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On the redox biology of the immuno-virological receptor CD4: biological function and applications in HIV-1 drug and vaccine development



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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg 2014

Declaration

I, Nichole Michelle Cerutti, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……

23rd day of September, 2014

Dedication

To my husband Carlo who brings colour into my life and is the glue that holds my pieces together

My beautiful daughters Isabella and Sophia who inspire me to be the best I can be

My mother Diana who has dedicated her life to her family, my only wish is that I am able to follow in your footsteps

My father Michael-John who would have been embarrassingly proud

*You are **BRAVER** than you believe*
__STRONGER__ than you seem
and __SMARTER__ than you think

~Christopher Robin to Pooh~

Publications and Presentations

The results of this study have been published in the following journal articles and conference proceedings:

Journal Articles

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1. **Cerutti N, Mendelow BV, Napier GB, Papathanasopoulos MA, Killick M, Khati M, Stevens W and Capovilla A** (2010). Stabilization of HIV-1 gp120-CD4 Receptor Complex through Targeted Interchain Disulfide Exchange. Journal of Biological Chemistry, **285**: 25743-25752. (**Chapter 2**)

2. **Cerutti N, Killick M, Jugnarain V, Papathanasopoulos M and Capovilla A** (2014). Disulfide Reduction in CD4 Domain 1 or 2 Is Essential for Interaction with HIV Glycoprotein 120 (gp120), which Impairs Thioredoxin-driven CD4 Dimerization. Journal of Biological Chemistry, **289**: 10455-10465. (**Chapter 3**)

Conference Proceedings

Cerutti N (2012). Disulphide Bond Shuffling and the Control of CD4 function at the Cell Surface, Faculty of Health Sciences Research Day & Postgrad Expo, Johannesburg, South Africa.

Capovilla A and **Cerutti N** (2012). Protein Disulphide Isomerase (PDI) shuffles CD4 Disulphide Bonds in vitro: putative role for PDI in HIV infection? South African Society of Biochemistry and Molecular Biology Congress, Drakensburg, South Africa.

Cerutti N (2010). Stabilization of the HIV-1 gp120-CD4 complex by targeted disulphide exchange, Faculty of Health Sciences Research Day & Postgrad Expo, Johannesburg, South Africa.

Abstract

Human receptor CD4 is a membrane-bound glycoprotein expressed on the surface of certain leukocytes where it plays a key role in the activation of immunostimulatory T cells. This function is diverted by the Human Immunodeficiency Virus (HIV) envelope glycoprotein (gp120), which uses CD4 as its primary receptor for cell entry. The requirement of CD4 for viral entry has rationalised the development of recombinant CD4-based proteins as competitive viral attachment inhibitors and immunotherapeutic agents. While growing evidence suggests that redox exchange reactions involving CD4 disulphides (potentially catalysed by cell surface-secreted oxidoreductases) play an essential role in regulating the activity of CD4, their mechanism(s), biological utility and structural consequences that may be applicable to the designs of novel antiviral therapies and vaccines remain incompletely understood.

Herein, a novel recombinant CD4 protein designed to bind gp120 through a targeted disulphide-exchange mechanism is described. This molecule contains a conservative Ser60 to Cys mutation on the CD4 domain 1 α-helix which, according to theoretical crystal structure modelling, positions a thiol in close proximity of the gp120 V1/V2 loop disulphide (126Cys–Cys196) resulting in the formation of an interchain disulphide bond. Experimental evidence for this effect is provided by describing the expression, purification, refolding, receptor binding and antiviral activity analysis of a recombinant two-domain CD4 variant containing the S60C mutation (2dCD4-S60C). This 2dCD4-S60C binds HIV-1 gp120 with a significantly higher affinity than wild-type protein under conditions that facilitate disulphide exchange and this translates into a corresponding increase in the efficacy of CD4-mediated viral entry inhibition.

To gain more insights into the importance of redox activity in the mechanism of HIV entry, a panel of recombinant 2-domain CD4 proteins (2dCD4), including wild-type and Cys/Ala variants, were used to show that Thioredoxin (Trx), an oxidoreductase found on the cell surface, reduces 2dCD4 highly efficiently, catalysing the formation of conformationally distinct monomeric 2dCD4 isomers, and a stable, disulphide-linked 2dCD4 dimer. HIV-1 gp120 was shown to be incapable of binding a fully oxidised, monomeric 2dCD4 in which

both domain 1 and 2 disulphides are intact, but binds robustly to reduced equivalents that are the products of Trx-mediated isomerisation. This Trx-driven dimerisation of CD4, a process believed to be critical for the establishment of functional MHCII-TCR-CD4 antigen presentation complexes, is shown to be impaired when CD4 is bound to gp120. Finally, preliminary, low-resolution structural analysis of individual CD4 domains 1 and 2 are suggestive of intrinsic metastability in domain 2, and reduction of its resident allosteric disulphide bond likely underpins the structural rearrangements in CD4 that are required for efficient interaction with gp120.

Overall, these findings emphasise the fundamental importance of redox pathways in the biochemical mechanism of HIV entry, and illustrate the feasibility of exploiting these for the development of novel antiviral ligands.

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Mr Vinesh Jugnarain for sub-cloning, expressing and purifying the two single domain (1dCD4-D1 and 1dCD4-D2) proteins used in this study.

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List of Abbreviations

Abbreviations used in this thesis describe the following unless otherwise indicated:

1dCD4	one-domain CD4
2dCD4	two-domain CD4
AIDS	acquired immune deficiency syndrome
ARV	antiretroviral
bnAbs	broadly neutralising antibodies
bp	base pairs
BSA	bovine serum albumin
CA	p24 capsid protein
CCR5	chemokine (C-C Motif) receptor 5
CD	circular dichroism
CD4	cluster of differentiation 4
CD4+	CD4 Positive
CD4bs	CD4-binding site
CD4i	CD4-induced
cDNA	complementary DNA
CXCR4	chemokine (C-X-C motif) receptor 4
Cystine	disulphide bonded cysteine
D1D2	domain 1 and domain 2 of CD4
D2D3	domain 3 and domain 4 of CD4
DNA	deoxyribonucleic acid
DSE	dihedral strain energy
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	ethylenediamine tetra acetic acid
EI	entry inhibitor
ELISA	enzyme linked immunosorbant assay
EMSA	electrophoretic mobility shift assay
Env	envelope glycoprotein
ER	endoplasmic reticulum
Fab	antigen binding fragment of an antibody
gag	group-specific antigen
gp120	glycoprotein 120
$gp120_{BaL}$	subtype B envelope glycoprotein 120
gp160	glycoprotein 160 (env precursor protein)
gp41	glycoprotein 41
Grx-1	glutaredoxin-1
GSH	reduced glutathione
GSSG	oxidised glutathione

HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
HR1	heptad repeat helix 1
HR2	heptad repeat helix 2
HRP	horseradish peroxidase
IAM	iodoacetamide
Ig	immunoglobulin
IN	integrase
INI	integrase inhibitor
IPTG	isopropyl β-D-1-thiogalactopyranoside
ITAM	immunoreceptor tyrosine activation motif
kDa	kilodalton
kJ	kilojoule
LB	Luria-Bertani broth
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LH	left-handed
LTR	long terminal repeat
MA	p17 matrix protein
mAb	monocolonal antibody
MHC	major histocompatibility complex
MHCII	major histocompatibility complex class II
MMP	methyl α-D-mannopyranoside
MPB	3-(N-maleimido-propionyl) biocytin
mRNA	messenger RNA
MS	mass spectrometry
MW	molecular weight
m/z	mass to charge ratio
NAb	neutralising antibody
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NC	p6 and p7 nucleocapsid
nef	negative effector
NHS	N-hydroxy succinimide
NIH	national institute of health
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline containing tween-20
PDB ID	protein data bank identification number
PDI	protein disulphide isomerase
PI	protease inhibitor
PIC	pre-integration complex
pol	polymerase
PR	protease
pr160	gag-pol protein precursor
pr55	gag protein precursor

rev	regulator of virion gene expression
RH	right-handed
RLU	relative light units
RMS	root mean square
RNA	ribonucleic acid
RT	reverse transcriptase
S60C	serine in position 60 mutated to cysteine
sCD4	soluble 4 domain CD4
SCOPs	structural classification of proteins
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFMII	serum free media II
-SH	thiol
S_N2	bimolecular nucleophilic substitution
SPR	surface plasmon resonance
TAR	transactivation response
tat	transcriptional transactivator
TCR	T-cell receptor
TRX	thioredoxin
TrxR	thioredoxin reductase
TS	transition state
T-TBS	tween-tris buffered saline
US-FDA	United States Food and Drug Administration
UV	ultraviolet
vif	viral infectivity factor
vpr	viral protein R
vpu	viral protein U
WT	wild-type
X4 virus	CXCR4 co-receptor using virus
X4/X5 virus	either CXCR4 or CCR5 using virus
X5	CCR5 co-receptor using virus

The IUPAC-IUBMB three and one letter codes for amino acids are used

Chapter 1: Introduction

1.1 HIV

Human Immunodeficiency Virus (HIV), the virus which causes Acquired Immune Deficiency Syndrome (AIDS), is a Lentivirus of the family *Retroviridae* and was first isolated in 1983 [1, 2]. Despite decades following the discovery of this virus, neither a preventative vaccine nor a therapeutic cure has been developed.

1.1.1 HIV Epidemiology and Treatment

Although worldwide efforts to prevent transmission of HIV-1 have been made, the number of individuals living with the disease still increases annually. By the end of 2012, an estimated 35.3 million people were living with HIV and 2.3 million people were newly infected in that year [3]. Most striking is the fact that 23.5 million HIV-1 infected individuals reside in sub-Saharan Africa, with 6.1 million of these individuals living in South Africa (approximately 17% prevalence), the highest number of individuals living with the virus in a single country [3]. Furthermore, in South Africa, 370 000 new infections were recorded by the end of 2012 with 240 000 AIDS-related deaths [3]. There are currently 37 US Food and Drug Administration (FDA) approved antiretroviral drugs (ARVs) targeting distinct steps of the viral life cycle, various combinations of which make up Highly Active Antiretroviral Therapy (HAART). HAART has been successfully implemented to reduce the number of AIDS-related deaths and is a combination of three antiretroviral drugs from different classes, including reverse transcriptase inhibitors (RTI – nucleoside RTIs and non-nucleoside RTIs) and protease inhibitors (PIs). Patients failing HAART therapy may be treated with integrase inhibitors (INIs) and/or entry inhibitors (EIs). Although there has been great success achieved with HAART, such as approximately 14 million lives have been saved since 1995 [3], pill burden and the resultant adherence issues, toxicity and the inevitable emergence of antiretroviral drug-resistant viruses remain major sources of concern.

1.1.2 Viral Genomic and Structural Organisation

The HIV-1 genome is approximately 9.8 kb in length and contains nine open reading frames [4, 5]. The group-specific antigen (*gag*), polymerase (*pol*) and envelope (*env*) genes encode the structural and enzymatic viral proteins. The four Gag proteins (matrix (MA), capsid (CA), nucleocapsid (NC, p7 and p6) and the envelope glycoprotein (Env) are the structural components that make up the viral core and outer membrane envelope [6, 7]. The Env precursor (gp160) is cleaved by host cell proteases into the viral surface gp120 subunit and the transmembrane gp41 subunit [8]. The four Pol proteins (reverse transcriptase (RT), RNase H, protease (PR) and integrase (IN) enzymes) are essential during the viral replication cycle [9]. Cleavage of both the Gag and Gag-Pol protein precursors during viral assembly is achieved by the viral PR. The transcriptional transactivator (*tat*) and regulator of virion gene expression (*rev*) genes code for two proteins involved in gene regulation. Tat binds to the transactivation response (TAR) element within the R domain of the 5' long terminal repeat (LTR) thereby regulating transcription and Rev mediates the transport of singly spliced and unspliced viral transcripts from the cell nucleus into the cytoplasm [10-12]. Four accessory genes which are responsible for viral virulence also encoded by the viral genome include negative effector (*nef*), viral infectivity factor (*vif*), viral protein R (*vpr*) and viral protein U (*vpu*) [13, 14].

1.1.3 HIV Replicative Cycle

The replicative cycle is intricate and proceeds through a series of steps beginning with recognition of and binding to the host cell surface receptors, culminating in the maturation of newly released viral particles into infectious virions. The cells targeted by HIV-1 include CD4+ T cells, macrophages, monocytes, dendritic cells and microglia [15-18]. The process of viral entry is initiated by the recognition and binding of the N-terminal immunoglobulin domain of CD4 by the CD4 binding site (CD4bs) on the trimeric viral envelope glycoprotein gp120. This interaction leads to structural rearrangements which expose/create the co-receptor binding site on gp120. The gp120 binds one of two members of the chemokine seven-transmembrane G-protein coupled co-receptor family, CCR5 (R5 tropic virus) or CXCR4 (X4 tropic virus) on the host cell surface [19-21]. R5 tropic viruses represent the major transmissible HIV-1 strains, whereas X4 viruses tend to emerge later on in the course

of the disease. While X4 tropic viruses are generally ill-adapted for propagation in healthy individuals, they may emerge as opportunistic HIV variants as an HIV-1 infected individual progresses to AIDS [22]. Despite the fact that several CD4-independent viruses have been described [23-26] co-receptor binding remains a critical step during viral entry.

Recognition and binding of the co-receptor by gp120 leads to the release of the "fusogenic" potential of the transmembrane gp41 [27] which then acts in two distinct steps: 1) casting: the fusion peptide (HR1 region) "missiles out" and inserts into the host cell membrane [28, 29]; 2) folding: the HR2 region in gp41 then folds into the HR1 region creating a six-helix bundle, leading to fusion of the viral and host cell membranes [30-32]. Following fusion, the HIV-1 capsid (containing two copies of plus strand viral genomic RNA and other essential proteins required for viral replication) [33], is injected and uncoated in the cytoplasm and its contents released [34-36]. Reverse transcription of viral RNA into cDNA by RT follows [37-40] and the resultant viral cDNA is transported, directed by the vpr nuclear localisation signal [41], to the cell nucleus as part of the pre-integration complex (including viral IN, MA, RT, Vpr and essential host proteins) [42, 43]. Viral cDNA is then irreversibly integrated into the host genome by the catalytic activity of IN [43, 44]. Following transcription, spliced and unspliced mRNA transcripts are then synthesised and transported out of the nucleus for translation in the cytoplasm. The newly synthesised viral structural Gag proteins and enzymes migrate to lipid rafts in the host cell membrane where immature virions are then assembled [45]. As the immature virus buds out of the cell, it acquires the lipid membrane from the infected host cell [29], within which the viral Env is embedded. Immature virus maturation is dependent on the complete proteolytic cleavage of the Gag and Gag-Pol protein products by the PR [29, 46, 47]. The mature virus is then capable of subsequent rounds of productive infection.

1.1.4 HIV Entry: Mechanisms and Major Protein Role Players

The process of HIV-1 entry involves a stepwise series of interactions with specific receptors that initiate multiple conformational changes in Env [48, 49] (Figure 1.1). Functionally active (fusogenic) Env spikes, composed of a trimeric arrangement of non-covalently linked heterodimers of gp120 and gp41 [50-56], dock onto the host cell surface CD4 receptor via the CD4bs on gp120 and a cascade of structural rearrangements ensue in both binding

partners. Initial attachment to the host cell may be facilitated by (but is not dependent upon) non-covalent interactions between Env and host cell attachment factors including heparan sulphate and mannose-binding lectins such as dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) [57-59]. Following CD4 binding, a bridging sheet forms between the inner and outer domains of the gp120 monomer and the co-receptor binding site on gp120 is exposed [60] thereby making way for the projection of one of five hypervariable loops (variable loop 3, V3). This gp120 conformation is known as the CD4 induced (CD4i) conformation [61-63]. Interestingly, CD4-independent Envs are triggered for fusion [25, 64-66], therefore the requirement for CD4 binding by CD4-dependent viruses is likely to prevent the irreversible triggering of viral fusion [49]. Specific regions within the V3 loop determine whether the virus uses the CCR5 or CXCR4 co-receptor for entry [67]. During formation of the CD4i conformation of gp120, movement in the hinge region between the second and third globular domains of CD4, positions the Env trimer in closer proximity to either CCR5 or CXCR4 [68]. Co-receptor binding then leads to relaxation of gp120 which then allows gp41 to undergo extreme structural rearrangements: the fusion peptide is inserted into the host cell membrane, this triggers reorganisation between trimerised N- and C-terminal heptad repeat sequences within gp41, a six helical hairpin structure then forms leading to apposition and ultimately fusion of the viral and host cell membranes [30-32].

1.1.4.1 Strategies for targeting HIV entry

Each step of the entry process is a potential target for antiretroviral compounds including neutralising antibodies (NAbs), small molecule inhibitors and synthesised peptides/polymers (Figure 1.1). A large number of broadly neutralising antibodies (bNAbs) have been identified from HIV-1 infected individuals which recognise specific Env epitopes but all ultimately prevent the entry of HIV-1 into target cells. Development of an effective preventative HIV-1 vaccine is one of the highest global health priorities and a key issue with respect to the development of such a vaccine is the ability to induce the production of neutralising antibodies that are able to neutralise the diverse array of circulating HIV-1 variants.

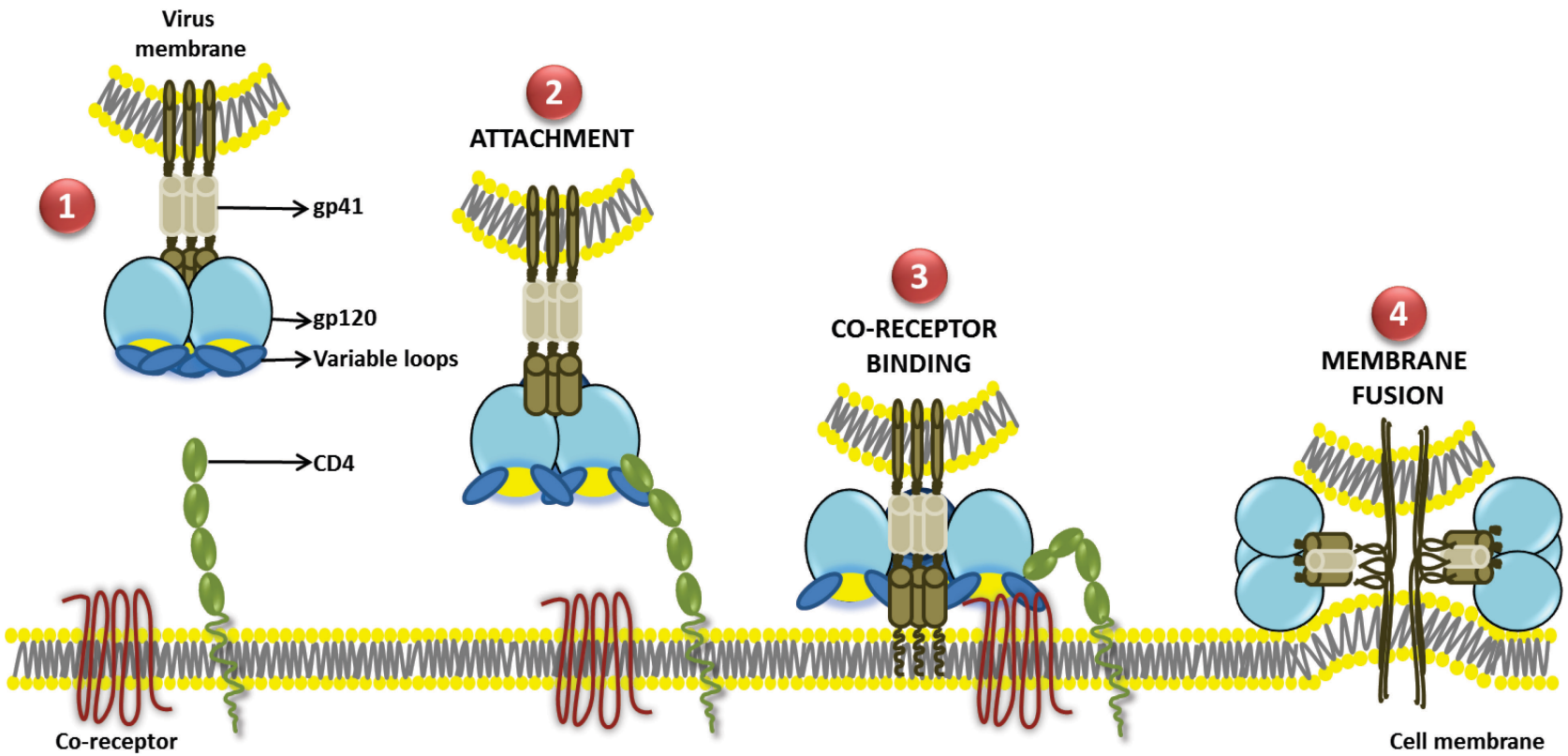

Figure 1.1: Schematic representation of the major steps of HIV-1 entry mediated by the viral envelope glycoprotein
First, gp120 binds to CD4 (attachment) leading to the formation of the co-receptor binding site on gp120 (CD4i conformation). Second, the newly exposed co-receptor binding site on gp120 recognises and binds either CCR5 or CXCR4 (co-receptor binding) – a process which triggers the process of membrane fusion. Last, the gp41 subunits of the trimer facilitate fusion of the viral and host cell membranes. Anti-entry target sites are indicated by the numbers 1, 2, 3 and 4: 1. Antibodies (Abs) target the variable loops; 2. Abs to CD4, Abs and small molecules that target the CD4bs of gp120; 3. Abs to co-receptors, small-molecule co-receptor ligands, Abs (Fabs) to CD4i binding site on gp120, Abs to V3 loop; 4. Abs to gp41 and gp41 derived peptides. Figure adapted from Doms 2000 [48].

