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Redox exchange of the disulfides of human two-domain CD4 regulates the conformational dynamics of each domain, providing insight into its mechanisms of control

Gavin R. Owen [a, *], Doris Le [a], Stoyan Stoychev [b], Nichole M. Cerutti [a], Maria Papathanasopoulos [a]

[a] HIV Pathogenesis Research Unit, Department of Molecular Medicine and Haematology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, 2193, Johannesburg, South Africa
[b] Council for Scientific and Industrial Research, Biosciences, Pretoria, 0001, South Africa

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ABSTRACT

CD4, a membrane glycoprotein expressed by specific leukocytes, plays a vital role in the human immune response and acts as a primary receptor for HIV entry. Of its four ecto-domains (D1–D4), D1, D2, and D4 each contain a distinctive disulfide bond. Whereas the disulfides of D1 and D4 are more traditional in nature, providing structural functions, that of D2 is referred to as an "allosteric" disulfide due to its high dihedral strain energy and relative ease of reduction that is thought to regulate CD4 structure and function by shuffling its redox state. While we have shown previously that elimination of the pre-stressed D2 disulfide results in a favorable structural collapse that increases the stability of a CD4 variant comprising only D1 and D2 (2dCD4), we sought to further localize and determine the nature of the biophysical modifications that take place upon redox exchange of the D1 and D2 disulfides by using amide hydrogen-deuterium exchange mass spectrometry (HDX-MS) to measure induced changes in conformational dynamics. By analyzing various redox isomers of 2dCD4, we demonstrate that ablation of the D1 disulfide enhances the dynamics of the domain considerably, with little effect on that of D2. Reduction of the D2 disulfide however decreases the conformational dynamics of many of the β-strands of the domain that enclose the bond, suggesting a model in which inward collapse of secondary structure occurs around the allosteric disulfide upon its eradication, resulting in a marked decrease in hydrodynamic volume and increase in stability as previously described. Increases in the dynamics of regions important for HIV gp120 and MHCII binding in D1 also result allosterically after reducing the D2 disulfide, which are likely a consequence of the structural changes that take place in D2, findings that advance our understanding of the mechanisms by which redox exchange of the CD4 disulfides regulates its function.

1. Introduction

The cluster of differentiation 4 (CD4) is an integral membrane glycoprotein expressed on the surface of T-cells and other immune cells, and plays an important role in the immune response. On T-cells, it functions primarily as a co-receptor to stabilize T-cell receptor interactions with peptide-major histocompatibility complex class II (MHCII) complexes on antigen presenting cells [1]. Its immune functions have also been studied in the context of human immunodeficiency virus (HIV) as it is the primary receptor for viral entry via interaction with the viral envelope gp120 glycoprotein [2].

Each human CD4 monomer comprises four immunoglobulin-

Abbreviations: CD4, cluster of differentiation 4; D1-D4, domain 1 to 4 of CD4; 2dCD4, two domain CD4 variant comprising both domain 1 and domain 2; D1A, 2dCD4 domain 1 disulfide-deficient mutant comprising a double Cys/Ala substitution at the cysteine residue pair that forms the disulfide in domain 1; D2A, 2dCD4 domain 2 disulfide-deficient mutant comprising a double Cys/Ala substitution at the cysteine residue pair that forms the disulfide in domain 2; HDX-MS, amide hydrogen-deuterium exchange mass spectrometry; MHCII, major histocompatibility complex class II; HIV, human immunodeficiency virus.

* Corresponding author.
E-mail address: Gavin.Owen@wits.ac.za (G.R. Owen).

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like ecto-domains (D1-D4, residues 1–371), a transmembrane segment (residues 372–395), and a cytoplasmic tail (residues 396–433) [3]. All of the ecto-domains but domain 3 have a single, distinctive disulfide bond located in their hydrophobic cores (Cys16-Cys84, Cys130-Cys159, and Cys303-Cys345 in D1, D2, and D4, respectively). The D1 and D4 disulfide bonds link β-strands in opposing β-sheets and have molecular configurations typical of conventional, stabilizing disulfides, whereas the D2 bond links β-strands within the same β-sheet [4,5]. This atypical arrangement results in a highly unstable disulfide due to the high dihedral strain energy in these bonds from both the torsional energy of the linkage and the energy of deformation in the β-sheet in which they occur [6]. The presence of these rare disulfides in proteins can thereby introduce metastable areas and decrease the overall stability of the protein. The high energy of these bonds has been shown to facilitate their reduction; in this way, they can act to regulate protein function in a non-enzymatic, allosteric manner by triggering conformational changes upon changing between redox states [6,7].

The binding sites for the CD4 ligands MHCII and gp120 have been mapped to its first two domains (2dCD4). We have previously studied the disulfide bonds in the first two domains of CD4 and have shown how the secondary structure of each of the single domains depends on the redox state of the corresponding disulfide. We have also illustrated the metastability of the allosteric disulfide bond in domain 2, showing that redox shuffling of the bond results in previously undescribed conformational changes in CD4 [8]. Through these studies we proposed that elimination of the D2 disulfide results in a favorable structural collapse that increases the stability of the protein. Here, we further compare the effects of D1 and D2 disulfide removal on the conformational dynamics of 2dCD4 via amide hydrogen-deuterium exchange mass spectrometry (HDX-MS). We show that elimination of the disulfide bond in domain 1 greatly increases the conformation dynamics of the domain, whereas it has little effect on the dynamics of domain 2. In contrast, reduction of the D2 bond decreases the conformational dynamics of the domain, despite allosterically increasing the dynamics of regions important to gp120 and MHCII binding in domain 1. These findings provide an understanding of the biophysical changes in CD4 upon redox shuffling, which may have important implications for anti-HIV drug development, as well as other immunomodulatory drugs.

