Short Communication

Snapshot of the interaction between HIV envelope glycoprotein 120 and protein disulfide isomerase

Zhiqiang Wang¹, Zhimin Zhou¹, Zhan-Yun Guo¹*, and Cheng-Wu Chi¹,²*

¹Institute of Protein Research, College of Life Sciences and Technology, Tongji University, Shanghai 200092, China
²State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences, Shanghai 200031, China

*Correspondence address. Tel: +86-21-65988643; Fax: +86-21-65988403; E-mail: zhan-yun.guo@tongji.edu.cn (Z.-Y.G.).
Tel: +86-21-54921165; E-mail: zwqi@sibs.ac.cn (C.-W.C.)

The human immunodeficiency virus-1 (HIV-1) envelope glycoprotein 120 (gp120) binds to cell surface receptors and mediates HIV entry. Previous studies suggest the cell surface protein disulfide isomerase (PDI) might interact with disulfide bond(s) of gp120 and thus facilitate HIV-1 entry. In the present study, a kinetic trapping approach was used to capture the disulfide cross-linking intermediate between gp120 and PDI. Active site mutant PDIs were prepared in which the C-terminal cysteine at the active site was replaced by a serine. The active site mutant PDIs were able to covalently cross-link with gp120 through a mixed disulfide bond in vitro. The cross-linking efficiency was enhanced by CD4 protein (primary receptor of HIV-1) and was inhibited both by bacitracin (a PDI inhibitor) and by catalytically inactive PDI. The present results suggested the cell surface PDI might play a role in HIV entry in vivo.

Keywords protein disulfide isomerase; glycoprotein 120; kinetic trapping; disulfide bond

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Introduction

The acquired immune deficiency syndrome (AIDS) is a global pandemic caused by infection with human immunodeficiency virus-1 (HIV-1). The first step of HIV-1 infection is viral entry, mediated by its trimeric envelope glycoprotein (Env). Each mature monomeric Env is composed of a surface protein, glycoprotein 120 (gp120), and a transmembrane protein, gp41, held together by non-covalent interactions. gp120 can bind to its primary receptor, CD4 protein, which presents on the host cell surface. This binding induces a conformational change in gp120 and enables it to bind to an appropriate coreceptor, CXCR4 or CCR5. Engagement of gp120 to the coreceptor triggers conformational changes in gp41, leading to exposure and insertion of the gp41 fusion peptide into the host cell membrane, which facilitates fusion of the virus envelope with the host cell membrane and release of the viral capsid into the host cytosol [1,2].

Aside from the primary receptor and coreceptor, other cell surface proteins/enzymes might facilitate HIV-1 entry. Sulphydryl modification agents can significantly inhibit HIV entry, suggesting that thiol-disulfide exchange might be involved in the process [3]. Consistent with this observation, gp120 contains nine conserved disulfide bonds, although its amino acid sequence is hypervariable. Previous studies showed that cell surface protein disulfide isomerase (PDI) is a probable candidate that might facilitate HIV-1 entry. Sulphydryl modification agents can significantly inhibit HIV entry, suggesting that thiol-disulfide exchange might be involved in the process [3]. Consistent with this observation, gp120 contains nine conserved disulfide bonds, although its amino acid sequence is hypervariable. Previous studies showed that cell surface protein disulfide isomerase (PDI) is a probable candidate that might facilitate HIV entry [4–7]. PDI is a prototype of the oxidoreductases [8–10]. It contains four thioredoxin-like domains (a, b, b′, and a′). Among these, both the a and a′ domains have a dithiol active site (CGHC motif), whereas the b′ domain is mainly responsible for substrate binding. The redox potential of the active site of PDI is −175 mV [11], a moderate value among oxidoreductases. Therefore, PDI can function as oxidase, reductase, and isomerase. Crystal structure shows that the four domains of PDI are arranged in a U-shaped structure, with the active sites facing each other across the long sides of the U-shape [12]. PDI is primarily located in the endoplasmic reticulum (ER) lumen and catalyzes oxidative protein folding [13,14]. PDI can also escape from the ER lumen through the secretary pathway and loosely bind to the cell surface [15–17].

