ANTIOXIDANTS & REDOX SIGNALING
Volume 5, Number 1, 2003

Review

Redox Control on the Cell Surface: Implications for HIV-1 Entry

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ABSTRACT

Proteins that work outside cells nearly always contain disulfide bonds. The prevailing view is that these bonds have been added during evolution to enhance protein stability. Recent evidence suggests that disulfide bonds can also control protein function. Certain secreted proteins contain one or more disulfide bonds that can control function by breaking and reforming in a controlled way. This review focuses on disulfide exchange events on the cell surface, with a particular reference to two proteins involved in HIV-1 infection. The primary HIV-1 receptor on immune cells, CD4, and the viral envelope glycoprotein, gp120, play a central role in HIV-1 entry. Redox change in a disulfide bond or bonds in one or both of these proteins appears to be important for HIV-1 entry. *Antioxid. Redox Signal.* 5, 133–138.

INTRODUCTION

THE CYSTEINE RESIDUES IN SECRETED PROTEINS are nearly always paired into disulfide bonds. The reason for their existence has been thought to be twofold.

First, disulfide bonds have consequences for protein folding (35, 48, 52). They stabilize the native conformation of a protein by destabilizing the unfolded form. Disulfide bonds lower the entropy of the unfolded form, making it less favorable compared with the folded form. Disulfide bonds can also decrease the stability of folded proteins due to bond enthalpy effects and strain.

Second, they help stabilize protein structure (*e.g.,* 25). The extracellular milieu is a fluctuating environment and can be harsh on proteins. Secreted proteins often need to work in an environment rich in oxidants and proteolytic enzymes that damage proteins. Disulfide bonds can protect proteins from damage and increase their working life.

It has been generally considered that the disulfide bonds in mature proteins are inert. Recent findings, however, have shown that this is not necessarily the case. Certain disulfide bonds can break and reform in a precise way, and when this happens, it has significant consequences for protein function (Fig. 1). This review focuses on disulfide exchange events on the cell surface, with a particular reference to two proteins involved in human immunodeficiency virus 1 (HIV-1) infection.

REDOX STATE OF THIOLS/DISULFIDES ON THE CELL SURFACE

The redox state of protein thiols/disulfides on the surface of fibroblasts (21) and lymphocytes (24, 46) is influenced by the activation state of the cell. Tagged thiol-reactive compounds were used to label cell-surface thiols, and the extent and/or pattern of labeled proteins was examined. Change in the labeling was correlated with perturbation of cellular function. It was observed that activation of cells resulted in an increase in cell-surface thiols. These findings implied that certain cell-surface protein disulfide bonds were reduced upon cell activation and suggested that a cellular factor was mediating this reduction. Protein disulfide isomerase (PDI) was implicated as the cellular factor in both cell types.

PDI is a thiol-disulfide oxidoreductase that facilitates proper folding and disulfide bonding of nascent proteins in the endoplasmic reticulum (16). This is achieved by two reactive dithiols/disulfides with the sequence Cys-Gly-His-Cys

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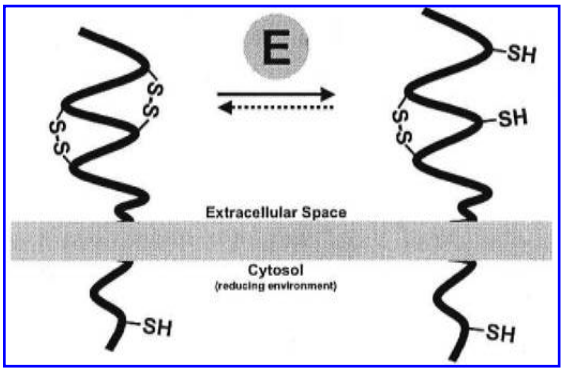

FIG. 1. Illustration of disulfide exchange on the cell surface. A transmembrane protein is shown, but the protein may be glycoslyphosphatidylinositol-linked or noncovalently bound to the cell surface. The protein contains a disulfide bond that can exist in either the oxidized (left-hand structure) or reduced dithiol (right-hand structure) form on the cell surface. The dotted arrow indicates that the dithiol may or may not be able to reform the disulfide bond. The redox event will usually require facilitation by another compound, such as thioredoxin or PDI. The notion is that the oxidized and reduced forms of the protein have different functions.