To date, eliciting these types of antibodies *in vivo* by active vaccination has not been successful. Specific epitopes on Env targeted by NAbs include: the CD4bs of gp120 (b12 [69], HJ16 [70], VRC01 and VRC03 [71], NIH45-46 [72], 3BNC117 and 3BNC60 [72], PGV04 and CH30-34 [73]); the membrane proximal external region of gp41 (2F5 and 4E10 [74] and 10E8 [75]); V1/V2/glycan site on gp120 (PG9 and PG16 [76], CH01-CH04 [77] and PGT141-145 [78]); V3/V4/glycans on gp120 (PGT121-123 [78, 79], 10-1074 [79] and PGT135-137 [78]); N-linked high mannose glycan cluster on the gp120 surface (2G12 [74]) and glycan-V3 β-strand on gp120 (PGT125-131 [78, 79]).

In addition to targeting Env, NAbs targeting host receptors have also been described. Ibalizumab (a humanised monoclonal NAb) targets the second domain of CD4 and locks CD4 once bound thereby inhibiting subsequent conformational changes in the viral protein [80, 81]. The anti-CCR5 antibody, PRO-140 (a humanised murine antibody) neutralises a wide range of clades [82, 83].

The use of bNAbs as preventative therapeutics has limitations such as high cost of production, poor oral bio-availability and incomplete/poor absorption following intramuscular or subcutaneous administration. However, there has been recent renewed interest in their use in passive immunisation strategies, and their efficacy has provided proof-of-concept that pre-existing bNAbs can prevent HIV-1 transmission [84, 85].

Despite many decades of research during which numerous compounds were developed for clinical use, only two EIs have obtained US FDA approval. Even so, research in this area continues since small molecule entry inhibitors are safe, well tolerated and generally orally bio-available [86]. Attachment inhibitors (BMS-378806, BMS-488043, NBD-556 and NBD-557) inhibit HIV entry by binding gp120 thereby preventing the interaction between CD4 and gp120 [87-89]. CXCR4 co-receptor antagonists/inhibitors include AMD3100 and TAK-779, and CCR5 antagonists include Maraviroc (currently the only US FDA-approved CCR5 antagonist [90]), Vicriviroc, Aplaviroc and TD-0680 (reviewed in [86]). As with the development and use of established antiretroviral drugs in HAART regimens, drug resistant viruses do emerge/evolve after exposure to all the attachment inhibitors and co-receptor antagonists [91-97].

Synthesised peptides and polymers include recombinant antibody-like peptides, synthesised polymers, modified chemokines and structural mimic oligomers (reviewed in [86]). These compounds inhibit HIV-1 infectivity by various mechanisms. To date, the only US FDA approved compound in this class is Enfuvirtide or T20. Although an approved treatment for AIDS patients, its clinical use is limited due to relatively low potency, short half-life, inconvenient subcutaneous administration and rapid induction of drug-resistant virus [86]. Sifuvirtide, like T20, targets gp41 but contains a pocket binding domain. This inhibitor was designed such that its three dimensional structure shares features with gp41 which results in increased affinity and potency [98]. PRO-542 (CD4-IgG2) is a recombinant antibody-like heterotetramer which mimics the CD4 receptor and is a competitive inhibitor of cell surface CD4 [99, 100]. PRO2000 is a naphthalene sulphonate polymer that blocks CCR5 and CXCR4 infection by binding CD4 and competitively inhibiting binding of gp120 [101]. Trials on both PRO-542 and PRO2000 were discontinued due to insignificant effects on patient viral loads. Research and development of this class of inhibitor compounds is limited by difficulties in synthesis and chemical modification, high cost of expression and purification in the case of recombinant proteins and peptides as well as the inconvenient methods of administration due to low oral bioavailability.

1.1.4.2 gp120 structure

Crystallographic analysis of core, monomeric gp120 (with truncated variable loops) in complex with the first two domains of CD4 and the Fab fragment of the mAb 17b [62] revealed that this glycoprotein contains inner and outer domains that correspond to portions of gp120 which are closer to the inner and outer regions of the trimeric spike respectively. These two domains are linked by a four-stranded β-sheet (bridging sheet) (Figure 1.2). Although the fold is complex, comparison of the primary protein sequences of numerous virus isolates reveals that the protein contains five constant (C1-C5) and five variable (V1-V5) regions [62]. Subsequent crystallographic studies of the variable regions [102-105] show that residues within the V1-V5 loop regions have structural variability and the precise conformations of these loops remain vague [106]. The CD4bs is located in the carbohydrate-free depression formed at the domain interface [62].

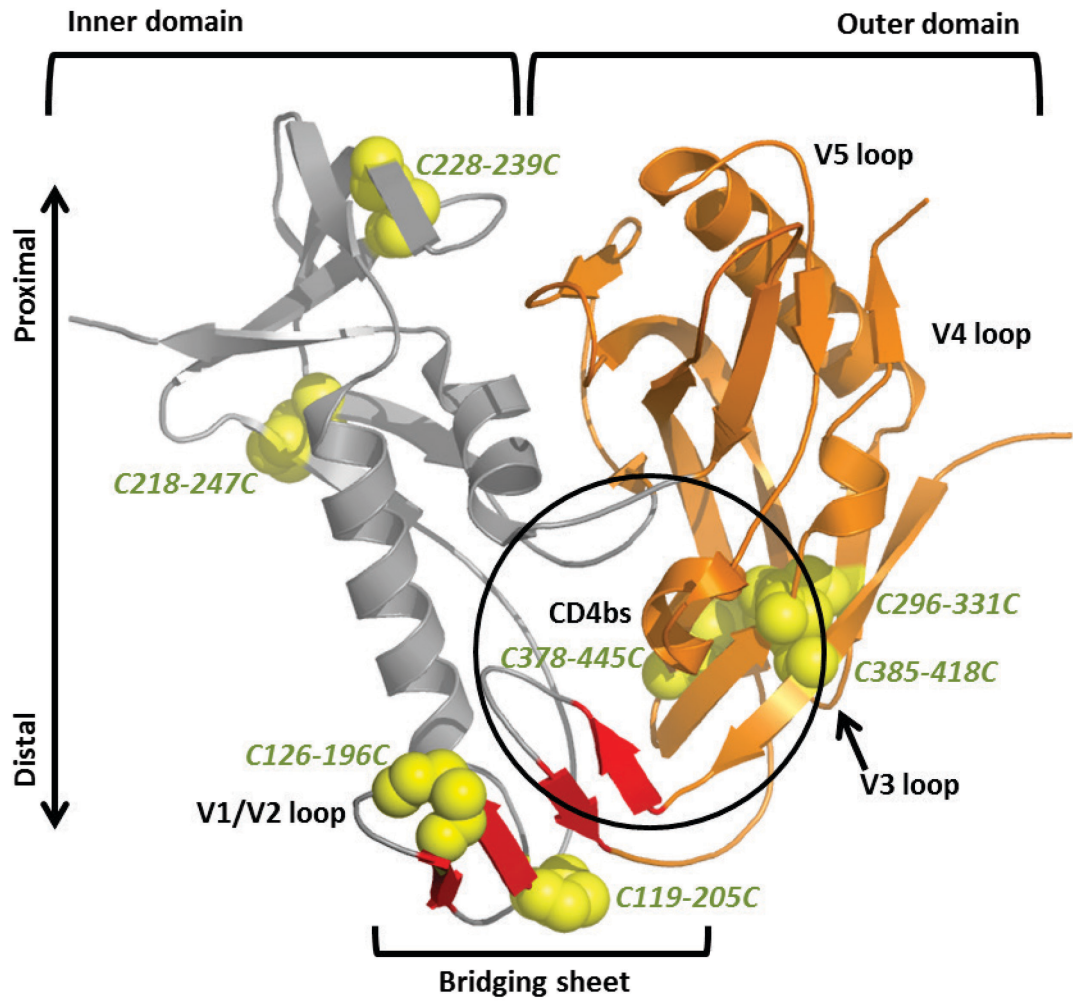

Figure 1.2: Core structure of gp120

The core structure of gp120 is shown in ribbon representation. In this particular orientation, the viral membrane would be orientated above the structure and the host cell membrane below (membrane proximal and distal labels are shown, respectively. The inner (grey) and outer (orange) domains give reference to the portions of gp120 which are closer to the inner and outer regions of the trimeric spike, respectively. The four β-sheets which compose the bridging sheet are shown in red, packing primarily in the inner domain. The CD4 binding site (CD4bs, circled) is formed at the domain interface and in this particular orientation, the CD4 molecule would be coming out of the page with the second domain furthest away from the binding site. Cysteine residues of the disulphide bonds are shown in space filling representation and labelled in green. This figure was adapted from Kwong *et al* [62] and was generated using PyMOL and PDB ID 1G9M. Crystals were obtained following multiple modifications of gp120 including truncation of the V1/V2 loop, V3 loop and C- and N-terminal segments (with resultant deletion of C57-74C and C131-157C) enzymatic removal of glycans as well as stabilisation via complex formation with CD4 and the Fab fragment of the CD4i antibody 17b (CD4 and 17b not shown).

A combination of cryo-electron tomography with image averaging and fitting of known X-ray structures (gp120 conjugates) has provided details of the quaternary conformations of trimeric Env complexes displayed on intact viruses or as soluble ectodomains [107-114].

A striking observation is the highly flexible core trimeric structure, and despite extreme conformational changes induced by CD4 binding, the structural integrity of gp41 is maintained [61, 115, 116]. The locations of the structurally dynamic variable loops, to date elusive by crystallography, have been determined using Cryo-electron microscopy [113, 117]. In the unliganded state or in the presence of antibodies which inhibit structural rearrangements (e.g. VRC01), the envelope is in a "closed" conformation with the V1V2 loops located at the tip of the spike structure. When bound to CD4 or antibodies that recognise the CD4i state of gp120 (e.g. 17b), the trimeric gp120 adopts an "open conformation". In this conformation, rearrangements in each monomeric unit of the trimer position the V1V2 loops at the trimer edge. When bound to the monoclonal antibody b12, the trimer partially opens due to slight perturbations in the envelope structure.

The viral envelope has employed various mechanisms which result in effective evasion of the host immune responses. Firstly, the envelope is heavily glycosylated, with gp120 and gp41 containing approximately 28 potential N-linked glycosylation sites [118, 119]. This glycan shield in conjunction with the variable loops (which themselves are glycosylated) mask the more conserved epitopes which are potential targets for neutralising antibodies (NAbs). In addition, genetic variability introduced by the error prone RT during viral replication enables the Env to escape any potentially NAbs due to the hypervariable nature of the Env sequences, and in turn structures presented on the surface of the virion. The hypervariable, hyperglycosylated and hyperflexible nature of gp120 is contrasted by the conserved architecture and constant presentation of its CD4bs.

1.1.4.2.1 gp120 disulphides

In the background of its notoriously hypervariable sequence, HIV-1 Env contains 10 highly conserved disulphide bonds (9 in gp120: C57-74C, C119-205C, C126-196C, C131-157C, C218-247C, C228-239C, C296-331C, C385-418C and C378-445C, Figure 1.2) [120]. Although the

antigenic shield of Env is variable, the disulphide bonded structure and in turn the basic Env structural arrangement remains constant. Three of these disulphides are located within the V1/V2 loop, one at the base of the V3 loop and one within the V4 loop [62]. In addition, all five of these disulphide bonds are in close proximity to at least one amino acid of gp120 that makes contact with CD4. In order to systematically assess the role of each individual disulphide bond in either the folding or function of gp120, van Anken *et al* [121] analysed a complete series of mutants where all Cys were replaced with alanines, both individually and pair-wise. Five of the ten disulphides were essential for folding (C57-74C, C218-247C, C228-239C, C296-331C (previously shown [122-124]), C385-418C (previously shown [124-127]). Surprisingly, when mutated, the C385-418C disulphide was dispensable for folding but important for function as mutant virus showed only residual infectivity. Two of the ten disulphides were shown to be dispensable for both folding and function (C119-205C and C378-445C). Most importantly, with regards to function, the C126-196C and C131-157C mutants showed folding identical to that of wild-type but showed complete loss of function (infectivity).

1.1.4.3 CD4

Receptor CD4, a differentiation marker primarily expressed on the surface of T lymphocytes, is a 55 kDa type I integral membrane glycoprotein that plays a substantial role during adaptive immune responses. In addition to T-cells, CD4 has also been shown to be expressed on monocytes, monocyte-derived macrophages, Langerhans cells, B lymphocytes, dendritic cells, eosinophils, megakaryocytes and mast cells [128-133]. In T-cells, CD4 stabilises T-cell receptor (TCR) interactions with peptide-major histocompatibility complex class II (MHCII) complexes on antigen presenting cells and mediates intracellular T-cell signalling [134]. Both the TCR and CD4 are required to make contact with the peptide-MHCII complex in order to transduce the appropriate proliferative signal to the antigen-specific T-cell, which is then able to co-ordinate various effector activities critical for the maturation of a robust, antigen-specific immune response. In contrast, binding of the TCR to MHCII without a CD4-MHCII co-stimulatory signal leads to T-cell anergy and apoptosis [135, 136]. There is a general consensus that the main function of CD4 is to recruit the Src tyrosine kinase Lck to the TCR-MHCII complex upon co-receptor binding to MHC which leads

to the formation of a TCR-MHCII-CD4 complex [137-139]. Lck recruitment, which occurs via its association with the cytoplasmic tail of CD4, promotes phosphorylation of the immunoreceptor tyrosine activation motifs (ITAMs) located within the cytoplasmic tails of the TCR, thus resulting in signal amplification. By subverting the critical immunological function of CD4, a possible contribution of gp120-mediated anergic effects on $CD4^+$ T cells to the pathogenesis of AIDS was demonstrated [140] soon after the discovery of CD4 as the receptor for HIV [141-143].

Human CD4 is composed of an extracellular region (residues 1-371), a transmembrane segment (residues 372-395) and a cytoplasmic tail (residues 396-433) [144-147] (Figure 1.3, the extracellular region of CD4). Characterisation of the receptor was initiated with the production of a soluble, recombinant CD4 molecule containing the extracellular residues 1-369 [148, 149]. Due to extensive crystal polymorphism, low diffractive resolution and high solvent content (indicative of an extended flexible structure) [148], structural characterisation of the molecule was focused on the first two domains of human CD4 (2dCD4-D1D2 [144, 145]) and the last two domains of rat CD4 (2dCD4-D3D4 [150]). Following the crystallisation of the first two and last two domains of CD4, Wu and co-workers [151] were able to use these models to solve and refine the structure of a recombinant soluble CD4 molecule (sCD4) containing all four extracellular domains (Figure 1.3). The N-terminal extracellular region of CD4 is comprised of a linear repeat of four immunoglobulin-like domains (D1-D4). These domains have sequence and structural homology with immunoglobulin (Ig) light chain variable and constant regions [146, 147], a common structural feature being two β-sheets that oppose one another in a barrel or sandwich-like structure. Each β-sheet comprises seven to nine β-strands and has a distinctive disulphide bond that primarily functions to stabilise the structure by linking opposing sheets. D1 is composed of nine anti-parallel β-strands arranged in a two layer, β-sheet sandwich. In what seems to be an extension of D1, D2 is composed of seven anti-parallel β-strands (resembling Ig constant domains) where the last strand of D1 continues straight into the first strand of D2. The tandem association of domains gives the molecule a "rod-shaped" appearance and in addition, the unique linkage between the two domains results in a large hydrophobic interface with tight domain association. Despite the similarity in the overall folds of D2 and Ig constant domains, there are two striking distinctions.

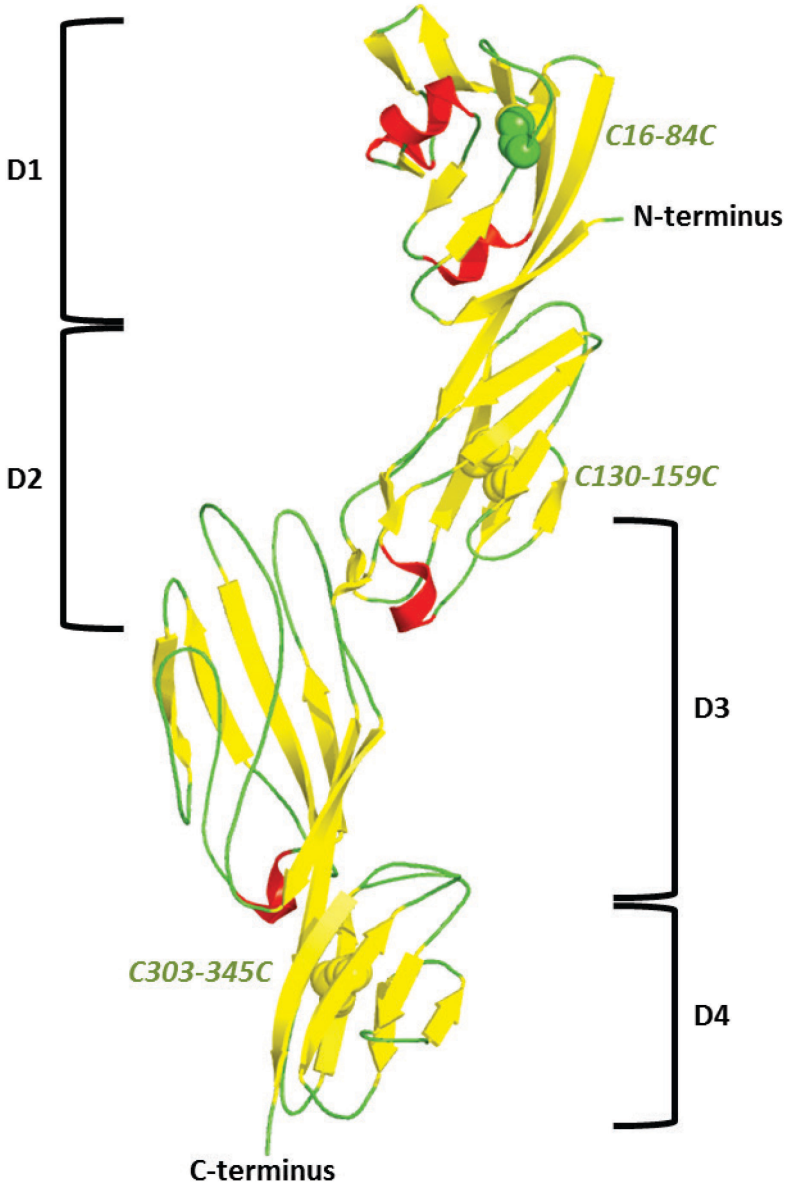

Figure 1.3: Ribbon representation of extracellular CD4
The extracellular portion of CD4 containing domains 1, 2, 3 and 4 (D1-D4) is shown in ribbon representation. β-sheets are shown in yellow, loops in green and helices in red. The disulphide bonds in D1, 2 and 4 are shown in space filling mode. The figure was generated using PDB ID 1WIQ [151] and PyMOL.

First, the D2 domain contains 75 amino acid residues compared with approximately 100 residues found in Ig constant domains, translating into shorter strand lengths overall. Second, the disulphide bond in D2 links strands in the same sheet rather than strands between sheets as is the case in the typical Ig-fold. Even with this difference there is structural conservation of the hydrophobic core wherein the disulphide bond is located. Both the antiparallel sheeted structure and characteristic Ig fold observed for D1 and D2 is also observed for the D3 and D4 domains [150].

The surfaces of D1 and D2 are highly charged with positive amino acid residues giving these two domains a positive potential at neutral pH, however, there are also two small prominent patches of negative potential in both domains (associated with residues Glu85, Glu87 and Asp88 in D1 and Asp105, Asp153 and Asp173 D2) [144]. These charged regions have the potential to allow for self-association between CD4 molecules at the cell surface. The strand arrangement in D3 is similar to that of D1, however the disulphide bond linking the two β-sheets is absent in D3 resulting in an increased β-sheet separation in this domain. As seen in D2, the D4 domain has seven strands and despite significant differences in sequence (e.g. disulphide bond arrangement) these two domains superimpose readily. Finally, rigid domain association as observed between D1 and D2 is also observed for D3 and D4.

1.1.4.4 The gp120-CD4 interaction

D1 of CD4 binds to gp120 in a depression formed by the interface between the inner and outer domains of gp120 (Figure 1.4). CD4 residues that contact gp120 include amino acids 25-64 (an exposed loop located at the tip of domain 1 of CD4) and these residues are distributed over six segments of gp120. The most important contacts are those between Phe43 of CD4 and Glu370 and Trp427 of gp120 as well as electrostatic interactions between Arg59 of CD4 and Asp368 of gp120. Interestingly, this interaction seems to mimic the interaction observed between CD4 and MHCII [152] where both Phe43 and Arg59 play important roles.

