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Review

Immunoregulation through membrane proteins modified by reducing conditions induced by immune reactions

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Selected disulfide bonds in membrane proteins are labile and are thus susceptible to changes in redox potential and/or the presence of thiol isomerase enzymes. Modification of these disulfide bonds can lead to conformational changes of the protein that in turn may alter protein activity and function. This occurs in the entry of several enveloped viruses into their host cells, e.g. HIV, hepatitis C virus and Newcastle disease virus. Labile disulfide bonds are also important in platelet activation, cytokine signalling and in a variety of diseases including cancer and arthritis. In this review we will concentrate on recent advances in understanding the conditions that lead to disulfide bond reduction in membrane proteins and their effects in regulating immune function.

Keywords: Disulfide redox · Immunoregulation · Membrane protein · Virus fusion

Labile disulfide bonds

A role in regulating membrane protein function

Cysteine is a unique aa in that it contains a sulfydryl group (–SH) that can covalently bond with a sulfydryl group of a separate cysteine residue to form a disulfide bond. Disulfide bonds can form between cysteines within the same polypeptide chain (intramolecular disulfide bond) or between cysteines on different polypeptide chains (intermolecular disulfide bond). The formation of disulfide bonds requires oxidation of the constituent cysteine residues. Once formed disulfide bonds are not chemically inert, they can be reduced back to their constituent cysteine residues. Although disulfide bonds are clearly important structurally, there is increasing evidence that they can be reduced under certain physiological conditions and that this can affect the activity of the protein itself with downstream functional consequences. In this review we discuss how these redox events may be important in regulating the immune system and we review recent data on the characterisation of membrane proteins that contain labile disulfide bonds.

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Structure and modification

The disulfide bonds formed between cysteine residues in proteins play an important structural role such as stabilising Ig-like domains in the harsh extracellular environment by bridging the beta sheets at the core of the fold, stabilising dimer formation for example, bridging between the light and heavy chains of Ig. Some disulfide bonds have a catalytic role notably in thioredoxin and protein disulfide isomerases (PDIs). In addition other disulfide bonds can be reduced and these can lead to changes in protein structure and have been termed 'allosteric'. The disulfide bonds themselves are heterogeneous in geometry and this is discussed in detail by Schmidt et al. [1] who studied the geometry of about 7000 disulfide bonds from known structures. In this review we use term labile to describe those disulfide bonds that can be reduced under physiological conditions. It seems likely that they will cause conformational changes but, in the absence of such data, we use the simple term 'labile' meaning easy to break. Recent screening studies have shown that a surprisingly large number of different

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membrane proteins have labile disulfide bonds that can be reduced by mild reducing conditions in vitro and that many are reduced in vivo during inflammation [2]. The proteins identified include integrins, adhesion proteins, cytokine and chemokine receptors, Ag receptors and transporters.

PDI is an enzyme involved in catalysing disulfide bond formation, reduction and isomerization [3–5]. As a member of the thioredoxin superfamily, it contains regions with high aa sequence identity to the thioredoxin active site, which is comprised of a double-cysteine motif (CXXC, C refers to cysteine and X any aa). Disulfide bond reduction by PDI and thioredoxin superfamily members is a catalytic process where one molecule of enzyme can reduce many disulfide bonds. However co-enzymes such as thioredoxin reductase are needed to supply electrons to PDI to allow continuous turnover. This is controlled by the NADPH pathway [6].

Reducing conditions at the cell surface and extracellularly

The cytoplasm of cells is maintained under reducing conditions that are highly controlled, for example, in disease situations and in processes such as the oxidative burst. This area is beyond the scope of this review and is extensively reviewed elsewhere [7]. The extracellular space is generally oxidising but decreases in redox potential occur in inflammation and immune activation. For example, activation of DCs leads to the production of free extracellular cysteine [8], in vivo immunisation leads to increased free thiol production and induction of thioredoxin expression in lymphoid organs [9] and levels of extracellular thioredoxin are increased in lung injury [10]. Enzymes such as the PDI members are generally associated with the ER where they assist in the proper folding of proteins but they are also found in other locations such as the cell surface, the extracellular space, the cytosol and the nucleus [4]. Localisation of PDI and related proteins on the cell surface or the extracellular space can cause the reduction of labile disulfide bonds in membrane proteins, which may be important for viral infection, disease or cell signalling [4, 5]. Thus the presence of thioredoxin and PDI provides the mechanism to alter disulfide bonds in membrane proteins as discussed later in a variety of different situations.