that undergo cycles of oxidation/reduction with dithiols or disulfides in the protein substrate resulting in formation, reduction, or isomerization of disulfide bonds in the substrate. PDI is recycled back to the endoplasmic reticulum from the Golgi and intermediate compartment through interaction with the KDEL receptor via its C-terminal KDEL motif. Despite this retrieval mechanism, PDI is exported from cells and binds to the cell surface. Secreted PDI retains the KDEL anchor (47, 54). Cultured rat hepatocytes (47) and pancreatic cells (1) secrete PDI, which associates with the cell surface, and murine fibroblasts secrete PDI in response to treatment with calcium ionophore (6). PDI is also on the surface of B cells (23, 46) and platelets (10, 14).

PDI has been directly implicated in reduction of disulfide bonds in some cell-surface proteins. Cleavage of disulfide bond(s) in the human thyrotropin (12) and L-selectin (4) receptors by PDI appears to mediate their shedding. PDI has also been implicated in transport of diphtheria toxin (28) and nitric oxide (37, 55) into cells and redox control of a thiol/disulfide in GP1bα on the platelet surface (8).

Two proteins involved in HIV-1 infection have been suggested to undergo redox changes. These proteins are the primary HIV-1 receptor on immune cells, CD4, and the HIV-1 envelope glycoprotein, gp120. Both proteins play a central role in HIV-1 infection, and redox change in a disulfide bond or bonds in one or both of these proteins appears to be important for HIV-1 entry.

CD4 CONTAINS A STRAINED DISULFIDE BOND THAT IS REDOX-ACTIVE ON THE CELL SURFACE

CD4 is a member of the immunoglobulin (Ig) superfamily of receptors that is expressed on most thymocytes and on peripheral helper T lymphocytes. Binding of HIV-1 to CD4 and a chemokine receptor triggers fusion of the viral and cell membranes leading to HIV-1 entry and infection (44).

CD4 is a transmembrane glycoprotein with a molecular mass of 45 kDa (27). The extracellular portion consists of four Ig-like domains, D1 to D4 (7, 19, 41, 49). The D1, D2, and D4 domains each contain a disulfide bond. The backbone of Ig domains are defined by seven β strands. One β sheet of the barrel-like structure contains strands A, B, and E, and the other sheet has strands C, C′, F, and G. The disulfide bonds in Ig folds are nearly always between a Cys in strand B and one in strand F (38). The disulfide bond in the D2 domain, however, is between a Cys in strand C and one in strand F. This means that the disulfide bond in D2 is between strands in the same sheet rather than between sheets as is usual.

The D2 disulfide bond is right- rather than left-handed, has a short $C_\alpha - C_\alpha$ distance, and makes the least contribution to overall stability from enthalpy calculations (Table 1). Notably, the dihedral strain energy of the D2 bond is approximately twice that of the D1 and D4 disulfide bonds (30). It has been demonstrated that the more strain on a disulfide bond, the more readily it is reduced (22, 36, 50, 51, 52).

Matthias *et al.* (30) showed that the D2 disulfide bond could exist in the reduced dithiol form on the cell surface. Cys to Ala mutants of the three pairs of Cys residues in domains 1, 2, or 4 of CD4 were expressed on the surface of human fibrosarcoma cells. The wild-type and D1 and D4 disulfide-bond mutant CD4s formed disulfide-linked dimers, and the monomers were labeled with a biotin-linked maleimide that alkylates free thiols at neutral pH. The D2 disulfide-bond mutant, however, did not form dimers and was not labeled by the maleimide.

THE REDOX STATE OF CD4 APPEARS TO BE CONTROLLED BY THIOREDOXIN

Activation of blood T cells with phytohemagglutinin approximately doubled the fraction of total cell-surface CD4 that contained free thiols (30). This result implied that the redox state of CD4 was controlled by the activation state of the cell and perhaps by a factor secreted by the T cells. Thioredoxin is a member of the PDI superfamily with an active site dithiol/disulfide in the sequence, Cys-Gly-Pro-Cys

TABLE 1. CHARACTERISTICS OF THE CD4 DISULFIDE BONDS

Domain disulfide bond	*C_α–C_α bond length (Å)*	*Dihedral strain energy (kcal/mol)*	*Number of residues in disulfide crosslink*	*Change in enthalpy (kcal/mol)*
D1	6.58	2.28	67	4.36
D2	3.92	4.74	30	3.65
D4	6.56	1.71	43	3.97

The C_α–C_α bond length, dihedral strain energy, and change in enthalpy were calculated from the crystal structures of the D1-D2 (41, 49) and D3-D4 domains (7), and the energy equations by Weiner *et al.* (50) and Katz and Kossiakoff (22).