In the present study, a kinetic trapping approach was used to capture the disulfide cross-linking intermediate between gp120 and PDI. The present results showed that PDI preferably reacted with certain disulfide bond(s) of gp120, suggesting the cell surface PDI might play a role during HIV entry in vivo.
Materials and Methods

Materials
The expression construct of the wild-type human PDI (with a 6 × His-tag at its N-terminal) was a generous gift from Prof. L.W. Ruddock (Biocenter, University of Oulu, Finland). HIV-1 gp120 protein (SF162 strain, cat# 4615) and soluble CD4 protein (cat# 7363) were generously provided by the NIH AIDS Research and Reference Reagent Program.

Construction and expression of the active site mutant PDIs
The active site mutant PDIs were generated by a MutanBEST site-directed mutagenesis kit (Takara, Dalian, China) using the construct of wild-type human PDI as template. The expected mutations were confirmed by DNA sequencing. A short DNA fragment encoding a hemagglutinin (HA)-tag was chemically synthesized, digested by restriction enzyme NdeI, and ligated into the construct of [CS:CS]PDI that was pretreated with the same restriction enzyme. The correct insertion of HA-tag was confirmed by DNA sequencing. The DNA fragment encoding the b or b′ domain was amplified from the wild-type human PDI construct by polymerase chain reaction, and subsequently cloned into a pET vector with a 6 × His-tag. The mutant PDIs or PDI fragments were expressed in Escherichia coli BL21(DE3) strain and purified by metal-ion affinity chromatography (Ni²⁺-chelating Sepharose™ Fast Flow resin; GE Healthcare, Uppsala, Sweden). The purified PDIs were treated with 5 mM dithiothreitol (DTT) overnight at room temperature, and the excess amount of DTT was removed by gel filtration (Sephacryl S-100 HR; GE Healthcare). The eluted reduced PDIs were stored at −80°C for later use.

In vitro trapping the PDI–gp120 intermediate
HIV-1 gp120 protein and the active site mutant PDI (0.5 μM each) were co-incubated in the reaction buffer (100 mM Tris–HCl, 2 mM ethylenediaminetetraacetic acid, pH 8.0) with or without redox potential (2 mM reduced glutathione and 1 mM oxidized glutathione) in the absence or the presence of soluble oxidized CD4 protein (0.5 μM) at 25°C for 60 min. For inhibition studies, inhibitors at different concentrations were added. Then the N-ethylmaleimide (NEM) stock solution was added to the reaction mixture to the final concentration of 50 mM. The alkylated mixture was then subject to an 8% non-reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to a polyvinylidene difluoride membrane after electrophoresis and detected by western blot analysis using the anti-HA or anti-6 × His-tag monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA).

Results and Discussion

Preparation of the active site mutant PDIs
As shown in Fig. 1(A), the reduction in an existing disulfide bond by the reduced PDI is a two-step reaction. The first step is a nucleophilic attack on the substrate disulfide bond by the sulfhydryl group of the N-terminal active site cysteine, leading to the formation of a PDI–substrate intermediate covalently cross-linked with a mixed disulfide bond. The second step is a nucleophilic attack on the mixed disulfide bond by the sulphydryl group of the C-terminal active site cysteine, leading to the separation of the mixed disulfide bond. The isomerization of an existing disulfide bond by the reduced PDI is a multiple-step reaction, but the first step also forms a substrate–PDI intermediate cross-linked with a mixed disulfide bond. In general, the disulfide cross-linked PDI–substrate intermediate is short-lived and difficult to detect. As shown in Fig. 1(B), if the C-terminal active site cysteine of PDI is mutated, the reaction will be kinetically trapped at the intermediate stage, and therefore the resultant intermediate has a longer half-life and can be detected more easily.

As shown in Fig. 1(C), a series of the active site mutant PDIs were constructed by site-directed mutagenesis, recombinantly expressed in E. coli cells as soluble cytosolic proteins, and purified to essential homogeneity using immobilized metal ion affinity chromatography. To keep

![Figure 1](https://academic.oup.com/abbs/article-abstract/42/5/358/789)
the active site cysteine(s) in a reduced form, the purified mutant PDIs were treated with DTT and subsequently applied to a gel filtration column to remove excess DTT.