Viruses

HIV

Enveloped viruses infect host cells by firstly binding to specific host cell receptors and then fusing with the host cell membrane. Virus-cell membrane fusion is a consequence of conformational changes in the envelope (ENV) proteins. These changes can be triggered by acidification, as in influenza virus infection [11], or by rearrangement of disulfide bonds by PDI family members as shown in HIV and other viruses such as, HCV, Newcastle disease virus and rotavirus as discussed in this and subsequent sections.

HIV is a major human pathogen that causes Acquired Immune Deficient Syndrome (AIDS). During HIV entry into the host cell, HIV ENV gp 120 (gp120) binds the CD4 receptor on T cells or macrophages [12]. HIV uptake can be blocked by preventing disulfide bond reduction by several agents including the PDI inhibitor bacitracin, the membrane-impermeable sulfhydryl reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and Abs against PDI (reviewed in [13]). Various disulfide bonds in gp120 have been implicated as being labile using thioredoxin, PDI and glutaredoxin [14–17]. A recent comparison of PDI, thioredoxin and DTT indicates reduction to some extent of five disulfide bonds in gp120 [18, 19], and complexes of PDI and gp120 have been identified [16]. It is proposed that the conformational change that occurs due to disulfide bond reduction leads to structural changes in gp41 which, in turn lead to the fusion of the viral envelope with the host cell [20].

CD4, the receptor for HIV, also contains a labile disulfide bond in domain 2 of the four extracellular Ig-like domains [21]. There is evidence that CD4 may exist as disulfide-linked multimers on the T- and myeloid-cell surfaces and that multimer formation increases upon activation of a myeloid cell line with PMA [22]. It is proposed that HIV has a preference for binding to the monomeric, reduced form of CD4 [23]. Interestingly thioredoxin but not PDI was effective in reducing the labile disulfide bond of CD4 whereas PDI is clearly involved in HIV uptake [13]. The role of disulfide exchange in the various stages of HIV uptake and shedding together with possibilities for therapy has been extensively studied and is reviewed in [13].

HCV

HCV, which primarily infects the liver, is the causative agent of hepatitis C. In severe cases patients develop chronic liver infections and subsequent liver cirrhosis, carcinoma, hepatitis and liver failure [24]. Entry of HCV is a slow and complex multistep process that involves initial attachment of the virion to the host cell, interaction of the envelope proteins with the host cell surface receptors and subsequent internalisation by membrane fusion (reviewed in [25]). The two viral envelope proteins E1 and E2 are expressed as inactive precursors [26] which associate as large covalent complexes [27]. The two envelope proteins E1 and E2, contain 8 and 18 cysteine residues, respectively [28]. Alkylation of free cysteine residues on viral particles containing E1 and E2 has been shown to block the uptake of the virus [28]. This process, however, could not be rescued by addition of reducing agents as is the case for other retroviruses [28]. Recent mutagenesis studies on E2 showed that all nine disulfide pairs were strictly required for infection. The role of cysteines is complex as mutagenesis of individual cysteines had differential effects on assembly with E1 and binding to the tetraspanin membrane protein CD81 [29].

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Newcastle disease virus

Newcastle disease virus infection is extremely rare in humans and usually only occurs in people in close contact with infected birds. Entry into the host cell is mediated by viral haemagglutinin neuraminidase (HN) and fusion (F) gp attachment to the host cell. Free thiols in the F gp, which are required for virus entry into the cell [30], are only present after binding of the virus to the target cell surface [31]. This suggests that labile disulfide bonds are reduced before major conformational changes in the F protein occur and before activation by HN. How the appearance of free thiols influences F protein activation or conformational changes is as yet unknown [30]. The enzyme responsible for catalysing the reduction of disulfide bonds in F protein is likely to be PDI due to the observation that membrane-impermeable inhibitors of PDI (DTNB, bacitracin and anti-PDI Ab) could suppress the formation of free thiols and inhibit virus entry [30]. Further support for this is the observation that over-expression of PDI resulted in significantly increased cell–cell fusion mediated by F and HN proteins [32].