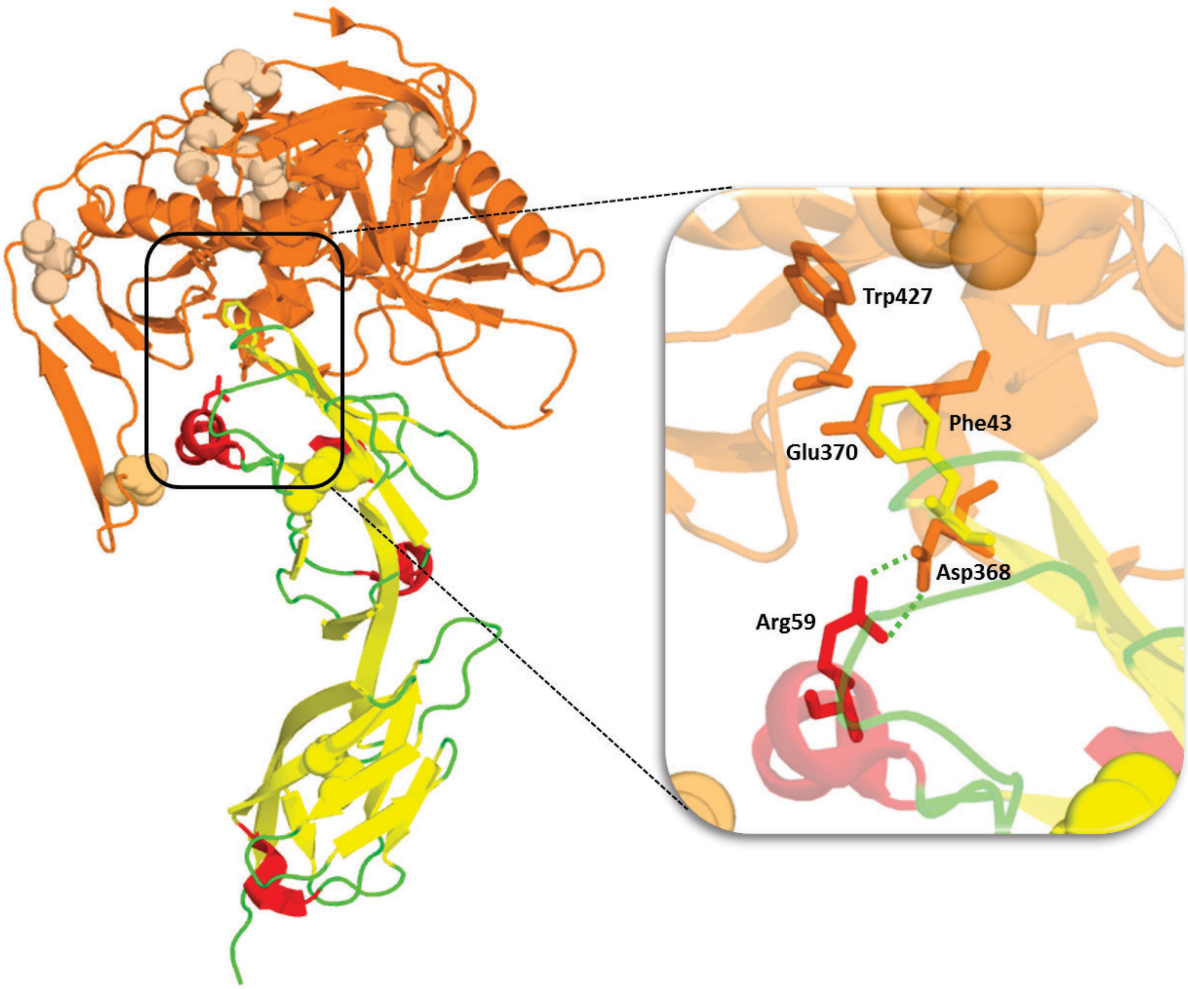

Figure 1.4: The CD4-gp120 interaction
Ribbon representation of gp120 (orange) binding to CD4 (yellow sheets, red helices and green loops). Important residues involved in the interaction are shown in stick representation and labelled. Disulphide bonds in both receptors are shown in space filling mode. The binding pocket in which the Phe43 residue of CD4 is inserted is shown. The inset shows the gp120-CD4 interface with important interacting residues between gp120 (Asp368, Glu370 and Trp427) and CD4 (Phe43 and Arg59). The electrostatic interaction between Arg59 of CD4 and Asp368 of gp120 are shown as green dashed lines. Hydrophobic interactions are found between Phe43 of CD4 and Trp427 and Glu370 of gp120. This figure was generated using PDB ID 1G9M [62] and PyMOL.

Antibody binding to residues outside of the exposed loop in D1 of CD4 as well as mutations of residues in D2, D3, and certain buried residues in D1 have been found to interfere with the interaction between gp120 and CD4 [153-155] as well as other steps during the entry process [80, 156]. The importance of the non-D1 residues as well as those buried in D1 and not at the gp120-CD4 interface suggests the existence of significant structural effects exerted by the residues buried within D1 (intradomain effects) as well as interdomain communication between D1 and D2/D3 which contribute to the binding and recognition between gp120 and CD4 [157].

Crystal structure data as well as detailed biochemical analysis of the gp120-CD4 complex have defined the key residues involved during this interaction. However, recent biochemical investigations have revealed that complex biophysical rearrangements of disulphide bonds in both receptors are involved before, during and after the binding event, and evidence now suggests that redox regulation of these bonds plays a fundamental role in modulating immuno-virological receptor function.

1.2 The Redox Biology of Disulphide Bonds during Protein Function

Disulphide bonds, the product of post-translational protein processing, are covalent linkages between the sulphur atoms of cysteine pairs. Protein disulphides are classically known to be responsible for assisting in the process of protein folding [158] as they stabilise the native conformation of a protein by destabilising the unfolded form. Once formed, disulphides may influence the stability of the native protein conformation and in turn protect proteins from damage caused by oxidants and proteolytic enzymes in the extracellular environment [159, 160]. Contrasting this, disulphides may be destabilising by restricting any energetically favourable conformational changes in proteins. Less common is the participation of these bonds in protein function.

1.2.1 Cysteine

Cysteine (Cys) is one of two sulphur-containing amino acids. When comparing the closest relating or matching sequences in the entire protein sequence database, this residue was found to be the second most conserved amino acid after tryptophan [161, 162]. The side

chain thiol (-SH, pK_a of 8.6) gives Cys a wide range of functions including disulphide bond formation (both intra- and intermolecular disulphides), metal binding, electron donation and redox catalysis. Two important factors which influence the reactivity of Cys are the exposure of this residue to surrounding solvent (either at the protein surface or in the solvated polar microenvironment found in protein pockets and cavities) as well as the thiol protonation status.

The structural and functional importance of this residue is reflected by the increase in Cys frequency within orthologous proteins encoded by triplets of closely related genomes from 15 Taxa representing Bacteria, Archaea and Eukaryota [163, 164]. In a comparative study of disulphide bonded Cys, Cys alone, tryptophan and all amino acids combined, Wong *et al* [165] were able to demonstrate that disulphide-bonded Cys (cystines) were significantly more conserved than Cys not involved in disulphide bonds. Moreover, cystines were shown to be even more conserved than tryptophans, a phenomenon which most likely reflects the requirement for individual Cys to be acquired or lost in pairs [165]. One of the most striking observations made by Thornton [158] is that in all cases where a disulphide bond was not conserved, both Cys in the bond were simultaneously replaced. This observation has been supported by subsequent analyses using larger data sets. One study mapped disulphide conservation across five distinct protein families and found that Cys involved in disulphide bonds were invariably replaced in pairs [166]. Soon after, Rubinstein and Fiser [167] analysed the disulphide bond conservation pattern across all domains from the structural classification of proteins (SCOPs) [168, 169]. Following multiple sequence alignment, it was found that Cys pairs were replaced in concert in 99% of cases. These findings suggest that it is very rare for one of two free cysteines to remain through protein evolution most likely due to the reactivity of the free thiol groups. This has formed the basis for disulphide bond prediction within members of the same protein families [167].

1.2.2 Disulphide Bond Formation and Classification

Disulphide bond formation between the thiol groups of two Cys residues in a protein chain occurs during the process of protein maturation [170]. In eukaryotic cells, protein maturation occurs in the endoplasmic reticulum (ER), Golgi complex, post-Golgi complex vesicles and mitochondrial intermembrane space [171]. Although disulphide bond

formation may occur spontaneously (in the presence of a redox couple most commonly reduced and oxidised glutathione) this process may also be controlled by protein catalysts known as oxidoreductases. When recently examining the UniProt database, Cook and Hogg [172] identified 1 204 disulphide bond containing proteins secreted or functioning outside the ER, Golgi and endosome spaces (e.g. cytoplasm or nucleus), environments not typically believed to be supportive of disulphide bond formation.

In the absence of the oxidoreductases, thiol-disulphide exchange between the protein and the thiol groups of reduced glutathione (GSH), present in millimolar concentrations [173], results in disulphide bond formation. GSH predominates over oxidised glutathione (GSSG) in the ER by a factor of up to three but by a factor of approximately 100 in the cytosol [174], making disulphide bond formation more favourable in the ER. In the reduced, highly unfolded state of a protein, initial disulphide bond formation is a random event that is primarily dependent upon the proximity of the Cys thiol groups [175]. Only once the protein begins to adopt non-random conformations will certain bonds be included or excluded [173]. Non-random conformations include: the non-specific molten globule [176]; partly folded proteins where certain parts of the polypeptide are specifically folded and the remainder are either unfolded [177, 178] or in a compact molten globule conformation [176]; or the protein reaches a completely native-like conformation where only one or two native disulphide bonds are absent [178]. These so called "quasi-native" conformations occur when the native state of the protein, with all the correctly paired cysteines, is so stable that even in the absence of one or more disulphide bonds, the native state is maintained [173].

1.2.2.1 Disulphide bond geometry and configuration

Disulphide bond geometry may be defined by the six atoms linking the two α-carbons (C_α) of the Cys residues namely: C_α-C_β-S_γ-S_γ'-C_β'-C_α' (Figure 1.5A). These six atoms and the amide nitrogen attached to the two C_α are used to define the five χ-angles (chi-angles) of a disulphide bond, calculated by the rotation around the bonds linking the individual atoms [179]. The first χ^1 angle is defined by the atoms N-C_α-C_β-S_γ; the second χ^2 angle is defined by the atoms C_α-C_β-S_γ-S_γ' and so on. These χ angles can be positive or negative, defining 20

possible combinations of disulphide bonds. The three main bond classifications, dependent on the angle of the three central bonds are the spirals, hooks and staples (Figure 1.5B). These may be further classified as either right- or left-handed (RH or LH) dependent upon the sign of the χ^3 angle (right and left defining positive and negative rotation, respectively).

The most prevalent type of disulphide is the -LHSpiral (approximately 25% of all disulphides) and is considered to be the primary structural disulphide [179, 180]. This configuration has the lowest mean dihedral strain energy (DSE) of 10 kJ.mol^{-1} in X-ray structures and an average C_α-C_α' distance of 5.7 Å [179, 180]. The low bond energy coupled with the C_α distance infers stability on the protein in which they are found. Two unusual disulphide bond types are the -RHStaple and +/-RHHook disulphides with mean DSEs of 18.1 kJ.mol^{-1} and 20.8 kJ.mol^{-1} compared to an average of 14.8 kJ.mol^{-1} for all disulphides as well as average C_α-C_α' distances of 4.3 and 5.7 Å, respectively [179]. These high energy bonds are documented to be more easily cleaved than bonds which have lower stored energy [181, 182]. The ability of these bonds to switch between bonded and unbonded states controls the function of the protein in which they reside. As such, these types of high energy bonds have been termed the functional disulphides. The best characterised functional disulphides are those found at the active sites of thiol-disulphide oxidoreductases [183]. By cycling between oxidised and reduced states with thiols or disulphides in protein substrates, these disulphide bonds are able to form, reduce or isomerise disulphides in the substrate and are therefore called the catalytic disulphides. Some of the highly strained disulphides, however, are able to regulate the function of the protein in which they reside in a non-catalytic fashion by causing changes in the intra- and intermolecular structure of proteins; these are known as the allosteric disulphides [184]. Allosteric disulphide bonds have been found in several proteins such as HIV-1 gp120 [185, 186], CD4 [187, 188], botulinum neurotoxin A and B [189] and von Willebrand Factor [190-192]. The allosteric bonds in these proteins have been shown to break and reform dynamically, with significant implications for their function.

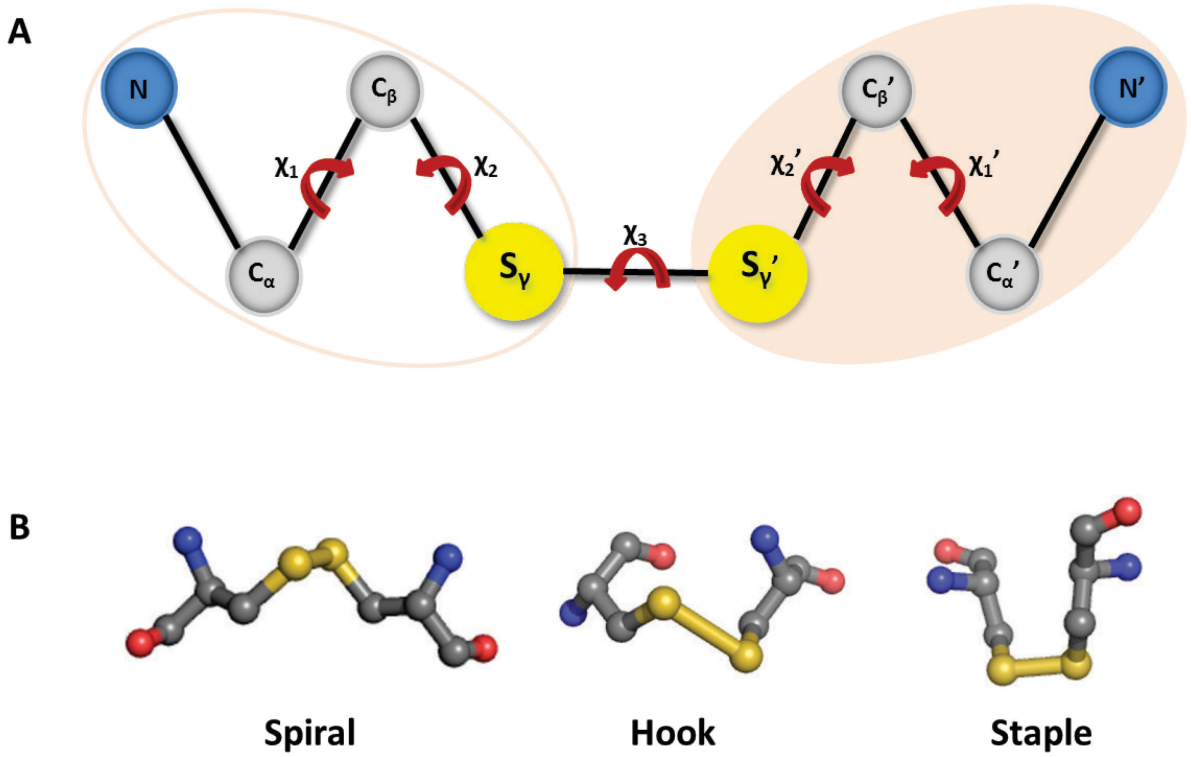

Figure 1.5: Disulphide bond geometry and configuration
(A) The disulphide bond geometry is described by the atoms linking the two C_α atoms. The eight atoms of the Cys residues define five χ angles. (B) The three types of disulphide bonds (spiral, hook and staple) are shown in ball and stick configuration (carbon grey, sulphur yellow, nitrogen blue and oxygen red). These configurations are defined by the angle of the central three bonds. The spiral configuration (-LHSpiral) was created using RNAse A 40C-C95 (PDB ID 1KF5), the hook configuration (+/-RHHook) was created using thioredoxin 30C-C33 (PDB ID 1R26) and the staple configuration created using 2dCD4 130C-C159 (PDB ID 3CD4). This figure was adapted from Schmidt *et al* [179] and created using PyMOL.

1.2.3 Allosteric Disulphides

The allosteric disulphides control the function of the protein in which they reside by inducing a structural change when reduced or oxidised [179, 180, 184]. The redox state of the allosteric disulphides may be controlled by the catalytic disulphides. The most common allosteric configuration is the -RHStaple, a defining feature of which is the close proximity of the C_α atoms of the two Cys residues (4.3 Å compared with a mean of 5.6 Å for all disulphides [179]). This distance is short due to the secondary structural elements linked by these disulphides with approximately 60% of these bonds linking adjacent strands in the same β-sheet rather than the more common linkage found between sheets. The resultant close proximity of these structural elements means that they need to pull together in order to accommodate the disulphide which results in significant bond strain [179, 193]. A classic example of an allosteric disulphide is the disulphide bond in the second domain of CD4 (Figure 1.3).

Observed allosteric disulphide bond cleavage has one of four potential outcomes: change in ligand binding, change in substrate hydrolysis, change in proteolysis or protein oligomer formation [172]. It should be noted that outcomes may be combined e.g. oligomer formation resulting in a change in ligand binding. The allosteric disulphides are cleaved by either oxidoreductases or by thiol/disulphide exchange within the protein containing the allosteric disulphide and in certain instances both cleavage mechanisms occur [172]. Thiol/disulphide exchange in the absence of an oxidoreductase occurs when the protein contains a sulphur ion nucleophile (i.e. a free Cys) that is activated when a conformational change brings the sulphur in line with the allosteric disulphide. Two such examples include CD4 and von Willebrand factor wherein allosteric disulphide cleavage by an oxidoreductase leads to the formation of two Cys thiolates which then cleave a second allosteric disulphide nearby the first. Reductive disulphide bond cleavage occurs via a bimolecular nucleophilic substitution (S_N2) reaction where the three sulphur atoms involved (the sulphur ion nucleophile and the two sulphur atoms in the disulphide bond) must form an approximately 180° angle [194, 195]. Due to the exacting stereochemistry required, stretching, twisting, pulling and shear forces influence the stability of the disulphide bond by changing the disulphide bond alignment [195-197]. It has also been suggested that cleavage of allosteric

disulphides may be influenced by the electrostatic environment of the cysteine involved [172].

Located at the active sites of oxidoreductases and thiol isomerases are the catalytic disulphides which mediate thiol/disulphide exchange in other proteins [183, 198]. These bonds cycle between reduced and oxidised states dependent on the oxidised and reduced states of the substrate protein, respectively. The redox state of the allosteric disulphides, as mentioned, may be controlled by the presence of the oxidoreductases.

1.2.4 Oxidoreductases

Oxidoreductase active-site disulphide bonds are nearly all +/-RHHooks, occurring in a conserved Cys-X-X-Cys motif at the end of an α-helix in a thioredoxin fold motif, which is present in all members of the Thioredoxin superfamily of proteins. This family includes the oxidoreductases such as thioredoxin (Trx), the protein disulphide isomerases such as protein disulphide isomerase (PDI) and other functionally different proteins such as the glutathione *S*-transferases (which catalyse the conjugation of GSH to xenobiotic substrates for the purpose of detoxification) and glutathione peroxidases (antioxidant enzymes which catalyse the detoxification of hydrogen peroxide). This thioredoxin motif consists of a four-stranded β-sheet surrounded by three α-helices (Figure 1.6) [199].

Trxs have several important cellular roles including: the regulation of enzyme activity by thiol-redox control (e.g. reduction of a single disulphide bond in ribonucleotide reductase allows this enzyme to convert ribonucleotides to deoxyribonucleotides essential for DNA synthesis [200]), protection of proteins from oxidative aggregation and inactivation [201-204], modulation of inflammatory responses [205] and protein folding [206]. Trx is secreted into the plasma from monocytes and is also present in a truncated form (Trx80) which acts as a growth factor inducing the T-helper-1 response in the presence of peripheral blood mononuclear cells (PBMC) via interleukin-12 [207]. Plasma levels of Trx have been used as a marker of inflammation, cancer and HIV infection [208, 209].

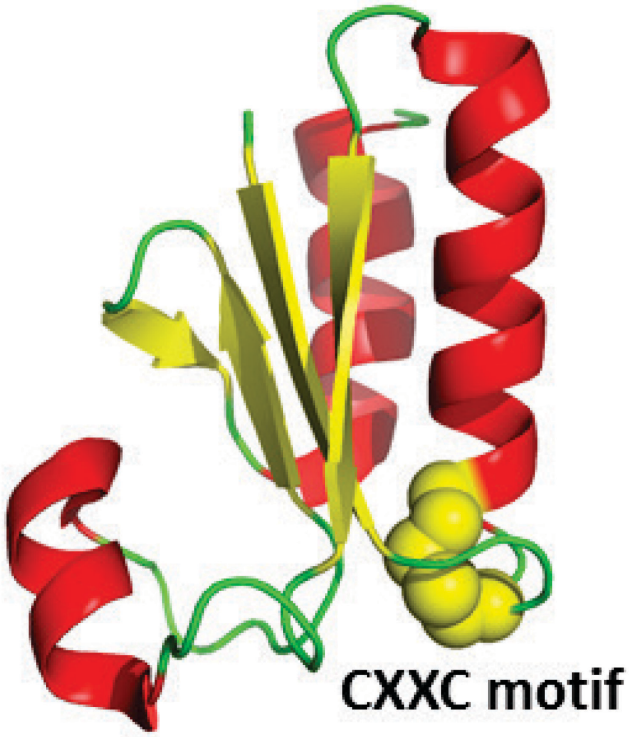

Figure 1.6: The thioredoxin (Trx)-fold
Three dimensional structure of glutaredoxin (PDB ID 1EGO) [210] showing the Trx fold – a four stranded β-sheet surrounded by three α-helices. The catalytic CXXC motif is shown at the N-terminal site of an α-helix with Cys residues in space filling mode shown in yellow.