**Disulfide cross-linking between gp120 and active site mutant PDIs**

A kinetic trapping approach was used to capture the disulfide cross-linking intermediate between gp120 and PDI under *in vitro* condition. To facilitate the detection of the gp120–PDI intermediate, an HA-tag was fused to the N-terminal of [CS:CS]PDI in which the C-terminal active site cysteine at both the a and a’ domains was replaced with serine. As shown in Fig. 2(A), after the purified gp120 and the purified [CS:CS]PDI were co-incubated in the buffer without redox potential (lane 1), an upper band with a molecular weight of ~175 kDa (indicated by a star) was detected in addition to the monomeric PDI band (~55 kDa). The ~175-kDa band was deduced to be a disulfide cross-linked gp120–PDI intermediate (120-kDa gp120 plus 55-kDa PDI). The band above the PDI–gp120 intermediate is probably an intermediate between the gp120 dimer and the mutant PDI. The purified gp120 contained a small amount of disulfide cross-linked dimers as analyzed by SDS–PAGE (data not shown). When gp120 and [CS:CS]PDI were incubated in a redox buffer (2 mM reduced glutathione (GSH) and 1 mM oxidized glutathione, lane 2), the intensity of the ~175-kDa band decreased significantly because GSH was able to reduce the mixed disulfide bond formed between gp120 and the mutant PDI. When soluble CD4 protein was added to the mixture, the ~175-kDa band intensity was greater than the corresponding band without CD4 (lanes 3 and 4). Thus, CD4 enhanced the disulfide cross-linking between gp120 and PDI. As a control (lane 5), the anti-HA antibody did not detect gp120 and CD4 protein that did not contain the HA-tag. As shown in Fig. 2(B), DTT treatment before electrophoresis made the ~175-kDa band disappear (lane 3), suggesting that the ~175-kDa band was indeed the disulfide cross-linking intermediate between gp120 and the PDI mutant. As shown in Fig. 2(C), both [SS:CS]PDI and [CS:SS]PDI were able to cross-link with gp120 (detected by anti-6×His-tag antibody), suggesting that either the active site of PDI could interact with the certain disulfide bond(s) of gp120.

The above results showed that the active site mutant PDI was able to cross-link with gp120 *in vitro*, especially in the presence of CD4 protein. Next, cross-linking specificity was determined using bovine serum albumin (BSA). BSA, like its homolog human serum albumin, is an abundant plasma protein containing 17 disulfide bonds. As shown in Fig. 2(D), the mutant PDI was able to cross-link with gp120, especially in the presence of CD4 (lanes 1 and 2); in contrast, the BSA–PDI cross-linking intermediate could not be detected at all (lanes 3 and 4). Therefore, the active site mutant PDI could only cross-link with an unstable disulfide bond with high energy.

**Inhibitory effect of bacitracin and catalytically inactive PDI**

The above results show that the active site mutant PDI was able to interact with certain unstable disulfide bond(s) of gp120. It is known that bacitracin, a cyclic polypeptide, can inhibit the oxidoreductase activity of PDI [18]. As shown in Fig. 3(A), when bacitracin was added, the