Other viruses

Labile disulfide bonds are likely to be important in other viruses. For instance in the rotaviruses that are a major cause of diarrhoea, in addition to proteolytic cleavage of the surface proteins, labile disulfide bonds are implicated in virus entry by the blocking of virus uptake by reagents such as DTNB, bacitracin and Abs to PDI [33]. DTNB also inhibited the uptake of the Sindbis alphavirus that causes fever in humans and, interestingly, virus fusion could be enhanced by exogenous reducing agent [34]. Although the SARS (severe acute respiratory syndrome) coronavirus contains free cysteine and labile disulfides, cysteine blocking reagents such as DTNB and bacitracin had no effect on virus uptake [35].

The extracellular redox state affects immune regulation, disease and clotting

Immune regulation

A reducing environment is important in the generation of immune responses as naive T cells do not express a cystine transporter and require a source of extracellular cysteine. X_C^- is a major transporter for cystine that is comprised of a common CD98 chain and a specific chain for cysteine, xCT. The DCs provide the source of cysteine for T cells. Although it was originally thought that, on activation, DCs secrete thioredoxin that in turn can reduce cystine to cysteine (Fig. 1) [8], further analysis suggests this is not the main cysteine source for T cells [36]. Blocking the X_C^- transporter, for example, prevents cysteine accumulation in DCs indicating the importance of cystine uptake by the DCs and secretion of cysteine [36]. When Tregs act on DCs, this process is inhibited [36]. This

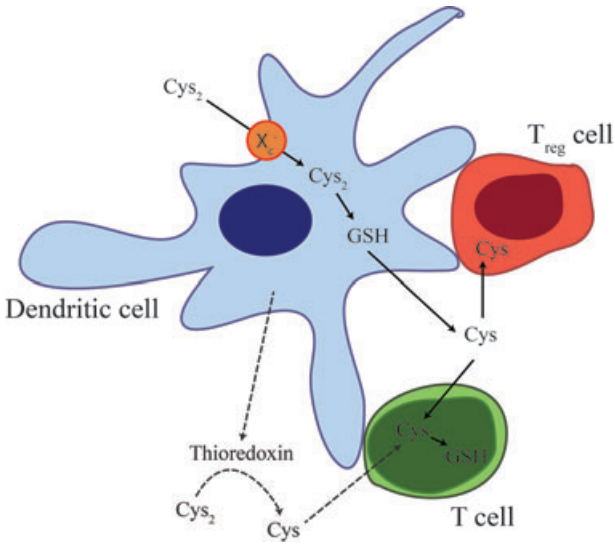

Figure 1. Tregs can limit redox changes. Resting T cells require a source of extracellular cysteine (Cys) as they lack the cystine (Cys_2) transporter X_C^- [8, 36, 37]. This transporter is, however, expressed by DCs meaning that these cells can generate glutathione (GSH) (cystine is rate-limiting for glutathione production); this, in turn, leads to the secretion of Cys that can be utilised by T cells. Thioredoxin secretion from DCs can be induced by activation but in the absence of a source of electrons, which are required for conversion of cysteine from cystine by thioredoxin, may not be a major source of extracellular Cys for T cells. The interaction of Tregs with DCs leads to inhibition of extracellular Cys [37].

inhibition depends on cell contact between the DC and the Treg and the modulation of glutathione metabolism in the DC [37].

The importance of the cystine transporter system for the immune system is illustrated in the gut, where lamina propria macrophages in mice do not normally express the X_C^- transporter which is, in contrast, expressed in peripheral blood monocytes. Gut lamina propria macrophages are therefore unable to take up cystine and hence cannot provide cysteine for the neighbouring T cells [38]. This may be important for maintaining an environment in the healthy gut that is more tolerant than that found in inflammatory bowel disease, a disease state in which there is increased expression of the X_C^- transporter and higher T-cell activity [38].

In addition to the metabolic effects of cystine transport and reduction, the reducing environment has effects on membrane and secreted proteins. This is illustrated by the breadth of membrane proteins on leukocytes that have labile disulfide bonds that are susceptible to the reducing conditions that are typical of both T-cell activation and innate activation in an LPS model of inflammation [2]. In one case a labile disulfide bond was identified in CD132 (see Fig. 2), the common gamma chain of several cytokine receptors, which on reduction prevented IL-2 signalling [39]. Signalling of other cytokine receptors seems likely to be affected [40] with possible additional effects on cytokines as well as IL-4 has a labile disulfide that leads to loss of activity upon reduction [41]. The implication is that the effects of the pro-inflammatory cytokines may be ameliorated by the redox changes resulting from