A number of homologues of Trxs have been identified in organisms ranging from archae to mammals, each having a specific location e.g. cytosol [211], nucleus [212, 213], cell membrane [214] and the extracellular space [215, 216]. In mammals, two types of Trx isoforms have been identified namely Trx-1 (present in both cytosol and extracellular environment) and Trx-2 (present in the mitochondria). Despite the wide range of Trxs across organisms, they all have a common catalytic motif (CGPC) located on the highly conserved Trx fold [217] with Cys as the major role players during catalysis. The S_N2 reaction catalysed by Trx together with NADPH and thioredoxin reductase (TrxR) results in reduction of the protein substrate (Figure 1.7). TrxR is a selenoenzyme with TrxR1 present in the cytosol, TrxR2 in mitochondria and TrxR3 (also known as thioredoxin glutathione reductase) present mainly in the testis [218]. During this reaction, a disulphide bond is transferred from the substrate protein to Trx (i.e. Trx electrons are shuttled to the substrate protein). Oxidised Trx is more stable than reduced Trx which provides the reaction driving force.

Another important role player in the redox control of protein function is protein disulphide isomerase (PDI). PDI is a member of the thiol isomerase family (and Trx Superfamily) and is an essential enzyme and chaperone that assists protein folding in the ER [219-223]. Despite active-site motifs and domain structure being a common feature, members of this family show low sequence identity [224, 225]. These proteins contain three major domain types which can be found in different orders and combinations: a thioredoxin-like, catalytically active domain, a catalytically inactive thioredoxin fold domain and a highly acidic domain believed to be involved in peptide binding. PDI is 55 kDa and catalyses the formation (oxidase activity) and rearrangement (isomerase activity) of disulphide bonds in proteins and the reducing activity is dependent on electron transfer from either GSH or TrxR1 [226]. Each of the two catalytic domains contains two Cys in the sequence -CGHC- [227]. The N-terminal Cys residue of the first active site is stabilised partly by the histidine imidazole group leading to a much lower pK_a value for this Cys than that of free Cys [224]. This allows the thiolate anion generated to initiate nucleophilic attack on disulphide bonds, making this Cys highly reactive.

In addition, there is a C-terminal ER retention sequence (-KDEL) which binds to -KDEL receptors and thereby ensures retention of PDI in the ER [228, 229]. This motif also serves as a retrieval signal for proteins normally present in the ER that have been transported beyond this compartment [230]. This was first demonstrated by Pelham [230] who attached an ER retention signal to the lysosomal protein cathepsin D, a protein modified by enzymes that do not act in the ER. Although this protein was shown to accumulate in the ER, it continued to be modified thereby supporting the idea that ER proteins are continuously retrieved from a post-ER compartment. Since this -KDEL motif serves as both a retention and retrieval signal for maintaining proteins in the ER, the presence of thiol isomerase enzymes in locations other than the ER (e.g. on the surface of cells) could be expected. Indeed, PDI has been identified on the surface of resting platelets [231] and a number of other cell types, including bovine aortic endothelial cells [232] as well as in the extracellular milieu of rat hepatocytes [233, 234] and human B cells [235, 236]. Studies profiling cell-surface proteins have found PDI present on the surface of cancer and leukaemia cell lines [237, 238] as well as endothelial and fibrosarcoma cell lines [239]. In addition to the presence of PDI on the surface of cells, this enzyme is also secreted at low levels from a variety of cell types including hepatocytes, pancreatic exocrine cells, endothelial cells and activated platelets [240]. Two distinct mechanisms for the pathway resulting in cell-surface exposure have been suggested: secretion into the media followed by re-association with the cell membrane (which would allow for greater degree of interaction with extracellular components), or direct placement into the cell membrane as part of a transport process [223]. No reports have detailed the presence of -KDEL receptor proteins on the cell-surface and it has been suggested that cell-surface PDI binds through electrostatic interactions [234].

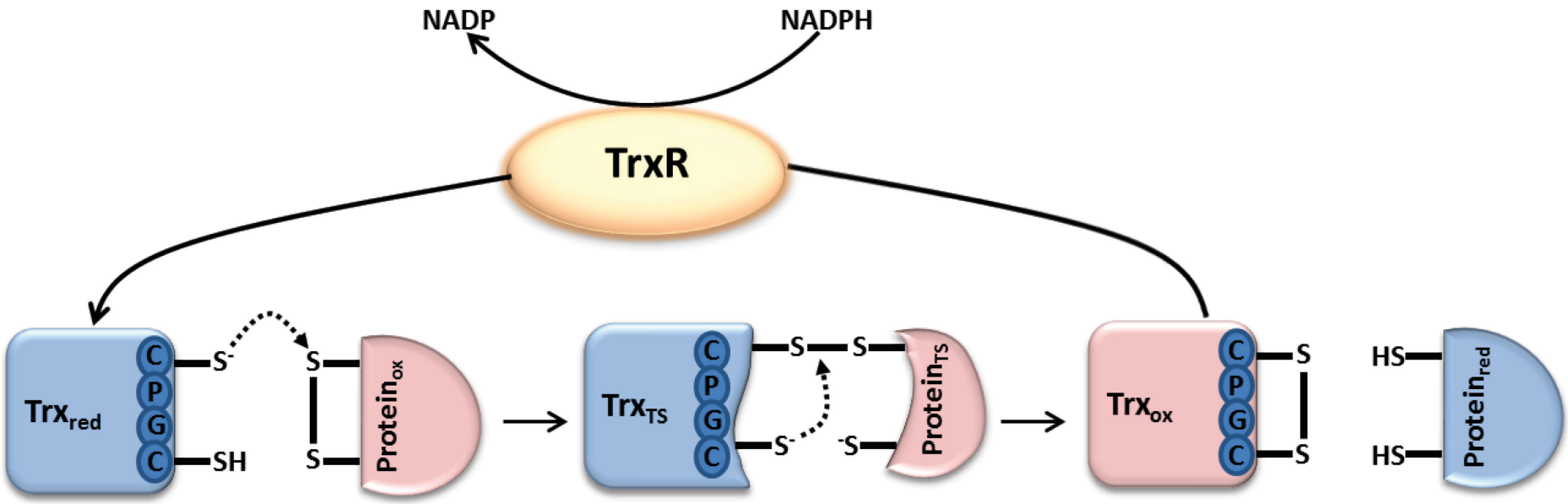

Figure 1.7: Schematic representation of the reaction mechanism of substrate disulphide reduction by Trx
Nucleophilic attack of the substrate thiol by the conserved N-terminal Cys in the -CPGC- motif starts the reaction. This results in the formation of a transition state (TS) mixed disulphide between Trx and the substrate protein which is subject to nucleophilic attack by the C-terminal Cys of Trx. The abbreviations ox and red stand for oxidised and reduced, respectively. This figure was adapted from Collet and Messens [241].

The tendency for either one of these enzymes to catalyse reduction or oxidation of a particular substrate is dependent upon their respective redox potential (a measure of the propensity of a molecule/protein to gain or donate electrons). The redox potential of Trx1 is -270 mV [242] and for PDI is -175 mV [243] which makes the active site of PDI far more oxidising than that of Trx1. Additionally, the redox potential for the allosteric disulphide of CD4 is -241 mV [244]. This puts the redox potential of CD4 closer to that of the strong protein reductant Trx suggesting that the most likely catalyst of disulphide bond reduction in CD4 would be Trx.

1.3 Redox Biology and HIV

Having discussed the biology of HIV entry as well as the redox biology of disulphide bonds during protein function, the question that remains is: how do these two fundamental principles integrate? The conformational changes in both viral and host receptors classically thought to be driven by their interactions, are believed to be aided by redox active enzymes present at the cell surface [185, 186, 245-247]. The allosteric disulphides in both gp120 and CD4 are substrates for one or more of the cell surface oxidoreductases (see below) and the structural changes that result from disulphide exchange lead to either promotion or inhibition of the gp120-CD4 interaction.

1.3.1 gp120 Redox Biology

Dynamic rearrangement of the highly conserved disulphide bonds within the core structure of gp120 [120] have been shown to be critical during entry [121, 186, 248-250]. Four of these disulphide bonds are located within the variable loop structures and two within the CD4bs on gp120 [62] (Figure 1.2). Moreover, rearrangements of these disulphides have been shown to be catalysed by cell surface active PDI [185, 186, 246, 249-251], Trx [249, 252] and Glutaredoxin-1 (Grx-1) [253]. More recently, the disulphide bonds targeted by PDI and Trx have been identified: C54-74C, C126-196C, C131-157C, C378-445C and C385-418C [254]. All five of these disulphides have been previously identified as allosteric disulphides [172]. Although the configuration and secondary structures linked by C54-74C and C131-157C are unknown, C126-196C, C378-445C and C385-418C are in the -RHStaple, -RHSpiral and -RHStaple configurations linking β-strands. Using a mechanism-based kinetic trapping

approach followed by mass spectrometric analysis, Azimi *et al* were also able to identify the C296-331C disulphide bond (–RHStaple), constraining the V3 domain β-loop structure, as the target for Trx-mediated reduction of gp120 [252]. Although the potential disulphide substrates for Trx and PDI in gp120 have been identified, the exact number of disulphides reduced for optimal viral entry has not been fully revealed. Previous studies have indicated that up to four disulphides in gp120 may be reduced [180] and that at least two bonds are susceptible to cleavage during entry [185, 255].

Reduction of gp120 disulphides is critical for Env-mediated cell-cell fusion, HIV entry and infection, since these processes are blocked by both small thiol alkylators and protein inhibitors of cell-surface oxidoreductases [185, 186, 246, 250]. It seems that ligand binding may influence the cleavage of gp120 allosteric disulphides since this cleavage is enhanced when gp120 is bound to CD4 [252]. Previous studies have indicated a complex role for Trx1 in HIV infection, possibly acting as both promoter and inhibitor of HIV infection [214, 256, 257]. Azimi *et al* [258] have suggested that the binding of HIV gp120 to CD4 which leads to co-receptor binding also functions to bring gp120 into close proximity of a cell surface oxidoreductase. Conformational changes in gp120 then facilitate the reduction of the V3 disulphide bond by the oxidoreductase. This disulphide bond cleavage then triggers a conformational change in the V3 domain, chemokine co-receptor dissociates from gp120 and finally gp41 refolds and viral and host cell membranes fuse. This hypothesis highlights the important role that oxidoreductase activity plays in the dynamic rearrangements of gp120.

1.3.2 CD4 Redox Biology

In addition to the oxidoreductase activity directed towards disulphides in the gp120 subunit of Env, the D2 disulphide of CD4 (-RHStaple allosteric disulphide) has been shown to be reduced by Trx-1 which is secreted by $CD4^+$ cells [188, 257]. Specific isoforms and oligomeric states of CD4 have been detected on the cell surface, each believed to have distinct utility in HIV-1 gp120 binding and antigen presentation [259-261]. Reduction of the D2 disulphide bond is thought to lead to the formation of covalently linked, domain-swapped dimers (between Cys130 in one monomer and Cys159 in the other) on the cell surface [188, 262-264]. The disulphide-linked dimeric CD4 is the preferred form for binding MHC class II

molecules (MHCII) expressed on the surface of antigen-presenting cells such as macrophages [187], and the reduced monomeric form is the preferred form for binding gp120 during HIV-1 entry [265]. Although crystallographic data suggests that only residues within D1 make contact with the CD4bs in gp120 [62], it seems that the redox activity of the disulphide in D2 plays a vital role in HIV entry. The interaction of CD4 with HIV-1 gp120 appears to involve CD4 monomers, with CD4 dimerisation-defective mutants [266], and specifically reduced monomeric forms of CD4 [244], supporting higher levels of HIV infection in cell culture. In contrast, it has been shown that CD4 oligomerisation is necessary for stable MHCII binding [260], and that effective T-cell activation requires CD4 dimer formation [267, 268], a process which evidence suggests involves redox exchanges in CD4 Cys [187, 188, 263].

There is an abundance of data demonstrating the effects of manipulating the activities of cell surface-active oxidoreductases such as PDI and Trx on HIV infection *in vitro* [186, 245, 246, 249, 250], although considerable debate remains on the biological utility and physiological relevance of such effects, and the extent of involvement of these factors *in vivo*. Furthermore, and complicating the elucidation of the biochemical importance of these enzymes for the mechanism of HIV entry, are the observations that gp120, itself containing nine disulphide bonds, is also a substrate for PDI-, Trx- and Grx-1-mediated reduction in *vitro* [186, 253, 269], and that inhibitory antibodies to PDI or Trx have different effects in the context of macrophage- or lymphocytic HIV infection [270].

1.4 Thesis Objectives

The importance of disulphide bonds for the function of both the HIV-1 Env glycoprotein and receptor CD4 as well as the potential consequences of redox activity directed towards these moieties has been highlighted herein. By gaining insights into the redox biology of these receptors during the process of HIV-1 entry, novel approaches in the development of redox-active antagonists may be revealed. Since extensive research has focused on the redox biology of gp120, the main objective of this thesis was to obtain a clearer understanding of the redox biology of the CD4 receptor with regards to its biological function as well as potential applications of this molecule in HIV-1 drug and vaccine development.

The approach to the study involved the use of recombinant two-domain CD4 (2dCD4) molecules (containing the first two N-terminal domains of CD4): one engineered to target a disulphide in the CD4bs of gp120 and a panel of Cys to Ala mutants to investigate the effects of Trx-mediated regulation of CD4 on HIV-1 entry. These studies are presented in Chapters 2 and 3 as published research articles. In Chapter 2, a rationally designed 2dCD4 containing a Ser to Cys mutation (2dCD4-S60C) was used to target the V1/V2 disulphide bond (126C-C196) of gp120. Qualitative and quantitative evidence for disulphide targeting was obtained using gel shift analysis, surface plasmon resonance and an enzyme-linked immunosorbant assay where the gp120-2dCD4-S60C interaction was compared to the gp120-2dCD4-wild-type interaction. These results, which revealed a significant increase in the gp120-binding capabilities of CD4 containing the S60C mutation were investigated *in vitro* using a phenotypic viral inhibition assay. In Chapter 3, a panel of Cys to Ala mutant 2dCD4 molecules were used to investigate the catalytic effects of both PDI and Trx on 2dCD4 using non-reducing analytical SDS-PAGE analysis and an insulin reduction assay. These results revealed preferential reduction and consequential dimerisation of 2dCD4 mediated by Trx. Following this, the effects of gp120 on this Trx-mediated dimerisation were investigated. The tendency for recombinant 2dCD4 to present itself in three distinct isomeric forms is also highlighted in Chapter 3 and the biological utility of these isomers is discussed. In an addendum to Chapter 3, liquid chromatography coupled to tandem mass spectrometry is used to confirm the oxidation state of the observed and characterised isomers presented in the published article. Building on this, Chapter 4 describes a preliminary study that is the current focus in our laboratory wherein the effects of disulphide bonds on the structural conformation of 2dCD4 are being investigated. This basic study generated preliminary insights into possible consequences of disulphide reduction, and provides a platform for further investigations on the effects of disulphide bonds on CD4 structure. As a conclusion to this thesis, Chapter 5 summarises the highlights of the preceding chapters and puts these results into the perspective of current and planned research interests in our laboratory.

Chapter 2

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Stabilization of HIV-1 gp120-CD4 Receptor Complex through Targeted Interchain Disulfide Exchange*

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HIV-1 enters cells via interaction between the trimeric envelope (Env) glycoprotein gp120/gp41 and the host cell surface receptor molecule CD4. The requirement of CD4 for viral entry has rationalized the development of recombinant CD4-based proteins as competitive viral attachment inhibitors and immunotherapeutic agents. In this study, we describe a novel recombinant CD4 protein designed to bind gp120 through a targeted disulfide-exchange mechanism. According to structural models of the gp120-CD4 receptor complex, substitution of Ser^{60} on the CD4 domain 1 α-helix with Cys positions a thiol in proximity of the gp120 V1/V2 loop disulfide (Cys^{126}–Cys^{196}), satisfying the stereochemical and geometric conditions for redox exchange between CD4 Cys^{60} and gp120 Cys^{126}, and the consequent formation of an interchain disulfide bond. In this study, we provide experimental evidence for this effect by describing the expression, purification, refolding, receptor binding and antiviral activity analysis of a recombinant two-domain CD4 variant containing the S60C mutation (2dCD4-S60C). We show that 2dCD4-S60C binds HIV-1 gp120 with a significantly higher affinity than wild-type protein under conditions that facilitate disulfide exchange and that this translates into a corresponding increase in the efficacy of CD4-mediated viral entry inhibition. We propose that targeted redox exchange between conserved gp120 disulfides and nucleophilic moieties positioned strategically on CD4 (or CD4-like scaffolds) conceptualizes a new strategy in the development of high affinity HIV-1 Env ligands, with important implications for therapy and vaccine development. More generally, this chalcogen substitution approach provides a general means of stabilizing receptor-ligand complexes where the structural and biophysical conditions for disulfide exchange are satisfied.

The first step of HIV-1[2] entry into host cells involves the interaction between the viral envelope protein (Env) and the host cell receptor CD4. Functionally active (fusogenic) Env exists on the surface of virions as a trimer composed of three heterodimeric gp120-gp41 complexes (1). The latter are established through noncovalent association of monomeric gp120 with membrane-embedded gp41 (2–4), such that gp120 is presented on the exterior of the viral lipid membrane for interaction with CD4. The development of both Env-directed therapeutic compounds (5–10) and Env-based immunogens (11–20) has been frustrated by the significant sequence heterogeneity known to exist among Envs, even from viruses that are phylogenetically closely related, which limits the cross-reactive breadth of anti-Env antibodies and therapies. Furthermore, Env is extensively glycosylated and conformationally flexible, which compounds the problem of Env ligand accessibility and reactivity. In the background of this extraordinary array of strategies evolved to enable viral escape from effective immune-mediated neutralization, and apart from a very small number of studies that have suggested the presence and relevance of CD4-independent viruses in the periphery during natural infection (21), the fundamental importance of CD4 for HIV-1 attachment and infection has dictated that at least one site on gp120, the CD4-binding site (CD4bs), is structurally conserved and constitutively accessible to its cognate receptor. For this reason, much research has focused on the development of both immunogens that present stabilized forms of the CD4bs (22–25), and recombinant protein or small molecule ligands targeting the CD4bs as attachment inhibitors or immunotherapeutic agents (5, 26–30).

The challenge in this regard is substantial, however; after showing promising antiviral effects in cell culture against laboratory-adapted HIV-1 strains (9), recombinant soluble CD4 (sCD4) was shown to be ineffective against primary viral isolates (31), and very large doses of sCD4 were required to achieve modest reductions in viral loads *in vivo* (32). The most likely explanation for these observations, provided by pioneering structural studies on gp120 in complex with CD4 (33) and a CD4bs-directed antibody (IgGb12) (34), is that the initial interaction between CD4 and gp120 on the native Env trimer is relatively weak and that a more stable complex requires contact with surfaces that are constituted only after substantial conformational rearrangement of gp120. During natural infection, coordination of target cell CD4 molecules enables multiple contact events per trimer, and this avidity overcomes the kinetic barriers presented by the requirement for sequential sampling to a conformation competent for binding CD4 stably.

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[2] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Env, envelope; PBS, phosphate-buffered saline; DTT, dithiothreitol; WT, wild type; sCD4, soluble CD4; mAb, monoclonal antibody; SPR, surface plasmon resonance; ELISA, enzyme-linked immunosorbent assay.

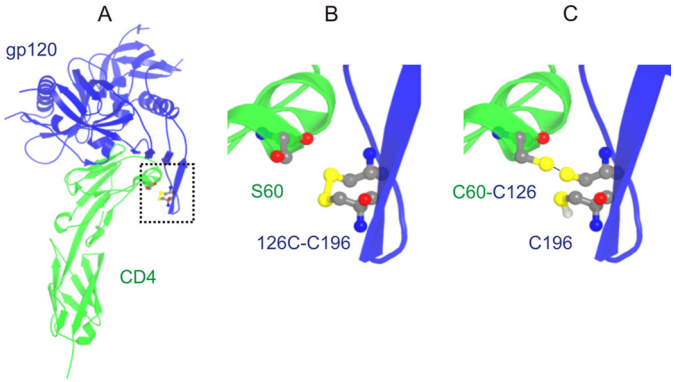

FIGURE 1. **Molecular model of CD4-gp120 receptor complex (Protein Data Bank code 1G9M).** *A,* gp120 (*blue*) and CD4 (*green*) are shown in *ribbon* representation, with Ser^{60} on the CD4 D1 α-helix shown in ball-and-stick representation. The *inset* (enlarged in *B*) shows the apposition of Ser^{60} with the Cys^{126}–Cys^{196} disulfide of the gp120 V1/V2 loop. *C,* molecular modeling of the CD4 S60C variant calculates the rotomeric positioning of the Cys^{60} side-chain thiol to within 2 Å of the target gp120 disulfide and predicts the formation of the interchain CD4 Cys^{60}–Cys^{126} gp120 disulfide bond. The atomic structures of the CD4 S/C60 and gp120 Cys^{126}–Cys^{196} residues are shown in *ball-and-stick* configuration (*yellow,* sulfur; *gray,* carbon; *red,* oxygen; *blue,* nitrogen; *white,* hydrogen).