(20). Thioredoxin is constitutively secreted by T cells and binds to the cell surface (29, 39, 45, 53) and can reduce cell-surface and soluble CD4 (30).

$CD4^+$ T cell activation is associated with increased secretion of thioredoxin (39, 45), which is in accordance with the increased reduction of peripheral blood T cell-surface CD4 upon phytohemagglutinin activation (30). Notably, plasma concentrations of thioredoxin in HIV-1-infected individuals are inversely correlated with $CD4^+$ cell numbers and survival in AIDS patients (33, 34). These observations are consistent with thioredoxin being the D2 disulfide-bond reductant. It is possible, however, that reduction of the D2 disulfide may occur indirectly through redox control of another cell-surface protein by thioredoxin.

It is not known if thioredoxin functions as a single turnover reductant at the cell surface or if it acts catalytically to reduce several CD4 molecules. For thioredoxin to act catalytically, a mechanism is required to reduce the oxidized form of the protein. Thioredoxin reductase and its cofactor, NADPH, reduce oxidized thioredoxin inside the cell (20). Interestingly, thioredoxin reductase is secreted by peripheral blood mononuclear cells and is present in plasma (43). It seems unlikely, however, that sufficient NADPH would exist in the extracellular milieu to enable thioredoxin reductase to catalyze reduction of thioredoxin. Another possibility is the cell-surface NADH-oxidoreductase system (5), which has been implicated in reduction of extracellular protein disulfide bonds. The reduction of cell-surface CD4 by thioredoxin and the consequences for CD4 structure are summarized in Fig. 2.

PDI CLEAVES DISULFIDE BOND(S) IN GP120

gp120 is the HIV-1 envelope glycoprotein that binds to D1 of CD4 (44). PDI has recently been shown to cleave, on average, two disulfide bonds in gp120 (3, 17).

Gallina *et al.* (17) reported that purified PDI and $CD4^+$ cells cleave disulfide bonds in recombinant gp120, and Barbouche *et al.* (3) showed that the thiol content of gp120 increased from 0.5–1 mol of SH/mol of gp120 to 4 mol of SH/mol of gp120 after interaction with cells expressing CD4 and the chemokine receptor, CXCR4. Reduction of cell-bound gp120 was prevented by PDI inhibitors in both studies. These results show that probably two of the nine gp120 disulfides are reduced by PDI upon interaction of gp120 with $CD4^+$ cells. Barbouche *et al.* (3) suggest that two of the three disulfide bonds that straddle the V1/V2 (Cys^{126}-Cys^{196} and Cys^{119}-Cys^{205}) and V4 loops (Cys^{385}-Cys^{418}) are the bonds most likely reduced by PDI.

It appears, therefore, that thioredoxin and PDI mediate separate redox events during HIV-1 entry. Interestingly, these oxidoreductases do not appear to cross-catalyze. Thioredoxin cleaves disulfide bonds in CD4 but not gp120 (30), whereas PDI cleaves disulfide bonds in gp120 but not CD4 (17).

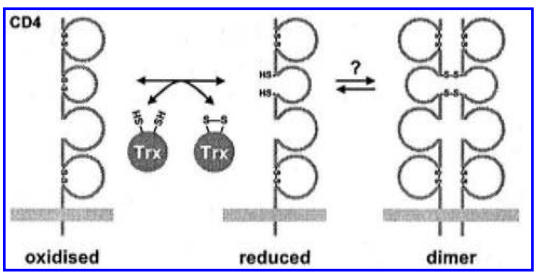

FIG. 2. Illustration of the redox change in CD4 and dimer formation. The thioredoxin dithiol/disulfide represents the active site Cys pair. Disulfide-dependent dimer formation may or may not be facilitated by a cellular factor. Trx, thioredoxin.

