of three states: helix, strand and coil.

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