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**Figure 2 Specific disulfide cross-linking between gp120 and the active site mutant PDIs** (A) Disulfide cross-linking between gp120 and [CS:CS]PDI in the presence of soluble CD4 protein. Purified recombinant gp120 and purified HA-tagged [CS:CS]PDI (0.5 μM each) were co-incubated in the reaction buffer (100 mM Tris–HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) with or without redox potential (2 mM GSH and 1 mM GSSG) in the absence or the presence of purified soluble CD4 protein (0.5 μM) at 25 °C for 60 min. After incubation, the mixture was treated with NEM (50 mM) and then subjected to a non-reducing 8% SDS gel. The proteins were detected by western blot analysis using the anti-HA monoclonal antibody. The asterisk indicated the disulfide cross-linked gp120–PDI intermediate. (B) DTT treatment analysis. The [CS:CS]PDI and gp120 were co-incubated in the buffer without redox potential in the absence of CD4 protein as described in (A). Before loading onto the SDS gel, the samples were further treated by 100 mM DTT for 20 min or not. Others were same as described in (A). (C) Disulfide cross-linking between gp120 and [SS:CS]PDI or [CS:SS]PDI. The gp120 protein was co-incubated with either [SS:CS]PDI or [CS:SS]PDI in the buffer without redox potential in the absence of CD4 protein as described in (A). For western blot, the anti-6×His-tag antibody was used to detect the mutant PDIs that carry a 6×His-tag at the N-terminal. (D) Disulfide cross-linking specificity. The purified [CS:CS]PDI (0.5 μM) was either co-incubated with gp120 (0.5 μM) in the absence or the presence of CD4 (0.5 μM) or co-incubated with BSA (1.5 μM). Others were same as described in (A). These results are a representative of at least two independent experiments.
The amino acid sequence of gp120 is hypervariable, but its nine disulfide bonds are highly conserved. Disulfide bonds are usually thought of as structural elements that can stabilize the protein tertiary structure. However, recent studies have shown that some disulfide bonds can serve as a regulatory switch for regulating the protein/enzyme function [22–27]. During HIV entry, the conformation of gp120 undergoes significant changes induced by CD4-binding and coreceptor-binding. Reduction and/or isomerization of certain disulfide bond(s) of gp120 might facilitate gp120 conformational change. The regulatory disulfide bonds (also known as allosteric disulfide bonds) are usually maintained at a high energetic state [28] and therefore can easily be attacked by oxidoreductases. Crystal structure-based energy calculations indicate that the V1/V2 region disulfide bonds 119–205 and 126–196 and the V4 region disulfide bond 385–418 have high energy [29]. Cell surface oxidoreductases might reduce these unstable disulfide bonds and thus facilitate HIV entry.

In future work, we will map the unstable and PDI-accessible disulfide bond(s) of gp120 by various biochemical and proteomic approaches. However, all of these approaches need significant amount of purified gp120 protein, so we are trying to prepare purified gp120 using suspension CHO cells cultured in a serum-free medium. The high quantity of disulfide bonds (total nine disulfide bonds in gp120) and its highly glycolated nature make it quite difficult to map the PDI-accessible disulfide bond(s) of gp120. We also tried to cross-link cell-surface-anchored gp120 (mimicking the HIV-1 virus) with the active site mutant PDI, but found that the background was high, suggesting the active site mutant PDI could cross-link other cell surface proteins. So, it seemed that more functions of the cell surface PDI are waiting to be revealed in future.

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Figure 3 Inhibition of the disulfide cross-linking between gp120 and the active site mutant PDI

(A) Inhibitory effect of bacitracin, catalytically inactive PDI, and wild-type PDI. Purified recombinant gp120 and purified [CS:CS]PDI (0.5 μM each) were co-incubated with inhibitors in the reaction buffer (100 mM Tris–HCl, 2 mM EDTA, pH 8.0) in the absence or the presence of purified soluble CD4 (0.5 μM) at 25°C for 60 min. Others were same as described in Fig. 2. The concentration of bacitracin was 1 mM; the concentration of [SS:SS]PDI and wild-type PDI was 3 μM. Neither wild-type PDI nor [SS:SS]PDI contained HA-tag. The asterisk indicates the disulfide cross-linked gp120–PDI intermediate. (B) Concentration-dependent inhibitory effect of the catalytically inactive PDI. The reaction conditions were same as described in (A). The final concentrations of [SS:SS]PDI were 12.5 μM (+), 25 μM (+ +), and 50 μM (+ + +), respectively. The concentration of bacitracin was 1 mM. (C) Inhibitory effect of the b or b’ domain of PDI. The reaction conditions were same as described in (A). The final concentrations of b or b’ domain were 50 μM (+) and 100 μM (+ +), respectively. The concentration of bacitracin was 1 mM. These results are a representative of at least two independent experiments.
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