THIOL ALKYLATING COMPOUNDS AND PDI INHIBITORS BLOCK HIV-1 ENTRY

The thiol alkylating agents, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *p*-aminophenylarsenoxide (aPAO), and 4-[*N*-(*S*-glutathionylacetyl)amino]phenylarsenoxide (GSAO) (13) inhibit HIV-1 entry (15, 17, 30, 40). DTNB and aPAO also inhibit envelope-mediated cell–cell fusion (15, 17), although GSAO does not inhibit this event (P.J. Hogg, unpublished observations).

Ryser *et al.* (40) and Fenouillet *et al.* (15) reported that anti-PDI monoclonal antibodies inhibit HIV-1 infection and envelope-mediated cell–cell fusion, respectively, and Gallina *et al.* (17) showed that *N*-acetyltriiodothyronine, which binds to PDI and blocks its interaction with substrates, inhibits HIV-1 entry and cell–cell fusion.

These findings support the notion that dithiol/disulfide redox events are important for HIV-1 entry, and perhaps cell–cell fusion. It is not possible to distinguish between perturbation of the CD4/thioredoxin and/or gp120/PDI systems by the thiol alkylating compounds. The compounds are known to inactivate both thioredoxin and PDI, and GSAO binds to the reduced dithiol form of CD4 (30). It may be that perturbation of both systems is required to block HIV-1 entry. It is informative, though, to speculate on the mechanism of action of GSAO. The consequence that binding of GSAO to reduced CD4 has for CD4 structure will be an important clue to understanding the role of CD4 reduction in HIV-1 entry.

The mechanism of action of GSAO is perhaps twofold: inactivation of thioredoxin, which prevents further reduction of CD4, and binding to the reduced CD4. It may be that GSAO locks the reduced CD4 into an oxidized configuration by constraining the movement of the D2 thiols. Alternatively, GSAO may not hinder any conformational change, but simply prevent the D2 dithiol from reforming the disulfide bond (Fig. 3).

WHY IS THE REDOX CHANGE IN CD4 AND GP120 IMPORTANT FOR HIV-1 ENTRY?

The reason why redox activity of the D2 disulfide is important for HIV-1 entry is unknown. Binding of HIV-1 to D1 of CD4 is not affected by the redox change in D2 (30; L.J. Matthias and P.J. Hogg, unpublished observations). There is

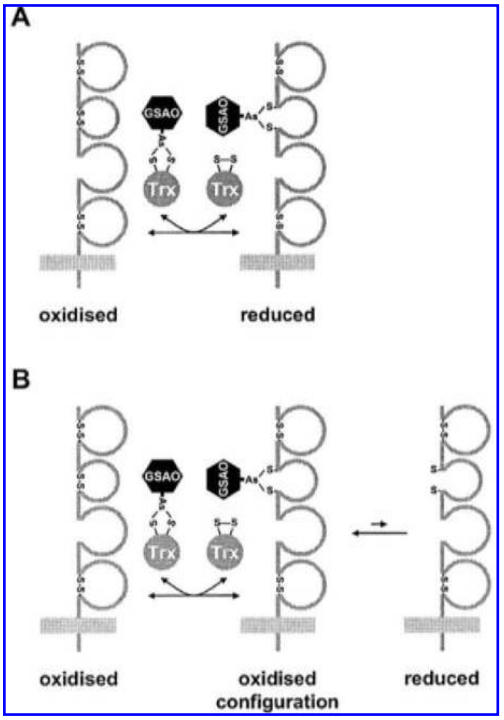

FIG. 3. Possible consequences of binding of GSAO for CD4 structure. GSAO inactivates thioredoxin, which prevents further reduction of CD4, and binds to reduced CD4. GSAO may simply prevent the CD4 dithiol from reforming the disulfide bond (**A**) or may lock the reduced CD4 into an "oxidized configuration" by constraining the movement of the free thiols (**B**). Trx, thioredoxin.

evidence that D2 undergoes a conformational change upon binding of HIV-1 to D1 (2, 9, 32, 42), which may be required for fusion of the viral and cell membranes. It is possible, therefore, that the redox activity of the D2 disulfide bond is involved in this conformational change. It is also possible that disulfide-dependent self-association of CD4 through the D2 domain plays a role in HIV-1 entry (26). In preliminary findings, we have observed that HIV-1 enters cells expressing a mutant CD4 in which the D2 disulfide has been eliminated by replacing both Cys with Ala. The efficiency of entry via the mutant CD4 was comparable to that for wild-type CD4. This finding is consistent with the notion that reduction of the D2 disulfide is involved in important conformational changes in D2 following HIV-1 binding to D1.