Thus, sCD4 *per se*, by virtue of a relatively lower overall affinity for Env, competes ineffectively with target cell-bound CD4. More recently, the development of various oligomeric CD4-immunoglobulin chain fusion proteins has resulted in improvements in both the overall affinity for gp120 and pharmacokinetic characteristics of the CD4 fusion proteins. The translation of these improvements into viable therapeutic compounds with the ability to mediate potent reductions in viral loads at low concentrations remains elusive, however.

With this in mind, we examined structural models of the CD4-gp120 complex and evaluated the potential biochemical effects of several conservative mutations of critical CD4 gp120-interacting residues. Our aim was to identify amino acid changes in the CD4 sequence that could be incorporated into the designs of soluble recombinant CD4 proteins, which could enhance the stability of their interactions with gp120 and correspondingly improve their competitive potencies. One particular CD4 mutation we studied was S60C, which resulted in a striking theoretical enhancement of the interaction with gp120 at this position. When modeled on a crystal structure of CD4 in complex with gp120 (Protein Data Bank code 1G9M), substitution of Ser^{60} on the CD4 domain 1 α-helix with Cys positions a reactive thiol in the proximity of the gp120 V1/V2 loop disulfide (Cys^{126}–Cys^{196}), satisfying the stereochemical and geometric conditions for redox exchange between the CD4 Cys^{60} and gp120 Cys^{126} and the consequent formation of an interchain disulfide bond (Fig. 1). Therefore, we proposed that the stereochemically conservative S60C CD4 mutation might result in negligible structural deviation from the wild-type sequence (35) and simultaneously promote a highly favorable interaction based on novel disulfide bond formation between the mutant sequence and the target gp120.

In this study, we present data showing that a functional recombinant 2dCD4 protein carrying the S60C mutation binds gp120 with a higher affinity than wild-type 2dCD4 and that this enhanced affinity is associated with targeted disulfide exchange. Furthermore, we show that 2dCD4 (S60C) competitively inhibits the binding of recombinant gp120 to CD4 *in vitro* more effectively than wild-type CD4, and potentiates a correspondingly more potent antiviral effect in cell culture. Considered with recent results by others, which have suggested critical functional (in addition to structural) roles for resident Env disulfides (36–40), and noting the striking conservation of these disulfides in the context of a hypervariable sequence, we propose that targeted redox exchange between conserved gp120 disulfides and nucleophilic moieties (such as Cys) positioned strategically on recombinant CD4 scaffolds represents a revolutionary strategy in the development of high affinity HIV-1 Env ligands.

EXPERIMENTAL PROCEDURES

Recombinant Proteins and Expression Plasmids—The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health (contributor in parentheses): recombinant $gp120_{BaL,}$ mAb 2G12 (Dr. Hermann Katinger); mAb 17b (Dr. James Robinson); soluble CD4 (4dCD4/sCD4; Progenics Pharmaceuticals Inc. (New York)); $pSG3^{renv}$ (Dr. John C Kappes and Xiaoyun Wu); and CAP210 Env-expressing clone (Drs. David Montefiori, Feng Gao, Salim Abdool Karim, and Gita Ramjee). DNA cassettes encoding the first two amino-terminal domains of human CD4 (2dCD4 residues 1–183, wild-type sequence, S60C, and folding-defective variant ($2dCD4^{FD}$)) were synthesized by GENEART (Regensburg, Germany) following codon optimization for expression in *Escherichia coli*. The cassettes additionally contained a 3′-terminal 6× polyhistidine-encoding sequence to enable affinity purification of the expressed proteins, and NcoI and BamHI restriction endonuclease sites on the 5′ and 3′ extremities, respectively, which were used for subcloning into the corresponding sites in pET15b (Novagen, Germany) to yield pET15-2dCD4 ($WT/S60C/^{FD}$).

2dCD4 Expression, Purification, and Refolding—Recombinant pET15-2dCD4 ($WT/S60C/^{FD}$) vectors were used to transform BL21 (DE3) *E. coli*; protein expression was achieved using a "leaky" (isopropyl 1-thio-β-D-galactopyranoside-free) induction protocol by culturing freshly transformed bacteria in standard LB medium for 20 h at 20 °C with vigorous shaking. The recombinant 2dCD4s were purified from inclusion bodies using a denaturing purification protocol and refolded in glutathione-containing refolding buffers. Briefly, following centrifugation of 200 ml of an overnight culture, the bacterial pellet was resuspended in 25 ml of phosphate-buffered saline (PBS, pH 7.4) containing 0.5 mg/ml chicken egg lysozyme (53,000 units/mg, Sigma). The resuspension was incubated at 4 °C for 1 h with gentle shaking, and the cells then lysed by three cycles of freeze/thawing in liquid nitrogen and sonication. The lysate was centrifuged at 20,000 × *g* for 30 min at 4 °C, and the supernatant was discarded. The pellet was resuspended in 25 ml of solubilization buffer (PBS, pH 7.4, 8 M urea, 50 mM glycine, 2 mM β-mercaptoethanol, 20 mM imidazole) and homogenized using a tissue homogenizer (Omni International). The homogenate was centrifuged at 20,000 × *g* for 45 min, and the supernatant was recovered and filtered through a 0.45-μm syringe filter.

Recombinant 2dCD4 was purified from the filtered, solubilized inclusion bodies in the presence of 8 M urea, 50 mM glycine, 2 mM β-mercaptoethanol, and imidazole (20–500 mM) by standard Ni^{2+}-affinity chromatography procedures. The purified protein (20 ml) was immediately dialyzed overnight at 4 °C against 1 liter of folding buffer 1 (50 mM glycine, 10% sucrose, 1 mM EDTA, 1 mM reduced GSH, 0.1 mM oxidized glutathione (GSSG) and 4 M urea, adjusted to pH 9.6 with NaOH) and then changed into 1 liter of folding buffer 2 (50 mM sodium carbonate, pH 9.6, 10% sucrose, 1 mM EDTA, 0.1 mM GSH, 0.01 mM GSSG) for a second overnight dialysis at 4 °C. The purified protein was then dialyzed exhaustively against PBS, pH 7.4 (4 °C, three times with 2 liters, 4 h for the first two dialyses, and then overnight for the final dialysis), recovered, filtered through a 0.45-μm syringe filter, and concentrated using Centricon ultrafiltration tubes (Millipore). The concentrated pure protein sample was aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C. Using this method, we routinely purified between 0.5 and 1.0 mg of functional recombinant 2dCD4 (per 200 ml of bacterial cell culture).

Recombinant 2dCD4 Folding and Functionality Analysis—The purified, refolded 2dCD4s were analyzed by denaturing, nonreducing SDS-PAGE and CD spectroscopy to check structural disulfide bond formation and native folding patterns, respectively. For PAGE analysis, protein samples (2.0 μg) were resolved on precast 12% BisTris polyacrylamide gels (Invitrogen) according to the manufacturer's instructions in the presence or absence of 50 mM 1,4-dithiothreitol (DTT), and the gels were stained with Coomassie Blue R-250. Circular dichroism spectra of 2dCD4-WT, 2dCD4-S60C, or $2dCD4^{FD}$ (10 μM, in 10 mM sodium phosphate, pH 7.4, 140 mM NaCl) were collected using a Jasco J-810 spectropolarimeter with a 1-mm path length cuvette. Five scans were made at a scan rate of 100 nm/min, a bandwidth of 1 nm, and a response time of 2 s. All spectra were buffer-corrected, and normalized mean residue ellipticities (degrees·cm^2/dmol) were calculated as described previously (41). All spectra were recorded at room temperature from 250 to 200 nm. The functionality of the recombinant 2dCD4s was tested by checking the efficiency of gp120 binding using surface plasmon resonance (SPR) (see below).

gp120–2dCD4 (S60C) Complex Stabilization Analysis in Vitro—The ability of 2dCD4 (S60C) to bind gp120 and stabilize the interaction through targeted disulfide exchange was analyzed by denaturing, nonreducing PAGE and SPR. For gel shift analysis, binding reactions were set up in 20 μl and contained 2 μg of recombinant $gp120_{BaL}$, 6.5 μg of recombinant 2dCD4-WT or 2dCD4-S60C, and 1 mM β-mercaptoethanol in PBS, pH 7.4. The reaction was incubated at 30 °C for 2 h, before mixing with 4× lithium dodecyl sulfate (LDS) sample loading buffer (Invitrogen) in the presence or absence of 50 mM DTT. The samples were then placed in a boiling water bath for 5 min and resolved on a precast 12% BisTris polyacrylamide gel. For specific detection of gp120, and free and gp120-bound 2dCD4 forms, the resolved proteins were then transferred to a nitrocellulose membrane by Western blotting and probed with an anti-gp120 antibody (ab21179, Abcam) and an anti-CD4 antibody (MT310, Santa Cruz Biotechnology), respectively. Detection was performed by standard chemiluminescence methodologies using a secondary horseradish peroxidase-conjugated anti-IgG antibody (GE Healthcare). For SPR, all experiments were performed on a BIAcore® 3000 optical biosensor (Biacore Inc., Uppsala, Sweden) at 25 °C. For direct binding comparisons and kinetics, 2dCD4-WT, 2dCD4-S60C, or $2dCD4^{FD}$ were immobilized on separate flow cells within the same sensor chip using amine coupling according to the manufacturer's instructions. Briefly, a solution of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 0.05 M *N*-hydroxysuccinimide was used to activate carboxyl groups on the sensor surface at a flow rate of 5 μl/min for 10 min. The recombinant 2dCD4, dissolved in sodium acetate buffer (100 mM, pH 5.5) to a final concentration of 4.5 μg/ml, was passed over the sensor chip surface at a flow rate of 5 μl/min until the desired immobilization level (2500 response units) was achieved. Excess carboxyl groups were capped by injection of 1 M ethanolamine, pH 8.0, at a flow rate of 5 μl/min for 5 min. Unbound ligand was removed by injecting glycine (10 mM, pH 2.5) at a flow rate of 5 μl/min for 2 min. Flow cell four was left blank as a control for nonspecific binding and refractive index changes. Analyte solutions ($gp120_{BaL}$, 0–500 nM) were prepared in HEPES-buffered saline supplemented with 3 mM EDTA, 1 mM β-mercaptoethanol, and 0.005% surfactant P20 (HBS-EP, pH 7.4) at least 30 min prior to injection and passed over the 2dCD4 surfaces at 10 μl/min, with a 20-min association phase and either a 50-min (for kinetic analyses) or 10-h (for complex stability analysis) dissociation phase. To confirm disulfide linkage between 2dCD4-S60C and gp120, the surfaces from overnight dissociations were treated with increasing concentrations of DTT (10–100 mM). When checking the specificities of the 2dCD4-gp120 interactions, mAb 17b was passed over the bound complexes at 200 nM. The 17b epitope on gp120 (a so-called CD4-induced (CD4i) epitope) is characteristically only efficiently exposed following gp120 binding to CD4, and checking the efficiency of 17b uptake provides an additional control for the quality of the gp120-CD4 interaction measured by SPR. Buffer injections and control surface binding were subtracted for all reported data, and all binding experiments were performed in triplicate, on separate chips.

2dCD4-gp120 Competition ELISA—96-Well Maxisorp microtiter plates (Nunc, Denmark) were coated with sCD4 (Progenics, 100 ng/ml in PBS, pH 7.4) for 3 h at room temperature (100 μl per well). The coating solution was removed, and the plates were then blocked with 250 μl of PBS containing 0.05% Tween 20 and 10 mg/ml bovine serum albumin for 16 h at 4 °C. After removing the blocking buffer and rinsing once with PBS containing 0.05% Tween 20 (PBS-T), a mixture of 2dCD4 (WT/S60C) (0–1 μM) and 1 nM of $gp120_{BaL}$ was added to the wells (200 μl in PBS-T), and the plates were incubated at 30 °C for 2 h. When checking the effect of the reaction redox potential on relative potency of 2dCD4-mediated gp120-sCD4 binding inhibition, DTT was included in the binding reactions at 0, 10, and 100 mM. The wells were washed five times with 250 μl of ice-cold PBS-T, and bound gp120 was then probed with 100 μl of mAb 2G12 (1 μg/ml in PBS-T) for 1 h at 30 °C. After washing, bound gp120 was detected using 100 μl of a horseradish peroxidase-conjugated monoclonal anti-human Fc antibody (Amersham Biosciences), diluted 1:2000 in PBS-T and incubated for 1 h at 4 °C. Bound secondary antibodies were detected, follow-

ing washing, by chromogenic methods using TMB Ultra substrate (Pierce) and quantified spectrophotometrically by measuring A_{450} levels.

Viral Inhibition Assay—The inhibitory effect of purified 2dCD4 (WT/S60C) on viral entry was assessed by an *in vitro* phenotypic inhibition assay (42) using an HIV-1 pseudovirus backbone ($pSG3^{renv}$) complemented with a subtype C CAP210 envelope. Briefly, a 3-fold serial dilution of each 2dCD4 variant (in PBS, pH 7.4) was prepared in a 96-well plate (Nunc), with concentrations ranging from 22.0 to 0.03 μg/ml. The diluted compounds were incubated with 200 $TCID_{50}$ of pseudovirus for 1 h at 37 °C. Ten thousand TZM-bl cells (in Dulbecco's modified Eagle's medium containing 12 μg/ml DEAE) were then added to each well, and the cells were incubated at 37 °C for a further 48 h in a humidified CO_2 incubator. Nonvirus and virus controls were prepared in exactly the same manner, except for the omission of virus or test compounds where appropriate. Luciferase activity induced in the TZM-bl cells (measured in relative light units) was quantified using the Bright-Glo luciferase assay system (Promega) according to the manufacturer's instructions. Inhibition of viral replication was expressed as a percentage of the reduction in relative light unit values generated in the absence of any compound. Cell culture toxicity was measured using the CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer's instructions. The significance of the difference between 2dCD4-WT- and 2dCD4-S60C-treated samples (during ELISA and virus inhibition experiments) was evaluated using a paired, two-tailed Student's *t* test at a confidence level of 95%.

RESULTS

Purification of Native, Functional 2dCD4-WT and 2dCD4-S60C—Because the reducing environment of the *E. coli* cytoplasm often renders correct folding of structural disulfide-containing proteins expressed in these hosts problematic, previous studies on the expression of functional recombinant 2dCD4 in *E. coli* have either made use of technologies to secrete the expressed protein to the bacterial periplasm (43) or post-purification refolding protocols (44–45). We initially employed a periplasmic secretory vector (pET22a, Novagen) to express and purify both 2dCD4-WT and 2dCD4-S60C. Although we were able to purify an active wild-type 2dCD4 expressed by this vector from the periplasmic fraction, we were unsuccessful in this endeavor with the free thiol containing 2dCD4 variant (S60C) (data not shown), presumably because the presence of an unpaired cysteine in this polypeptide interferes with efficient periplasmic secretion and/or canonical disulfide pairing and oxidation in this milieu. We therefore decided to apply a controlled oxidative refolding protocol to the recombinant 2dCD4s affinity-purified from *E. coli* inclusion bodies. Because 2dCD4-S60C contains a single unpaired cysteine in addition to the single cysteine pairs in D1 (Cys^{16}–Cys^{84}) and D2 (Cys^{130}–Cys^{159}) of CD4, one potential problem in folding these proteins into the native 2dCD4 structure could relate to aberrant disulfide pairing during the refolding process. To qualify and quantify different disulfide-bonded intermediates generated during the oxidative refolding process and gain insights into the ability of the S60C variant to fold into a native 2dCD4 structure, we analyzed disulfide oxidation in the refolded proteins by reducing and nonreducing SDS-PAGE (46).

Under reducing conditions, both 2dCD4-WT and 2dCD4-S60C migrated as a single band of ~21 kDa, corresponding to the predicted size for the fully denatured, reduced 2dCD4 isoform (Fig. 2*A*). When the proteins were analyzed under nonreducing conditions, the 2dCD4 proteins resolved as three distinct species that migrated between 17 and 21 kDa, most likely representing reduced protein (R) and the predominant disulfide-bonded 2dCD4 isoforms (O^1/O^2) (Fig. 2*A*). To gain further insights into which of the oxidized species was likely to represent the 2dCD4 isoform containing the correct disulfide-bonded cysteine pairs (Cys^{16}–Cys^{84} and Cys^{130}–Cys^{159}), we generated a folding-defective 2dCD4 mutant ($2dCD4^{FD}$) containing a five-amino acid substitution proximal to the D1 α-helical loop (residues 65–69, WT sequence, -GNFPL; $2dCD4^{FD}$ sequence, -VPGLV). Under nonreducing conditions, this mutant, which was expressed, purified, and refolded using the same procedures as 2dCD4-WT and 2dCD4-S60C, resolves into three predominant species (Red, O^2, and O^3), with little or no O^1 isoform present in the mixture (Fig. 2*A*). When the functionality of the protein was analyzed by SPR assay, $2dCD4^{FD}$ was unable to bind gp120; in contrast, 2dCD4-WT and 2dCD4-S60C, which have the same pattern of bonding and for which O^1 is the predominant oxidized isoform, all bound gp120 efficiently (Fig. 2*B*).

To compare folding characteristics and further evaluate whether the variants had acquired native secondary structural elements typical of 2dCD4, we then analyzed the purified and refolded proteins by CD spectroscopy (Fig. 2*C*). Far-UV spectra for the 2dCD4 WT and S60C proteins were virtually identical and typical of the classical β-structure spectrum, mean ellipticity minima and maxima were at 212–214 and 200 nm, respectively, in line with crystallographic data showing the predominant contribution of β-strands to the 2dCD4 structure, and consistent with published CD data for 2dCD4 (47–48). Conversely, the spectrum generated by $2dCD4^{FD}$ contained a large leftward shift in ellipticity minima and maxima, suggestive of irregular β-stranded structure and confirming, as expected, the failure of the folding-defective mutant 2dCD4 to fold into the native β-stranded conformation.

Taken together, these data suggest that O^1 represents the 2dCD4 isoform containing the correct disulfide pairs and that 2dCD4-S60C, like the wild-type protein, folds into a native 2dCD4 structure (with demonstrably efficient gp120 binding) through oxidation of correctly paired cysteine residues.

2dCD4-S60C Binds gp120 Stably through Disulfide Exchange—We adopted two independent approaches to study the gp120-binding properties of purified 2dCD4-WT and 2dCD4-S60C. First, we used an electrophoretic mobility shift assay to detect gp120-bound 2dCD4 following resolution of the protein complexes on denaturing polyacrylamide gels. This method also enabled us to determine whether the mechanism of binding of 2dCD4-S60C could additionally involve establishment of disulfide bonds with the target gp120, by measuring the stability of bound 2dCD4 under reducing and nonreducing conditions.