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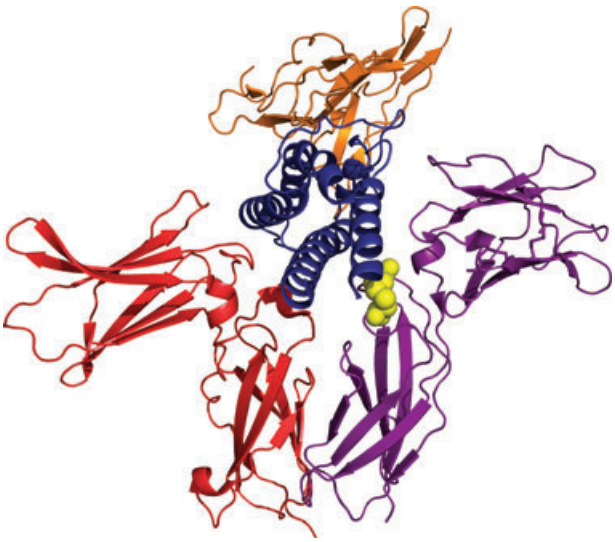

Figure 2. One of the disulfide bonds in CD132 is labile and lies at the interface with IL-2. Crystal structure of the IL-2/IL-2 receptor complex (PDB code 2ERJ). The labile disulfide bond (yellow spheres) in CD132 (purple) is in direct contact with the cytokine, IL-2 (blue). The IL-2 receptor alpha chain (CD25) is shown in orange and the beta chain (CD122) is shown in red.

the inflammatory response itself – a feedback control mechanism. It seems likely that other cytokine/chemokine interactions are affected by redox conditions. For example, thioredoxin affects the chemokine-induced chemotaxis of eosinophils although the molecular basis for this is unclear [42]. A different type of cytokine is the high mobility group box 1 (HMGB1) that is both a nuclear protein that regulates transcription and a secreted protein demonstrating extracellular inflammatory cytokine activity. The latter activity is dependent on one free cysteine and a disulfide bond that can be reduced by mild reducing conditions [43].

Redox changes, such as secretion of thioredoxin, occur to balance ROS production. For example, the induction of IL-1β processing in monocytes involves firstly an ROS response and then an anti-oxidant response but studies with inhibitors show that both are necessary for production of IL-1β [44]. As the effects of ROS on the immune system have recently been reviewed [45], this shall not be discussed further here.

Cancer

An important aspect of cancer resistance to chemotherapy is the up-regulation of anti-oxidant systems such as the cysteine/cystine redox cycle, PDI, thioredoxin, glutathione [46] and the X_C^- cystine transporter [47]. A possible link to the cell surface is indicated by a CD44 variant (CD44v) that interacts with the xCT component of the cysteine transporter; ablation of CD44v leads to inhibition of xCT and subsequent suppression of tumour growth [48].

The analysis of differential expression of proteins in invasive glioma cells and angiogenic glioma cells revealed that PDI is overexpressed in the invasive phenotype [49]. Interestingly, PDI was found in the tumour periphery but not in the angiogenic core. This led to the hypothesis that PDI has a functional role in glioma cell migration, which is supported by the observation that tumour cell migration is inhibited by bacitracin, a non-selective PDI inhibitor, and by a PDI Ab [49]. PDI can reduce disulfide bonds in many proteins, but one class of particular interest with respect to cancer is the metalloproteinase family of enzymes that can modulate the extracellular environment and is important in metastasis; PDI had recently been shown to regulate the activity and secretion of MMP-9 [50]. A key element in these enzymes is that the catalytic site contains a Zn ion coordinated to histidine residues and one cysteine; the site is in an inactive state until the cysteine coordination is broken – the 'Cys Switch' – by changes in the conformation of the protein that exposes the Zn ion to solvent [51, 52]. The potential for redox involvement in the immune regulation of cancer is large as illustrated by recent data indicating that chronic lymphocytic leukemia cells have high levels of surface PDI and thioredoxin that are associated with receptors for TNF, and blocking PDI and thioredoxin activity inhibits the production of the autocrine TNF [53].

Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory disease that is triggered by both environmental and genetic factors. A link between rheumatoid arthritis regulation and the ROS system is provided by the finding that neutrophil cytosolic factor 1 (Ncf1) is a regulator of rheumatoid arthritis [54]. Ncf1, is a component of the NADPH oxidase complex which catalyses the reduction of oxygen to ROS (reviewed in [55–57]). With regard to membrane proteins in particular, a decreased level of ROS has been shown to increase the number of reduced thiol groups (–SH) on the T-cell surface [58]. This resulted in increased activation and proliferation of T cells, as well as arthritis incidence and severity [58].