Gallina *et al.* (17) proposed that PDI associates with CD4 on the cell surface and reduces disulfide bonds in gp120 after the virus binds to CD4. They suggest that the conformational change in gp120 that accompanies reduction drives virus–cell and cell–cell fusion. The finding that gp120 and PDI interact both in solution and at the cell surface supports this proposal. Barbouche *et al.* (3) concluded that PDI-mediated reduction of disulfide bonds in gp120 occurs after chemokine receptor binding and is required for fusion. Both studies support an important role for PDI in gp120-mediated events in HIV-1 entry.

FUTURE DIRECTIONS

Our preliminary observations indicate that reduction of the CD4 D2 disulfide is important for conformational changes in D2 that are required for HIV-1 entry. This theory is being tested by engineering a new disulfide bond and/or a salt bridge into the D2 domain in an effort to restrict the conformational change in the domain that results from reduction of the cross-strand bond. This engineered CD4 is predicted not to permit HIV-1 entry. Comparison of the structure of oxidized and reduced CD4 would also be informative, as would the structure of reduced CD4 in complex with GSAO.

How thioredoxin reduces CD4 is also not known. The D2 disulfide is mostly hidden from solvent in the crystal structure of the D1-D2 fragment (41, 49). This observation implies that thioredoxin induces a conformational change in CD4 that exposes the D2 disulfide bond to reduction. The nature of the conformational change might be understood if an intermediate mixed-disulfide complex between thioredoxin and CD4 could be made and the structure determined. The conformational change in DsbDα, an Ig-fold thiol oxidoreductase, induced by DsbC, a disulfide-bond isomerase, was recently described using this approach (18).

It is not known whether reduction of gp120 disulfide bond(s) by PDI is an absolute requirement or facilitates HIV-1 entry. It would be informative to test the effect of PDI inhibitors on entry of a CD4-independent HIV-1. Entry of such a virus is through interaction of HIV-1 gp120 with the chemokine receptor. For example, GSAO blocked entry of CD4-dependent but not a CD4-independent virus (30). This was compelling evidence for an important role for CD4 reduction in HIV-1 entry. Gallina *et al.* (17) point out, however, that the gp120 mutations that confer CD4-independent entry may mimic the change in gp120 induced by PDI. Identification of the disulfide bond(s) in gp120 that are cleaved by PDI would shed light on this question. How PDI-induced conformational changes in gp120 influence chemokine receptor binding and activate the fusogenic properties of nearby gp41 are other important questions.

The current anti-HIV-1 drugs block HIV-1 maturation by targeting viral reverse transcriptase and protease. Problems with drug toxicity, however, and the emergence of drug resistance have highlighted the need for drugs with different modes of action. HIV-1 entry inhibitors like GSAO or the specific PDI inhibitors would complement those that interfere with viral maturation (31). T-20, a peptide entry inhibitor that is showing promising results in early-stage clinical trials, has an IC_{50} for inhibition of fusion of the viral and cell membranes of ~0.1 μM in cell culture (11). For a thiol-reactive HIV-1 entry inhibitor to be effective *in vivo,* therefore, it will most likely require an IC_{50} of at least 0.1 μM. We are taking two approaches to improve the affinity of GSAO for reduced CD4 and thioredoxin. Firstly, a nitro group is being situated *para* to the arsenic atom in GSAO, which is expected to activate the arsenic atom toward dithiols. This is because the nitro group withdraws electron density from the ring. Secondly, the arsenic atom in GSAO is being replaced by antimony. Antimony is more polarizable than arsenic and should bind the sulfur atoms of closely spaced dithiols more tightly.

ABBREVIATIONS

aPAO, *p*-aminophenylarsenoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSAO, 4-[*N*-(*S*-glutathionylacetyl) amino]phenylarsenoxide; HIV-1, human immunodeficiency virus type 1; Ig, immunoglobulin; PDI, protein disulfide isomerase.

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Received for publication October 8, 2002; accepted November 12, 2002.

This article has been cited by:

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