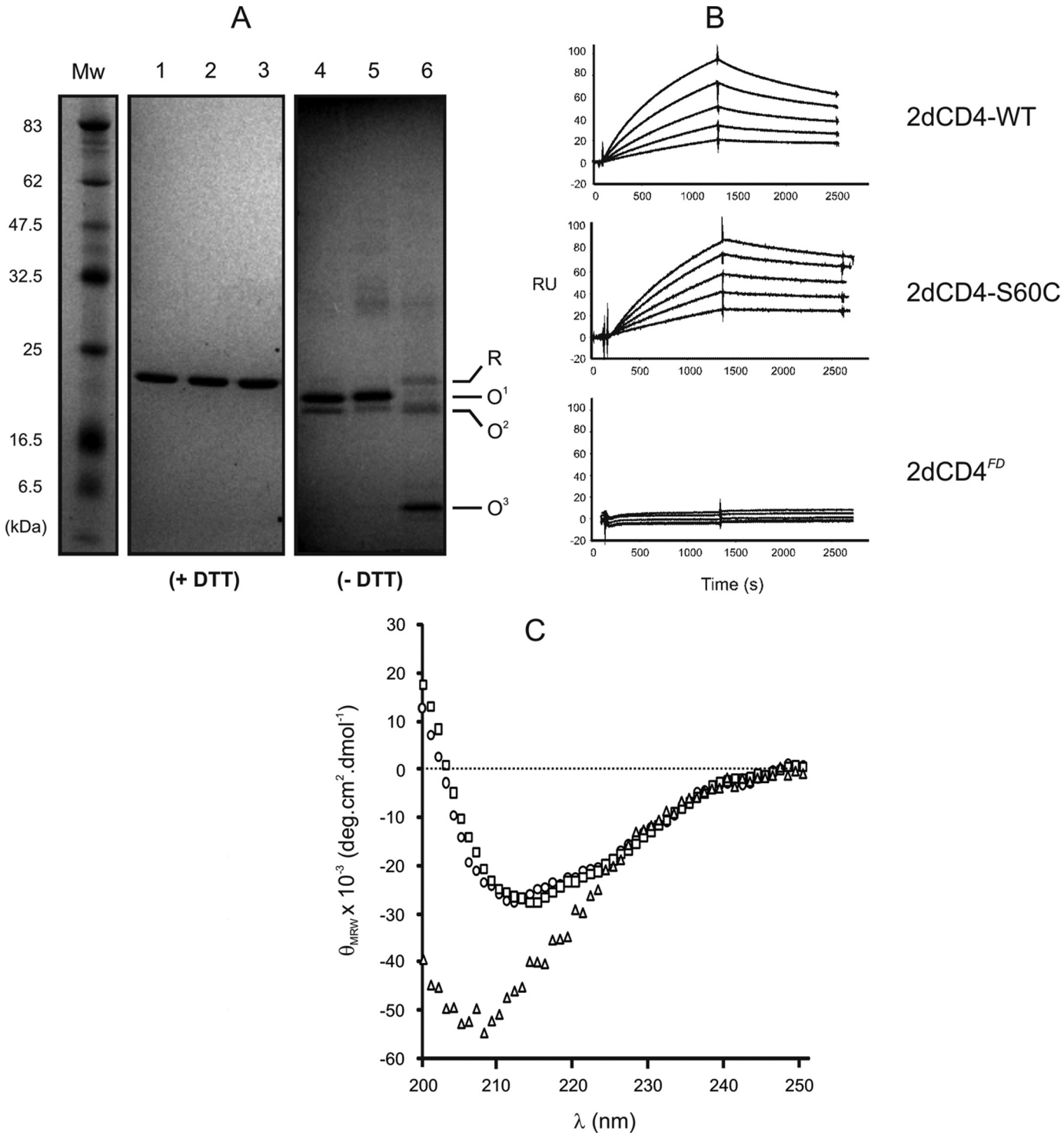

FIGURE 2. **2dCD4-WT or 2dCD4-S60C refolding and activity analysis.** *A,* reducing and nonreducing SDS-PAGE analysis. Purified, refolded 2dCD4-WT (*lanes 1* and *4*), 2dCD4-S60C (*lanes 2* and *5*), and 2dCD4^{FD} (*lanes 3* and *6*) were incubated with SDS-PAGE loading buffer with (*left panel*) or without (*right panel*) 50 mM DTT, resolved on a 12% BisTris polyacrylamide gel, and stained with Coomassie Blue R-250. *B,* gp120 binding activity analysis by SPR. Purified, refolded 2dCD4-WT, 2dCD4-S60C, or 2dCD4^{FD} was immobilized on sensorchips and sensorgrams generated by probing with recombinant gp120$_{BaL}$ (31.3. 62.5, 125, 250, and 500 nM). *C,* far-UV spectra of refolded 2dCD4-WT (○), 2dCD4-S60C (□), and 2dCD4^{FD} (△) (10 μM in PBS, pH 7.4). *RU*, response units.

Recombinant gp120 and either 2dCD4-WT or 2dCD4-S60C were reacted at 30 °C for 2 h. Immediately before loading on a 12% SDS-polyacrylamide gel, the reactions were mixed with SDS-PAGE loading buffer (without DTT) and placed in a boiling water bath for 10 min to disrupt any noncovalent quaternary interactions. In parallel, the gp120–2dCD4 complexes were treated with SDS-PAGE loading buffer containing 50 mM DTT. Under denaturing, nonreducing conditions, we were able to detect a significant quantity of 2dCD4-S60C that remained stably bound to gp120 following SDS and heat denaturation, although there was no detectable gp120-bound 2dCD4-WT (Fig. 3). Additional treatment of the complexes with DTT abol-

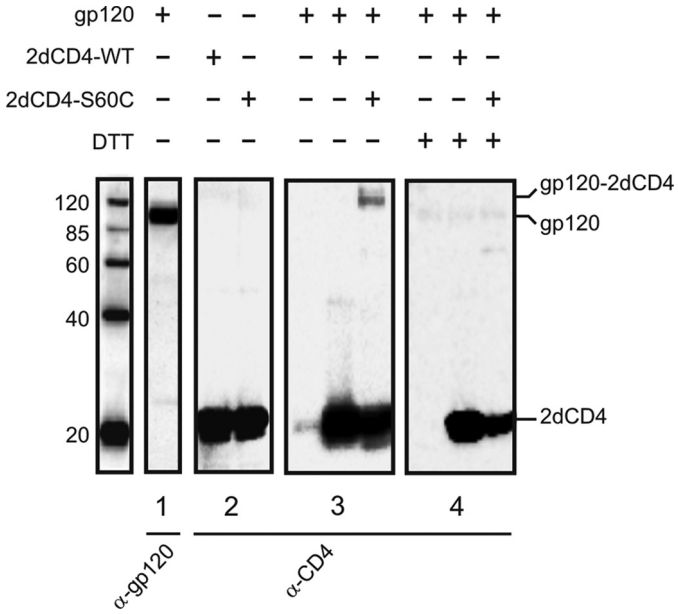

FIGURE 3. **Electrophoretic mobility shift analysis of 2dCD4-gp120 complexes.** gp120–2dCD4-WT and gp120–2dCD4-S60C complexes were heat- and SDS-denatured in the presence (*panel 4*) or absence (*panel 3*) of 50 mM DTT, resolved on a 12% denaturing polyacrylamide gel, and detected with an anti-CD4 antibody following immunoblotting. To confirm the specificity of antibody binding, the recombinant gp120 and 2dCD4 protein were resolved independently and detected with anti-gp120 (*panel 1*) and -CD4 (*panel 2*) antibodies, respectively.

TABLE 1
Kinetic parameters calculated for gp120–2dCD4-S60C binding

	K_D[a]	k_a[b]	k_d[c]	k_d^{+10}[d]	k_d^{+100}[e]	IC_{50}[f]
	nM	$M^{-1}s^{-1}$	s^{-1}	s^{-1}	s^{-1}	(μg/ml)
2dCD4-WT	53	5.0×10^3	2.6×10^{-4}		–	5.4
2dCD4-S60C	6	5.6×10^3	3.6×10^{-5}	1.2×10^{-4}	2.5×10^{-4}	1.6

[a] Equilibrium (affinity) constant is shown.
[b] Association rate constant is shown.
[c] Dissociation rate constant is shown.
[d] Dissociation rate constant in the presence of 10 mM DTT is shown.
[e] Dissociation rate constant in the presence of 100 mM DTT is shown.
[f] Concentration of protein required to effect a 50% reduction in viral infection is shown.

ished the ability of 2dCD4-S60C to remain bound during SDS and heat denaturation, suggesting that the stability of the gp120–2dCD4-S60C complex is related to intermolecular disulfide bond formation.

To elaborate on the gp120–2dCD4 binding data generated by gel shift assay and to define the kinetic parameters associated with the respective interactions, we performed 2dCD4-gp120 binding analysis by SPR using a BIAcore® 3000 instrument. Purified 2dCD4-WT or 2dCD4-S60C were immobilized on CM5 sensorchips and exposed to increasing concentrations of gp120 (0–500 nM). The association rate (k_a), dissociation rate (k_d), and equilibrium (K_D) constants for each reaction were calculated by fitting the generated sensorgrams directly to a 1:1 Langmuir binding model using the BiaEval software, and representative data from three independent kinetic analyses are shown in Table 1. The calculated affinity for the interaction between 2dCD4-WT and gp120 was 53 nM, consistent with data published by others (33). In contrast, the affinity of 2dCD4-S60C for gp120 was markedly higher, with a calculated value of 6 nM. This increase in affinity is attributed exclusively to the significantly slower off-rate kinetics of 2dCD4-S60C, whereas the rates of association between the latter and wild-type 2dCD4 are almost identical. To qualify further the difference in off-rate kinetics and to determine whether this could be related, as suggested by the gel shift analysis, to a redox exchange between the gp120 and 2dCD4-S60C, we made a direct comparison of the curves generated for each protein during an extended dissociation phase and checked the effect of DTT injection on complex dissociation (Fig. 4). gp120 remained stably bound to the 2dCD4-S60C surface for at least 10 h after withdrawal of the analyte solution; during the same time period, gp120 had almost completely dissociated from the 2dCD4-WT (Fig. 4*A*). Injection of 10–100 mM DTT over the surfaces resulted in a dramatic dose-related increase in the rate of dissociation of 2dCD4-S60C (Fig. 4*B*), with the k_d value approaching that calculated for 2dCD4-WT under normal conditions (Table 1). To confirm that the sensorgrams generated for gp120 binding to the 2dCD4 surfaces represented *bona fide* 2dCD4-gp120 interactions, we checked whether the binding was associated with induction of the epitope for mAb 17b on gp120, which is only efficiently and stably exposed following CD4 engagement. In line with this, efficient (and near stoichiometric) binding of 17b occurred only after binding of gp120 to both 2dCD4-WT and -S60C surfaces, qualifying the sensorgrams as representative of functional 2dCD4-gp120 complex formation (Fig. 4*C*). Taken together, these *in vitro* data confirm that 2dCD4-S60C binds gp120 with superior affinity to wild-type 2dCD4 through a mechanism that involves targeted intermolecular disulfide exchange.

2dCD4-S60C Is a Strong Competitive Inhibitor of gp120-CD4 Binding and Viral Replication—We next set up sCD4-gp120 competition ELISAs and pseudovirion inhibition assays to check whether the increased affinity of 2dCD4-S60C for gp120 resulted in enhanced competitive inhibition of gp120-CD4 binding and HIV-1 replication *in vitro*. Recombinant gp120 was challenged with increasing concentrations of 2dCD4-WT or 2dCD4-S60C and then probed over immobilized sCD4. 2dCD4-S60C mediated a significantly more potent inhibition of gp120 binding to immobilized sCD4 than 2dCD4-WT under the conditions tested (Fig. 5*A*), with 50% inhibition of gp120 binding achieved by 0.025 and 0.005 μg/ml of the wild-type and S60C proteins, respectively. This difference was significant across the entire range of 2dCD4 concentrations tested ($p \leq 0.05$). To confirm whether the observed increase in binding inhibition mediated by 2dCD4-S60C was related to thiol oxidation, we performed the competition assays in the presence of increasing concentrations of DTT. We calculated the fold-increase in gp120-binding inhibition mediated by 1.0 nM 2dCD4-S60C (relative to 2dCD4-WT) in the presence of increasing concentrations of DTT. Lowering the reaction oxidation potential with DTT reduced the relative potency of 2dCD4-S60C-mediated inhibition in a dose-dependent manner ($p \leq 0.05$), with the inhibitory effect of 2dCD4-S60C almost identical to that of the wild-type protein in the presence of 100 mM DTT (Fig. 5*B*).

We then set out to establish whether this enhancement in gp120 antagonism *in vitro* would translate into an improvement in viral inhibition mediated by 2dCD4-S60C. When a sub-

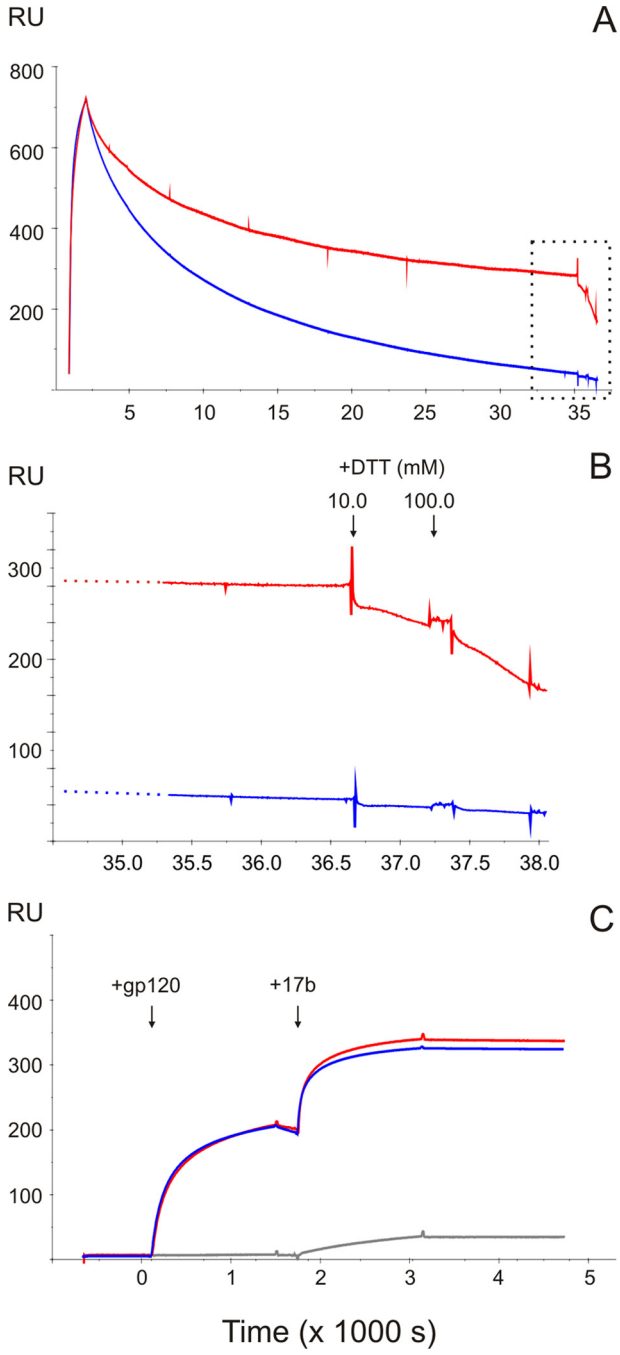

FIGURE 4. **gp120–2dCD4 interaction analysis by SPR.** *A,* recombinant gp120 (500 nM) was bound to immobilized 2dCD4 (20-min association phase) and allowed to dissociate over a 10-h period. *B,* at the end of this dissociation phase (*inset* of *A*), DTT (10–100 mM) was injected over the surfaces, which resulted in specific, concentration-dependent destabilization of the gp120–2dCD4-S60C complexes. *C,* quality of the interaction between gp120 and immobilized 2dCD4 was assessed by measuring CD4-induced (*CD4i*) epitope exposure, by injecting, following gp120 binding on the immobilized 2dCD4-WT or 2dCD4-S60C surfaces, mAb 17b at 200 nM. 2dCD4-WT, *blue*; 2dCD4-S60C, *red*; gp120 surface (no CD4 induction), *gray*. *RU*, response units.

type C HIV-1 envelope-pseudotyped virion (CAP210) was challenged with the recombinant 2dCD4 variants in a cell culture inhibition assay, 2dCD4-S60C effected a 50% reduction in replication of this virus at an average concentration of 1.6 μg/ml, although the corresponding IC_{50} value for the wild-type protein was over 3-fold higher (5.4 μg/ml) (Fig. 5*C* and Table 1). The observed effects were nontoxic and specifically related to the competitive effects of the 2dCD4, because VSV-G replication was not inhibited by the same challenge (Fig. 5*D*).

DISCUSSION

In this study, we describe the development of a recombinant two-domain CD4 protein containing a single S60C mutation (2dCD4-S60C), which is able to be folded as native 2dCD4 and binds recombinant gp120 with high affinity through the formation of an intermolecular disulfide bond. There are several potentially profound sequelae of these findings, for both anti-HIV therapy and vaccine research. In the first case, appropriate incorporation of oxidizable thiols (or other nucleophilic moieties) into the designs of existing CD4bs-targeted gp120-binding compounds may significantly enhance the affinities of such molecules for their target, correspondingly increasing the potency of their therapeutic effects. An example for which this may be highly relevant is that of the CD4-mimetic miniprotein CD4M33, which was first described by Vita and co-workers (8), and elaborated on more recently in a study that evaluated a structural analogue of CD4M33 with improved CD4 mimicry (F23) (49). The CD4M33/F23 compounds are composed of sequences resembling the CDR2-like loop of CD4 transplanted into the structurally similar region on the scorpion toxin charybdotoxin, a stabilized structure that acts as a scaffold for orienting and presenting this critical gp120-binding domain at the hydrophobic CD4-binding pocket. They exhibit slightly lower affinities for gp120 than CD4, but nonetheless they have broadly neutralizing activity against a range of laboratory-adapted and primary HIV-1 isolates. Moreover, these compounds induce structural changes in gp120 analogous to those that occur upon CD4 binding, exposing cryptic epitopes that may be vulnerable to attack by neutralizing antibodies. Thus, these compounds have potential applications as both therapies and as components of immunogens that could potentiate effective anti-HIV neutralizing antibody responses. In this regard, however, the relatively low affinity of the CD4-mimetic compounds is one of the fundamental obstacles in the development of these compounds for clinical use, and the potential utility of covalently linking F23 to gp120 has been articulated (49). As a potential therapy, lower affinity than CD4 limits the ability to compete effectively with the natural receptor *in vivo* at concentrations that are safe and realistically achievable. As a component of an immunogen, unstable binding between the ligand and Env target results in failure to stabilize the induced conformation in a form that constitutively exposes neutralization epitopes. Indeed, several studies have demonstrated that animals immunized with Env-CD4 complexes stabilized through chemical cross-linking develop strong neutralizing antibody responses (50, 51). The overall efficacy of this strategy may be limited, however, by modification of epitopes incurred through the chemical cross-linking procedure and by the potential of these immunogens to elicit autoreactive anti-CD4 antibodies. Targeted linkage of CD4-mimetic compounds as described here may, in turn, be an alternative means of stabilizing CD4-induced gp120 complexes.

Beyond representing a possible opportunity for complex stabilization through disulfide exchange, an increasing amount of

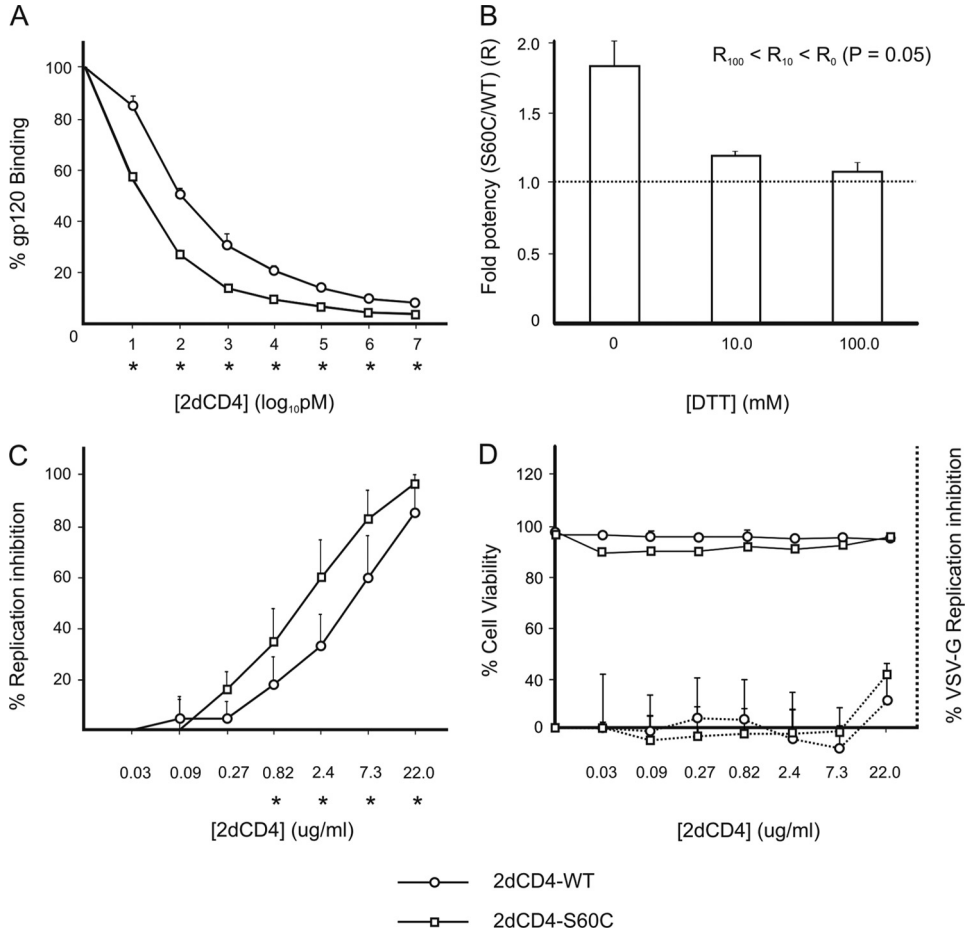

FIGURE 5. **2dCD4-S60C-mediated inhibition of sCD4-gp120 binding and HIV-1 replication.** *A,* competition ELISA. Recombinant gp120 was challenged with increasing doses of 2dCD4-WT or 2dCD4-S60C and allowed to react with sCD4 immobilized on a Maxisorp 96-well ELISA plate. Bound gp120 was detected with mAb 2G12 and a horseradish peroxidase-conjugated anti-human IgG, and the levels were expressed as a percentage of binding in the absence of any compound. The experiment was performed in triplicate on three independent occasions, and representative data from one of these is shown. *B,* relative potency of gp120-sCD4 binding inhibition mediated by 2dCD4-S60C, expressed as the ratio of the % gp120 bound in the presence of 2dCD4-WT:2dCD4-S60C (1.0 nM), decreases with increasing concentrations of the reductant DTT. Ratios were calculated from three independent experiments, and *error bars* represent positive standard deviations. *C,* pseudovirus inhibition assay. An HIV-1 subtype C Env-pseudotyped virus (CAP210) was challenged with increasing doses of 2dCD4-WT or 2dCD4-S60C and incubated with TZM-bl cells for 48 h at 37 °C. Entry inhibition was calculated by measuring viral tat-induced luciferase activity and is reflected as a percentage of the luciferase activity in the absence of any 2dCD4. The experiment was performed in triplicate on three independent occasions, and representative data from one of these are shown. *D,* VSV-G inhibition and cell viability assays. VSV-G was treated in the same way as the HIV-1 pseudoviruses and controlled for the specificity of the inhibitory effects (*dotted lines*), whereas the viability of the TZM-bl cells treated with 2dCD4-WT and 2dCD4-S60C (expressed as a percentage of the viability in the absence of any treatment, *solid lines*) evaluated the relative toxicities of the 2dCD4 compounds. In all experiments, *error bars* are shown for each concentration of recombinant protein tested and represent positive standard deviations. The significance of the differences between the 2dCD4-WT- and 2dCD4-S60C-treated samples was calculated at each concentration tested using a paired, two-tailed Student's *t* test, and significant differences ($p \leq 0.05$) are indicated by *asterisks*.

data points to the crucial structural, and probably functional, role of Env disulfides. It is now well established that the HIV-1 envelope undergoes extensive conformational change following engagement of the CD4 receptor, a process that is likely to require reduction of several of the nine disulfide bonds located within gp120. Indeed several reports have suggested the importance of certain redox proteins in the HIV entry process through their ability to reduce gp120 disulfides (37, 52). More specifically, van Anken *et al.* (39) systematically studied the contribution of each gp120 disulfide to the production of correctly folded and functional Env and found that several, including the Cys^{126}–Cys^{196} disulfide targeted in this study, are fundamentally important for the fusogenic activity of Env. Thus, HIV gp120 disulfide bonds are not only useful electrophilic handles for compounds targeting Env, they also represent critically important functional components of the viral entry apparatus. Their disruption through targeted reduction could thus represent a powerful new therapeutic approach, particularly so because they are strikingly conserved across most (if not all) functional envelopes sequenced to date.