2. Materials and methods

2.1. Hydrogen deuterium exchange mass spectroscopy (HDX-MS)

HDX-MS was used to analyze the effect that the redox state of the individual disulfide bonds in each domain of the first two domains of CD4 (2dCD4) has on the conformational dynamics of the protein. This was achieved using specific 2dCD4 variants that simulate the reduction of each disulfide bond separately, namely: wild-type 2dCD4, wild-type 2dCD4 that has been fully-reduced using 100 mM dithiothreitol (DTT), and two 2dCD4 mutants comprising double Cys/Ala substitutions at either of the cysteine residue pairs that form the disulfide bonds in each domain (referred to as 'D1A' and 'D2A' 2dCD4 variants, for the domains in which the double Cys/Ala substitution exists). The recombinant wild-type and Cys/Ala mutants of 2dCD4 were expressed and purified by standard denaturing metal-chelate affinity chromatography and refolded using oxidative refolding protocols according to methods described previously [9].

Deuterium labelling, quenching, and proteolytic cleavage were all performed in an automated manner using a LEAP PAL HDX system (Leap Technologies). LC-MSMS analyses were performed on an Agilent 1100 HPLC system coupled to an AB Sciex 6600 TripleTOF. To initiate on-exchange reactions, 4 μL of each 2dCD4 variant at a concentration of 1 mg/mL in 10 mM phosphate buffer (pH 7.4), 154 mM NaCl, 10% (w/v) sucrose, and 0.02% (w/v) sodium azide at 20°C was transferred to a vial containing 16 μL of 100% D_2O. After a set labelling time (15–3600 s), 20 μL of the protein-deuterium mix was transferred to a vial containing 30 μL quench buffer (1.7 M guanidinium hydrochloride, 20 mM TCEP, 0.1% (v/v) formic acid) and incubated at 0 °C. For the fully deuterated control, deuteration was conducted overnight. The non-deuterated control experiment was performed as described above except MilliQ H_2O was used in place of D_2O. Forty five microlitres of quenched samples were injected onto a Poroszyme immobilized pepsin column 2.1 × 5 mm (Life Technologies) at a flow rate of 100 μl/min using 0.1% formic acid. The resulting peptides were desalted on a Acclaim PepMap trap column (0.3 × 5 mm) for 2 min using 0.1% formic acid and separated at 200 μl/min using a linear 10 min gradient of 10–40% B (B: 80% acetonitrile/0.1% formic acid) on a Kinetex C18 column (2.1 × 5 mm). Proteolysis, desalting, and peptide separation were all performed in a column oven operated at 4°C. For peptide identification the 6600 TipleTOF mass spectrometer was operated in Data Dependant Acquisition (DDA) mode, while for deuterium labelling only a prescursor scan was collected. In DDA mode precursor scans were acquired from *m/z* 360–1500 using an accumulation time of 250 ms followed by 30 product scans, acquired from *m/z* 100–1800 at 100 ms each, for a total scan time of 3.3 s. Charge ions (1^+-5^+, that fall in the mass range 360–1500 *m/z*) were automatically fragmented in Q2 collision cells using nitrogen as the collision gas. Collision energies were chosen automatically as function of *m/z* and charge. Dynamic exclusion was set to 15 s. PEAKS 6 (Bioinformatics Solutions Inc.) was used for peptide identification using the following parameters: enzyme specificity none, precursor mass error 25 ppm, and product mass error 0.1 Da. The level of deuterium incorporation was calculated using HDExaminer 1.3 (Sierra Analytics).

3. Results & discussion

3.1. Conformational dynamics of wild-type 2dCD4

To simulate the various 2dCD4 redox states, we generated two mutants containing double Cys/Ala substitutions at the disulfide bond residues in either domain 1 or domain 2. These mutants, along with wild-type and fully-reduced 2dCD4, were incubated in D_2O for varying times, then subject to amide HDX-MS to study the role that each disulfide bond plays in the conformational dynamics of each domain and the protein as a whole. Exchange was reported for peptides covering 99% of the full-length 2dCD4 sequence (Suppl. Fig. S1) and the extent of back-exchange was on average 26%.

Deuterium incorporation patterns of wild-type 2dCD4 after 300 s were mapped to its crystal structure to examine its global conformational dynamics (Fig. 1). Though a small protein, 2dCD4 is comprised of eight β-strands and three α-helices in domain 1, and seven β-strands and one α-helix in domain 2. In domain 1, regions with high uptake (more than 50% deuteration) include the βB strand, part of the βC′ strand, αDE helix, and C′′ β-strand of the gp120 and MHCII binding site, part of the βF strand, and various loops in the domain. In domain 2, high deuteration levels were observed in parts of most β-strands, including βB, βC, βE, βF, and βG (Fig. 1). Areas of low exchange were comprised of the N-terminus, part of αDE and αEF in domain 1, the β-strand connecting the two domains, βC′ and αEF in domain 2, and the C-terminus. This local exchange behavior is consistent with what would be expected based on the structure of 2dCD4 [10]. Because of its size, most of the protein is exposed to solvent, corresponding to higher levels of

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