Platelet activation is associated with redox changes at the cell surface

Platelets are involved in haemostasis, wound healing and atherosclerosis. There are extensive data to suggest that platelet activation is associated with an increase in the extracellular redox potential and several PDI family members come to the cell surface [59–61]. A key protein that can be modulated by reduction is the fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$. This is the most abundant integrin on the platelet surface and its activation leads to increased binding of adhesive ligands such as fibrinogen, fibronectin and von Willebrand factor which in turn promote thrombus formation [59]. The β subunit of $\alpha_{IIb}\beta_3$ contains 56 highly conserved cysteine residues which all form disulfide bonds [62]. Interestingly, the active state of $\alpha_{IIb}\beta_3$ seems to be attained by the reduction of cysteines within the EGF-like domains $\alpha_{IIb}\beta_3$ [63]. Mutagenesis of a single disulfide in the β_3 chain leads to constitutively active $\alpha_{IIb}\beta_3$ [64] whereas a more extensive mutagenesis

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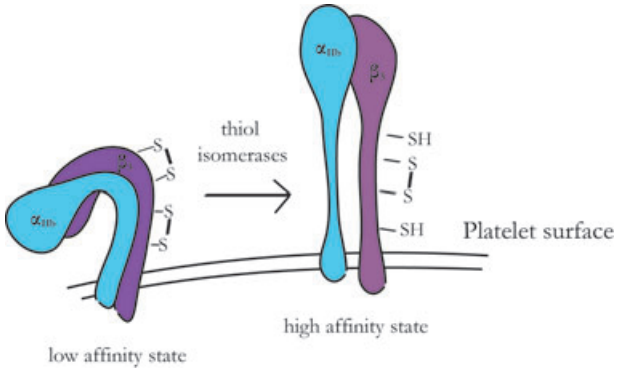

Figure 3. Scheme showing the redox regulation of the $\alpha_{IIb}\beta_3$ integrin on platelets. The resting state of $\alpha_{IIb}\beta_3$ has a low affinity for fibrinogen, $\alpha_{IIb}\beta_3$ being present in its oxidized state. The high affinity state is reached by reducing disulfide bonds on $\alpha_{IIb}\beta_3$.

of specific disulfide bonds in β_3 gave differential affects on the function of α_{IIb} and α_V integrins [65]. Integrins are unusual in containing disulfide bonds not only in the extracellular domains but also in their intracellular part and mutagenesis of the latter can also affect ligand binding [66]. The binding capacity of integrins can be modulated by various stimuli and the underlying concept is that the disulfide bonds maintain the integrin in a less active form (Fig. 3). This is evidenced by the finding that cysteine mutations and the addition of reducing agents result in increased ligand binding [67–69] and, more recently, a PDI inhibitor was shown to block thrombus formation [70]. In addition to the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$, other platelet surface proteins are known to have increased levels of free thiols, such as the $P2Y_{12}$ ADP receptor, gp Ib adhesion receptor α (GPIbα) and the gp VI collagen receptor (GPVI) on platelet activation [59]. Thus, redox changes at the surface of platelets seem likely to be important in regulating clotting.

Concluding remarks

Although the role of disulfide bonds in stabilising protein structures is well known, we have only recently started to understand the role of redox labile disulfide bonds. By changing from an oxidized to a reduced state, these bonds can control protein function. Labile disulfide bonds in membrane proteins play a key role in a number of viral infections, as well as in rheumatoid arthritis, cancer, platelet activation and cytokine signalling. Given the large numbers of membrane proteins with labile disulfides, it is likely that they may be important in immune responses, a variety of diseases and inflammation. These molecular mechanisms could be important for therapy based on four types of strategy: (i) prevention of the reduction of labile disulfide bonds by Abs that block PDI access to specific targets; (ii) the use of small molecular inhibitors or Abs to target specific PDIs; (iii) targeting the pathways that lead to activation of the cell surface PDIs; and (iv) targeting the transporters involved in the redox system.

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Abbreviations: **DTNB**: 5,5'-dithiobis (2-nitrobenzoic acid) · **HN**: haemagglutinin neuraminidase · **PDI**: protein disulfide isomerase

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