It should be noted that the ability of ligand-based thiols to engage in *trans*-disulfide exchange reactions with target disulfides on cognate receptors is theoretically dependent on several biophysical factors. In particular, the respective redox potentials of the *cis*- and *trans*-disulfides, the availability of reducing equivalents to facilitate the exchange reaction, and the overall stability of the disulfides within the context of the liganded and unliganded structures collectively define the favorability of *trans*-disulfide bond formation, and the corresponding propensity for reversion to *cis*. In the case of the gp120–2dCD4 (S60C) interaction *in vitro*, the reaction between the recombinant proteins proceeds favorably in the presence of low concentrations of β-mercaptoethanol (1 mM), which provides the reductive force required to produce a reactive Cys^{126}–Cys^{196} dithiol intermediate. Once the exchange reaction has taken place, we propose the *trans*-disulfide bond is stabilized by cooperativity with other canonical CD4-gp120 interactions. *In vivo*, the requirement for "priming" the target Cys^{126}–Cys^{196} disulfide is likely to be fulfilled by one of a number of redox enzymes that have been shown to act on gp120 disulfides (37, 40, 52), explaining the effectiveness of 2dCD4 (S60C) in viral inhibition assays without any reducing agent supplementation. Ongoing studies in our laboratory involve characterizing the gp120–2dCD4 (S60C) exchange reaction thermodynamically using isothermal titration calorimetry, testing the antiviral efficacy of 2dCD4 (S60C) across a broad range of diverse HIV-1 isolates, and the generation of 2dCD4 variants containing selenocysteine (Sec, U) point substitu-

tions (S60U), which should yield ligands with even higher affinities for gp120 by virtue of the extreme nucleophilicity of selenocysteine.

Structural studies have now identified the key features of HIV-1 gp120 and the mechanism of its interactions with the host cell receptor. These have shown the following: (i) prior to binding CD4, Env exists in a heavily glycosylated, conformationally flexible form that is highly resistant to antibody neutralization; (ii) CD4 binding occurs at a structurally conserved site that is vulnerable to neutralizing antibody and appropriately designed CD4-mimetic compounds and is accompanied by extensive structural rearrangements that expose additional neutralization epitopes. In turn, these pioneering studies have laid the platform for the rational design of a range of therapeutic and immunogenic compounds, which have already made significant contributions in the fields of antiviral and vaccine research. In this study, we describe a structurally conservative CD4 mutation (S60C) that effects a redox exchange reaction with the gp120 Cys^{126}–Cys^{196} disulfide and enhances the affinity of the gp120-CD4 interaction. We propose that this approach, in turn, represents the conceptualization of a revolutionary approach to enhancing the efficacies of gp120-targeted therapies and Env-based immunogens. Moreover, although we have discussed our findings in the context of HIV-1 therapy (and vaccine) development, it has not escaped our attention that quaternary structure stabilization by redox-active variants of natural ligands, produced in native form by strategic substitution of the structurally similar chalcogen-containing amino acids, may represent a generally applicable approach to receptor-ligand stabilization where structural and biophysical conditions for disulfide exchange prevail.

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Chapter 3

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Disulfide Reduction in CD4 Domain 1 or 2 Is Essential for Interaction with HIV Glycoprotein 120 (gp120), which Impairs Thioredoxin-driven CD4 Dimerization*

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Background: Interaction between HIV gp120 and cell CD4 initiates viral infection of host cells.
Results: Only CD4 with reduced disulfides in domain 1 or 2 binds gp120, which inhibits thioredoxin-dependent CD4 dimerization.
Conclusion: Cell surface oxidoreductases may prime CD4 for gp120 engagement, and impairment of redox-driven CD4 dimerization by gp120 may compromise CD4 function.
Significance: Redox-dependent isomerization of CD4 is critical for HIV entry.

Human CD4 is a membrane-bound glycoprotein expressed on the surface of certain leukocytes, where it plays a key role in the activation of immunostimulatory T cells and acts as the primary receptor for human immunodeficiency virus (HIV) glycoprotein (gp120). Although growing evidence suggests that redox exchange reactions involving CD4 disulfides, potentially catalyzed by cell surface-secreted oxidoreductases such as thioredoxin (Trx) and protein disulfide isomerase, play an essential role in regulating the activity of CD4, their mechanism(s) and biological utility remain incompletely understood. To gain more insights in this regard, we generated a panel of recombinant 2-domain CD4 proteins (2dCD4), including wild-type and Cys/Ala variants, and used these to show that while protein disulfide isomerase has little capacity for 2dCD4 reduction, Trx reduces 2dCD4 highly efficiently, catalyzing the formation of conformationally distinct monomeric 2dCD4 isomers, and a stable, disulfide-linked 2dCD4 dimer. Moreover, we show that HIV gp120 is incapable of binding a fully oxidized, monomeric 2dCD4 in which both domain 1 and 2 disulfides are intact, but binds robustly to reduced counterparts that are the ostensible products of Trx-mediated isomerization. Finally, we demonstrate that Trx-driven dimerization of CD4, a process believed to be critical for the establishment of functional MHCII-TCR-CD4 antigen presentation complexes, is impaired when CD4 is bound to gp120. These observations reinforce the importance of cell surface redox activity for HIV entry and posit the intriguing possibility that one of the many pathogenic effects of HIV may be related to gp120-mediated inhibition of oxidoreductive CD4 isomerization.

Human CD4 is a membrane-bound glycoprotein of ~55 kDa, which is expressed primarily on the surface of certain types of T-lymphocytes and phagocytic cells. It consists of C-terminal transmembrane and cytosolic segments, and an N-terminal extracellular region containing four domains (D1–D4)[2], three of which (D1, D2, and D4) contain a single cysteine pair (1). Physiologically, the function of CD4 is to cooperate with the T cell receptor (TCR) during engagement of major histocompatibility (type II) (MHCII) molecules, which present peptide fragments of antigens ingested and processed by antigen-presenting cells. Both the TCR and CD4 are required to make contact with the MHCII complex to transduce the appropriate proliferative signal to the antigen-specific T cell, which is then able to coordinate various effector activities critical for the maturation of a robust, antigen-specific immune response. In contrast, binding of the TCR to MHCII without a CD4-MHCII co-stimulatory signal leads to T cell anergy and apoptosis (2, 3). CD4 is also the primary receptor for HIV, the causative agent of AIDS, which interacts with CD4 via the viral surface glycoprotein gp120. By subverting the critical immunological function of CD4, a possible contribution of gp120-mediated anergic effects on $CD4^+$ T cells to the pathogenesis of AIDS was demonstrated (4) soon after the discovery of CD4 as the receptor for HIV (5–7).

Biochemical studies have revealed the existence of naturally occurring isoforms and oligomeric states of CD4, each believed to have distinct utility in HIV-1 gp120 binding and antigen presentation (8–10). One important mechanism by which these isoforms appear to be regulated is through redox exchange reactions effected on the CD4 D2 disulfide (Cys^{130}–Cys^{159}) (11). With its unusual geometric and thermodynamic characteristics, including a large angle between the planes of the C_β-S bonds of each cysteine (114.7°) and correspondingly large bond dihedral strain energy (4.7 kcal/mol), the D2 disul-

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[2] The abbreviations used are: D1, domain 1; TCR, T cell receptor; PDI, protein disulfide isomerase; Trx, thioredoxin; TR, thioredoxin reductase; IAM, iodoacetamide; MPB, *N*-(3-maleimidopropionyl)biocytin; gp120, glycoprotein 120.

fide bears the classical features of the so-called "allosteric" or "functional" disulfide bond (12). This type of bond is rare in proteins, and the pronounced torsional strain on the disulfide is thought to enable its relatively facile reduction, triggering local or global conformational changes that may be functionally significant.

The biological importance of CD4 allostericity inferred from this is poorly understood, although several lines of evidence suggest that redox-dependent isomerization of CD4 plays a role in the context of both HIV-1 gp120 receptor engagement and in the formation of stably associated CD4-TCR-MHCII complexes. The interaction of CD4 with HIV-1 gp120 appears to involve CD4 monomers with CD4 dimerization-defective mutants (13), and specifically reduced monomeric forms of CD4 (14), supporting higher levels of HIV infection in cell culture. In contrast, it has been shown that CD4 oligomerization is necessary for stable MHCII binding (9) and that effective T cell activation requires CD4 dimer formation (15, 16), a process that evidence suggests involves redox exchanges in CD4 cysteines (11, 17, 18).

How might redox-dependent isomerization of CD4 be achieved, and what role does this play in the mechanism of CD4-dependent HIV infection? There is now an abundance of data demonstrating the effects of manipulating the activities of cell surface-active oxidoreductases such as protein disulfide isomerase (PDI) and thioredoxin (Trx) on HIV infection *in vitro* (19–23), although considerable debate remains on the biological utility and physiological relevance of such effects and the extent of involvement of these factors *in vivo*. Furthermore, and complicating the elucidation of the biochemical importance of these enzymes for the mechanism of HIV entry, are the observations that gp120, itself containing nine disulfide bonds, is also a substrate for PDI-, Trx-, and glutaredoxin-1-mediated reduction in *vitro* (19, 24, 25) and that inhibitory antibodies to PDI or Trx have different effects in the context of macrophage- or lymphocytic HIV infection (26).

In this study, we show that although PDI has very low capacity for CD4 reduction, Trx reduces 2dCD4 disulfides robustly when activated by thioredoxin reductase (TR) *in vitro*, lending further support to the widely-held notion of Trx as the more likely physiological reductant of CD4. We further demonstrate that HIV gp120 binds specific reduced isomers of 2dCD4, the representation of which are significantly increased through reduction by Trx. In contrast, gp120 is completely incapable of binding a fully oxidized, monomeric CD4 in which both D1 and D2 disulfide bonds are intact. Moreover, Trx-mediated dimerization of CD4, which potentially plays a pivotal role in the establishment of functional TCR-CD4-MHCII complexes, is impaired when CD4 is bound to gp120. These data establish an additional possible rationale for the importance of cell-surface oxidoreductase activity in HIV-1 infection and simultaneously put forward another potentially important pathogenic consequence of CD4-gp120 binding for consideration.

EXPERIMENTAL PROCEDURES

Vectors and Proteins—DNA cassettes encoding the first two amino-terminal domains of wild-type human CD4 (2dCD4-WT, residues 1–183) and the following four Cys/Ala variants, C159A, C16A/C84A, C130A/C159A, and C16A/C84A/C130A/C159A, in-frame with C-terminal His_6 fusion tags were synthesized by GENEART (Regensburg, Germany) following codon optimization for expression in *Escherichia coli*. The recombinant 2dCD4 variants were expressed and purified by standard denaturing metal chelate affinity chromatography and refolded using oxidative refolding protocols according to methods described previously (27). Recombinant HIV-1 gp120 (BaL) was expressed in a stably transfected HEK293 cell line previously generated in our laboratory and purified by two-step affinity and size-exclusion chromatography. Following purification, both proteins were exhaustively dialyzed into phosphate-buffered saline (PBS, pH 7.4), concentrated, filter-sterilized, and stored in aliquots at −80 °C. The proteins were analyzed by reducing or non-reducing SDS-PAGE using precast 4–12% gradient NuPAGE gels (Invitrogen). Prior to gel loading, the samples were treated with LDS loading buffer with or without DTT (50 mM) and incubated at room temperature for 10 min. Iodoacetamide (IAM, Sigma-Aldrich) was then added to a final concentration of 50 mM. Gels were stained with Coomassie Blue R-250.

2dCD4 MPB Labeling Assay—To detect the free thiol content of 2dCD4-WT isomers, purified recombinant 2dCD4-WT was labeled with *N*-(3-maleimidopropionyl)biocytin (MPB, Sigma-Aldrich). Reactions contained 15 μM recombinant 2dCD4-WT and 150 μM MPB in PBS (pH 7.4), and these were incubated at room temperature for 1 h. Unconjugated MPB was removed by centrifugation through desalting columns containing Sephadex G10 equilibrated in PBS. Samples (100 ng) were then resolved by non-reducing SDS-PAGE and transferred to nitrocellulose membranes by standard Western blotting procedures. The membranes were probed with an HRP-conjugated anti-streptavidin antibody or an anti-His_6 antibody (Qiagen, Germany) followed by a secondary HRP-conjugated anti-mouse IgG (GE Healthcare). Bound antibodies were visualized by standard chemiluminescence procedures using a ChemiDoc imaging instrument and Quantity One software (Bio-Rad).

PDI/Trx Reduction Assay—Reduction of disulfide-bonded insulin is commonly used to measure the efficiency of disulfide reductase activity (28). We used a similar approach to assess whether PDI and/or Trx catalyzed the reduction of 2dCD4-WT. For the Trx reaction, 50 μM 2dCD4-WT, insulin (Novo Nordisk) or BSA (Roche Applied Science) was incubated with 0.2 mM NADPH (Calbiochem), 2 mM EDTA, and 4 μM human Trx1 (IMCO Corp., LTD AB, Sweden) in 0.1 M potassium phosphate (pH 7.4). The 100-μl reaction was started with 7 nM mammalian thioredoxin reductase (TR). For the PDI reduction reaction, 2dCD4-WT, insulin, or BSA were incubated with 4 μM PDI (Takara Bio., Inc., Japan), 2 mM EDTA in 0.1 M potassium phosphate, and the 100-μl reaction was started with 100 μM DTT (Sigma-Aldrich). All reactions were prepared in triplicate, and control reactions of either 2dCD4-WT alone or 2dCD4-WT with no activator (TR or DTT for the Trx and PDI reactions, respectively) were performed in parallel. A_{650} readings were taken every 10 s for 10 min.

Trx-mediated 2dCD4 Isomerization Analysis—To analyze the products of oxidoreductase-mediated 2dCD4 disulfide reduction, 2dCD4-WT or purified 2dCD4-$gp120_{BaL}$ complexes

(see below) were treated with Trx/TR/NADPH, and the reactions were stopped at various times between 0 and 60 min. Each isomerization reaction contained 0.1 nmol of 2dCD4 or purified 2dCD4-gp120$_{BaL}$ complex incubated with 4 μM Trx in 0.1 M potassium phosphate buffer (pH 7.4) and 2 mM EDTA at 37 °C. The reactions were started by adding TR (7 nM) and NADPH (2 mM). Control reactions were performed in parallel and included 2dCD4 or 2dCD4-gp120$_{BaL}$ complex alone, 2dCD4 or 2dCD4-gp120$_{BaL}$ complex with Trx only, and 2dCD4 or 2dCD4-gp120$_{BaL}$ complex with TR and NADPH only. The isomerization reactions were stopped by carbamidomethylation of free cysteine residues after the addition of IAM to a final concentration of 50 mM at 0, 10, 30, 40, and 60 min. The time-resolved reaction products were then electrophoresed by reducing and non-reducing SDS-PAGE, stained, and analyzed by densitometry using Quantity One software (Bio-Rad Laboratories).

gp120–2dCD4 Isomer Binding Analysis and Complex Preparation— gp120–2dCD4 complexes were formed by incubating 100 μg of 2dCD4-WT, -C16A/C84A, -C130A/C159A, or -CΔA with 100 μg of gp120$_{BaL}$ for 1 h at room temperature. Following incubation, the binding reactions were centrifuged at 13,000 × *g* and then loaded onto a HiLoad 16/600 Superdex 200 column (GE Healthcare) attached to an ÄKTA FPLC chromatography unit (GE Healthcare). Complexes were loaded onto the PBS-equilibrated column (pH 7.4) through a 2-ml loop at a flow rate of 1 ml/min. The gp120-bound and unbound 2dCD4 fractions were collected, treated with IAM (50 mM), concentrated using Amicon® ultra-filtration units (Merck-Millipore), and analyzed by reducing and non-reducing SDS-PAGE as described.

gp120–2dCD4 Binding ELISA—96-Well microtiter plates (Maxisorp) were coated with 100 μl of the anti-gp120 antibody D7324 (Aarto, UK, 1 μg/ml in PBS, pH 7.4) by overnight incubation at 4 °C. The coating antibody was removed, and the plates were blocked for 2 h with PBS containing 1% BSA and 0.05% Tween 20. Recombinant gp120 (200 ng/ml) was incubated with each 2dCD4 variant at concentrations ranging from 0 to 1 μg/ml for 1 h at room temperature, and the complexes then bound to the D7324-coated wells for 1 h at room temperature. The plates were washed five times with PBS containing 0.05% Tween 20 (PBS-T), and bound gp120-CD4 complexes probed with the monoclonal gp120 antibody 17b (0.3 μg/ml in PBS-T) for 1 h at room temperature. The plates were then washed, probed with a secondary HRP-conjugated anti-human antibody (GE Healthcare), and the bound antibodies were detected and quantified by chromogenic methods using TMB Ultra substrate (Thermo-Pierce).

RESULTS

2dCD4 Redox Isomer Analysis—We previously reported the production of a recombinant protein containing the first two N-terminal domains of human CD4 (2dCD4-WT) using an *E. coli*-based expression system (27). Following extraction from inclusion bodies, purification by nickel-nitrilotriacetic acid affinity chromatography and refolding under conditions that promote disulfide bond formation, functional 2dCD4-WT can be prepared to apparent homogeneity, resolving as a single band of ~28 kDa when analyzed by denaturing, reducing SDS-PAGE (Fig. 1*A*). When analyzed under non-reducing conditions, which would maintain disulfide bond integrity, the purified 2dCD4-WT resolves into two distinct isomers that have increased mobility compared with the fully reduced isoform (Fig. 1*A*). In general, reduction of disulfide bonds has been shown to decrease relative electrophoretic mobility of proteins by increasing the hydrodynamic volumes of the resultant structures (29), and this effect has previously been demonstrated on full-length CD4 expressed in mammalian cells (30). Accordingly, we reasoned that the differentially resolved 2dCD4-WT isomers represented distinct redox intermediates containing either both domain cysteines in the oxidized disulfide state or reduced cysteine pairs in either D1 or D2 or both.

To assign oxidation states of the cysteine pairs within these isomers, we generated a panel of identical 2dCD4 proteins containing double Cys/Ala substitutions in each domain (2dCD4-C16A/C84A and 2dCD4-C130A/C159A), as well as a variant in which all four cysteines were mutated to alanine (2dCD4-CΔA). As expected, under reducing, denaturing conditions each variant migrated as a single band of ~28 kDa (Fig. 1*B*, *top panel*). When the samples were alkylated with IAM and analyzed under non-reducing conditions, each variant resolved into one to three distinct isomers ranging from an estimated 22–28 kDa (Fig. 1*B*, *bottom panel*). Consistent with its inability to form disulfide bonds in D1 and D2, 2dCD4-CΔA migrates at a position identical to that of reduced 2dCD4-WT establishing the species at 28 kDa as a fully reduced 2dCD4 isoform in which both domain cysteines are reduced. In contrast, the predominant species found in preparations of 2dCD4-C130A/C159A, which is capable of forming only the D1 disulfide bond, is found at ~25 kDa, suggesting that the isoform which migrates to this position in 2dCD4-WT contains a stable disulfide in D1 and reduced cysteines in D2. Interestingly, the mobility of the main isoform found in the domain, one double Cys/Ala variant, 2dCD4-C16A/C84A, at 28 kDa identical to 2dCD4-CΔA and fully reduced 2dCD4-WT, implies that 2dCD4-C16A/C84A is unable to form the D2 disulfide, perhaps through the absence of cooperative contributions made by the D1 cysteines during folding of this protein. However, a previous study demonstrated marked differences in the effects that abolition of the D1 and D2 disulfides have on the mobility of full-length CD4 expressed in HeLa cells (30). In this study, it was shown that the migration rate of a full-length CD4 containing alanine mutations at Cys-16 and Cys-84 was pronouncedly retarded, whereas corresponding alanine substitutions at Cys-130 and Cys-159 had no effect in this regard. Clearly the context of these mutations, whether present in a full-length or two-domain CD4, is an important determinant of the effect they have on the structures, hydrodynamic volumes, and ultimately the migration rates of these molecules. Thus, we propose, and, as the gp120-binding data shown in Fig. 4 appear to confirm, that 2dCD4 in which both domain disulfides are reduced, and 2dCD4, in which only the domain 1 disulfide is reduced, each migrate with the same apparent molecular mass of 28 kDa.

Most importantly, only the 2dCD4-WT preparation contained the fast-migrating isoform at 22 kDa (Fig. 1*B*, *bottom panel*). In keeping with the exclusive ability of this construct to

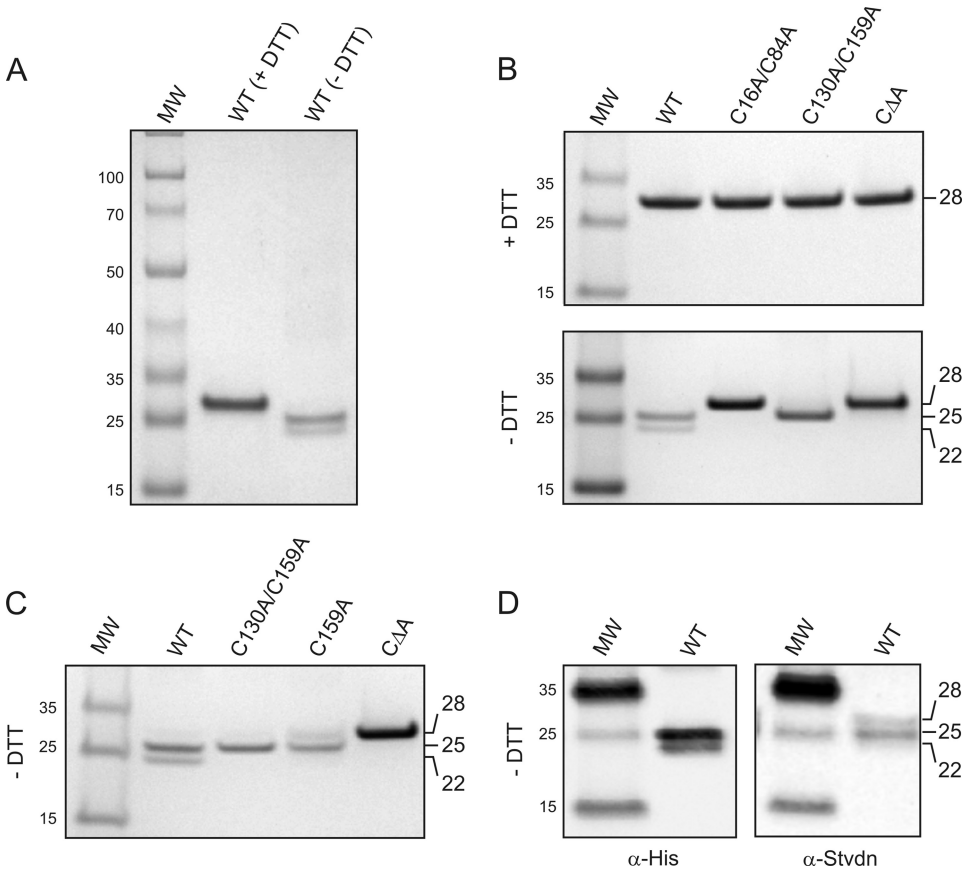

FIGURE 1. **Analysis of 2dCD4 redox intermediates.** *A–C*, purified recombinant 2dCD4-WT and the indicated Cys/Ala variants were treated with LDS loading buffer with or without DTT (50 mM) and then with IAM (50 mM) and resolved by SDS-PAGE. Gels were stained with Coomassie Blue R250. *D*, recombinant 2dCD4-WT was labeled with MPB, resolved by SDS-PAGE under non-reducing conditions, and transferred to nitrocellulose by Western blotting. 2dCD4 isomers were detected by probing either with anti-His_6 (*left panel*) or anti-streptavidin (*right panel*) antibodies. In all figures, *numbers* alongside the molecular mass markers are in kDa.

form both domain disulfides, we propose that this band represents a 2dCD4 in which both domain cysteines exist in the disulfide state. Because a theoretical argument could be made that this isoform of 2dCD4-WT might contain interdomain disulfide bonds, perhaps formed through kinetic end points encountered during the refolding process, we generated a 2dCD4 mutant containing a single Cys/Ala substitutions in domain 2 (2dCD4-C159A). Trans-domain oxidation between Cys-130 and either D1 cysteine in this molecule would be expected to have a dramatic effect on either the solubility or conformation, and thus mobility, of the resultant polypeptide. Gel analysis indicated that this is indeed unlikely to be the case because 2dCD4-C159A exists predominantly as a soluble isoform containing an intact D1 disulfide, as suggested by the identity of the mobility between this and 2dCD4-C130A/C159A (Fig. 1*C*).

To gain further confirmation regarding the proposed assignments of the oxidation states of the cysteines within each redox isomer, we used MPB to label any free thiols present on the various isomers present in the 2dCD4-WT preparation. Following Western transfer of the MPB-labeled protein, probing with anti-His detected both species at 22 and 25 kDa (Fig. 1*D*, *left panel*), whereas only the 25-kDa isoform and small amount of a fully reduced, 28-kDa isoform, which is occasionally present in 2dCD4-WT preparations (for examples, see Figs. 3–5) and not detectable with the anti-His probe, was detected with streptavidin-HRP (Fig. 1*D*, *right panel*). Taken together, these results provide compelling evidence that recombinant 2dCD4-WT exists predominantly as two distinct isomeric species, one of which contains intact disulfides in both D1 and D2. For ease of reference, we henceforth refer to the 2dCD4-WT species migrating at 28, 25, and 22 kDa under non-reducing conditions as $2dCD4^{R2}$, $2dCD4^{R1}$, and $2dCD4^{Ox}$, respectively.

PDI- and Trx-mediated 2dCD4 Reduction—We next set out to assess whether 2dCD4 disulfides were targets for enzymatic reduction by Trx and/or PDI *in vitro*. In this assay, intermolecular disulfide exchanges between thiols formed from reductase activity results in progressive precipitation of the target protein, the extent of which, determinable by measuring solution turbidity at A_{650}, provides an indication of the efficiency of target protein reduction. We treated the same concentrations of recombinant insulin, commonly used as a target for assessing reductase activity (28), as a positive control for the PDI and Trx activity, and BSA, which contains 35 cysteines, to control for nonspecific effects of reduction. Under these conditions, both Trx and PDI catalyzed reduction of insulin over a 10-min reaction course (Fig. 2*A*, *top panels*), whereas only Trx reduced 2dCD4 robustly (Fig. 2*A*, *middle panels*) over the same time interval. No reduction of BSA by either Trx or PDI was detected (Fig. 2*A*, *bottom panels*), and Trx-mediated reduction was spe-

Further supporting evidence for the assignment of oxidation states using Mass Spectrometry has been placed in the addendum to Chapter 3

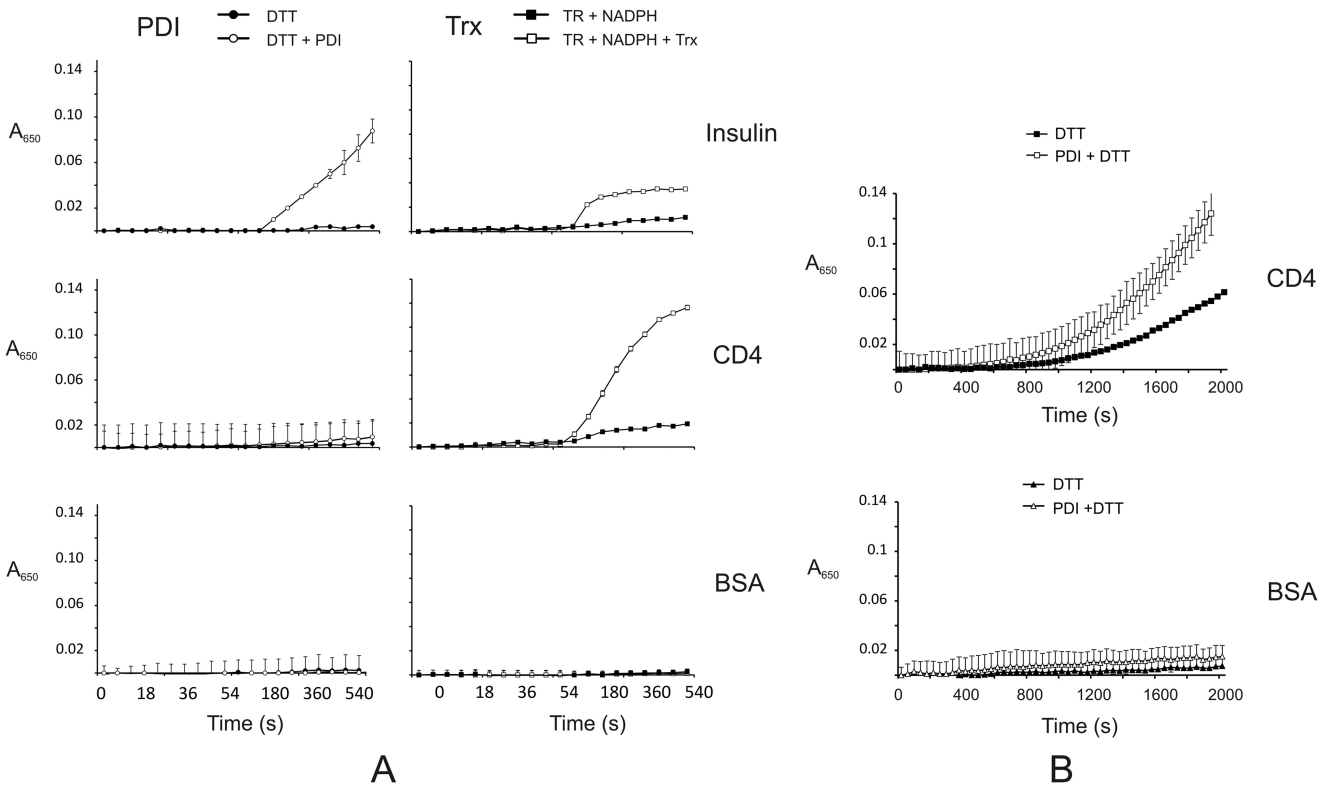

FIGURE 2. **PDI- and Trx-mediated 2dCD4 reduction activity.** *A*, recombinant 2dCD4-WT, insulin, or BSA (50 μM) were treated with either PDI or Trx and the respective activation factors and incubated at 37 °C in 96-well microtiter plates. Substrate reduction was determined by measuring $A_{650\ nm}$ at the indicated time points. Each experiment was done in triplicate, and S.D. at each time point are shown. *B*, time-extended analysis of the PDI-treated CD4 and BSA samples shown in *A*.

cifically attributable to the catalytic activity of the oxidoreductase because no increase in turbidity was observed in identical reactions that omitted Trx (Fig. 2*A*, *middle right panel*). Interestingly, an increase in turbidity in PDI-treated 2dCD4 samples was noted after an extended period of incubation (>15 min, Fig. 2*B*), which, together with the observation that both PDI and Trx are secreted on the surfaces of human lymphocytes (31, 32), suggests that certain conditions and contexts may exist under which PDI could have catalytic effects on CD4. However, calculations of the respective redox potentials of the CD4 D2 disulfide (14) and the active site dithiols of these enzymes (33, 34) have suggested that Trx is the more likely physiological reductant of CD4, and our data, which shows substantially more efficient reduction of CD4 by Trx, via a mechanism of activation that is more realistically feasible in the cellular context, appear to be consistent with this.

Trx-mediated 2dCD4 Isomerization—We then investigated whether the robust enzymatic reduction of CD4 effected by Trx resulted in observable isomerization of the constituent 2CD4-WT isomers. A time-resolved enzyme assay was performed in which Trx/TR/NADPH were incubated with 2dCD4-WT for 0–60 min, and the reactions were stopped at various intervals with 50 mM IAM. Samples were then resolved by reducing (Fig. 3*A*, *bottom panel*), and non-reducing SDS-PAGE (Fig. 3*A*, *top panel*), and the relative quantities of the 2dCD4-WT isomers determined by densitometry (Fig. 3*B*). Incubation of 2dCD4-WT with Trx treatment resulted in a time-dependent decrease in the amount of $2dCD4^{Ox}$, which was completely ablated after 10–30 min of enzymatic reduction. At the same time, $2dCD4^{R2}$ and a species of ~50 kDa, most likely representing a disulfide-bonded 2dCD4-WT dimer ($2dCD4\text{-}WT^{D}$) are formed. Although the quantity of CD4 dimer does not change significantly beyond this point, the amount of $2dCD4^{R2}$ increases progressively for the duration of the reaction. Conversely, although $2dCD4^{R1}$ remained at near constant levels for the first 10 min of treatment, the levels of this isomer declined toward the end of the reaction cycle. Considering the commensuration in the decline of $2dCD4^{R1}$ and increase in $2dCD4^{R2}$ from 10 to 60 min, and the observation that these changes continue long after $2dCD4^{Ox}$ has been depleted and steady-state levels of $2dCD4^{D}$ achieved, these findings imply that exchanges involving the D1 disulfide of CD4 may also be an important component of Trx-mediated CD4 isomerization.

To confirm that these isomerization events were exerted specifically through the catalytic function of Trx, control reactions were done at 0 and 60 min and included 2dCD4-WT alone, 2dCD4-WT in the presence of reducing equivalent only (TR-NADPH) and 2dCD4-WT in the presence of Trx alone. In each of these cases, no change in the composition of native 2dCD4-WT isomers was observed after 60 min. As final confirmation that the isomerization was related fundamentally to changes in the oxidation states and bonding patterns of 2dCD4-WT cysteines, and that the dimeric 2dCD4 species was in fact disulfide-bonded, the Trx-treated samples were resolved under reducing conditions. Under these conditions, the isomerized 2dCD4s resolved as a single, fully-reduced protein of 28 kDa (Fig. 3*A*, *bottom panel*). An acknowledged limitation of this assay is its inability to define the exact sequence of

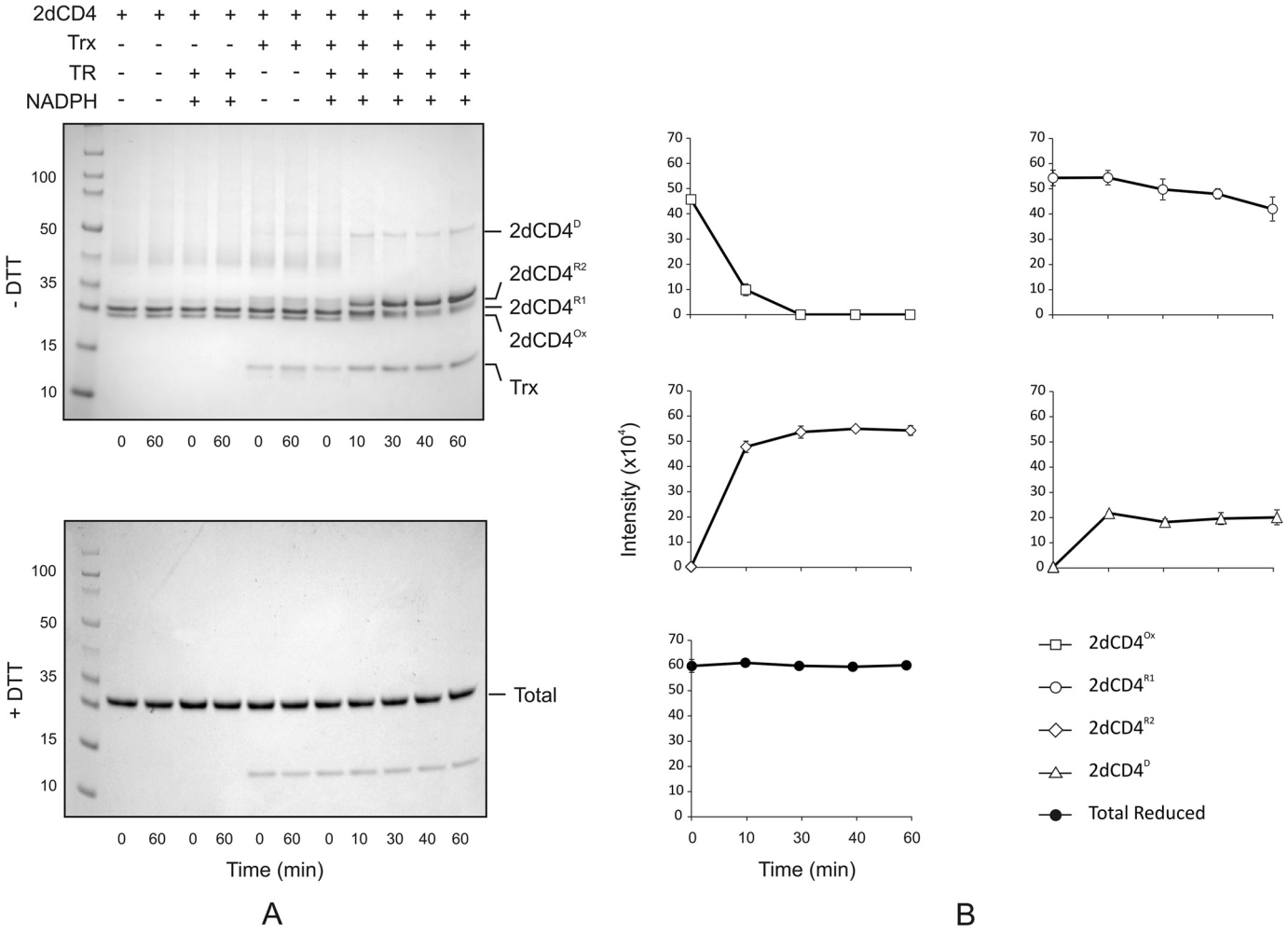

FIGURE 3. **Trx-mediated 2dCD4 Isomerization.** *A*, recombinant 2dCD4-WT, in the presence or absence of Trx, with or without TR and NADPH, was incubated at 37 °C for 1 h, and reactions were stopped at the indicated time points with IAM (50 mM). Samples from the same reaction were resolved by SDS-PAGE under reducing (*bottom panel*) or non-reducing (*top panel*) conditions. *B*, changes in the relative quantities (peak intensities) of the 2dCD4 isomers were determined by scanning densitometry. Representative data are shown, with *error bars* representing the standard deviations of peak intensities determined for the corresponding bands on three independent gels.

isomerization events, for example, which species represent the immediate precursors for 2dCD4 dimer formation, and ongoing experiments using various 2dCD4 Cys/Ala mutants should provide further insights in this regard. However, taken together, the data reported here allow us to conclude that $2dCD4^{Ox}$ is a substrate for Trx-mediated reduction, which may involve disulfides located in both D1 and D2, and this activity results in the formation of a disulfide-bonded 2dCD4 dimer.

2dCD4 Isomer-specifc gp120 Binding—Previous studies have shown that reduced forms of CD4 are the preferred receptor for HIV-1 gp120 (14). To gain further insights in this regard, we applied a chromatographic approach to analyze to what extent the constituent isomers within 2dCD4-WT, 2dCD4-C16A/C84A, and 2dCD4-CΔA are capable of binding HIV-1 gp120. First, recombinant $gp120_{BaL}$ was incubated with 2dCD4-WT, the bound gp120–2dCD4 complexes were separated from unbound ligands by FPLC, and fractions containing complexes and unbound proteins analyzed by non-reducing SDS-PAGE. The data showed that only $2dCD4^{R1}$ was able to bind gp120, whereas $2dCD4^{Ox}$ was found exclusively in the unbound fraction (Fig. 4*A*). We then sought to establish whether this finding would be corroborated by the gp120-binding capacities of 2dCD4-C16A/C84A and 2dCD4-C130A/C159A, proxies for 2dCD4 containing reduced cysteines in domains 1 and 2, respectively. Remarkably, both 2dCD4-C16A/C84A and 2dCD4-C130A/C159A, but neither $2dCD4^{Ox}$ nor 2dCD4-CΔA, bound gp120 robustly (Fig. 4*B*).

To check whether the 2dCD4 variants interacted with gp120 through specific, functional interaction, we performed an ELISA assay to establish whether their binding resulted in the exposure of coreceptor (CCR5/CXCR4) binding sites on gp120. These sites (so-called CD4-induced (CD4i) epitopes) are efficiently exposed on gp120 only when the latter is engaged with CD4 and can be detected by using CD4i-specific antibodies (of which the monoclonal antibody 17b is one of the best characterized). Recombinant gp120 was allowed to react with the 2dCD4 variants at a range of concentrations, and the complexes were captured on a gp120-antibody-coated microtitre plate. The captured complexes were then probed with 17b antibody. As expected, 2dCD4-WT, -C16A/C84A, and C130A/C159A, but not 2dCD4-CΔA, resulted in a robust, dose-dependent induction of the 17b-binding site on gp120 (Fig. 4*C*), confirm-

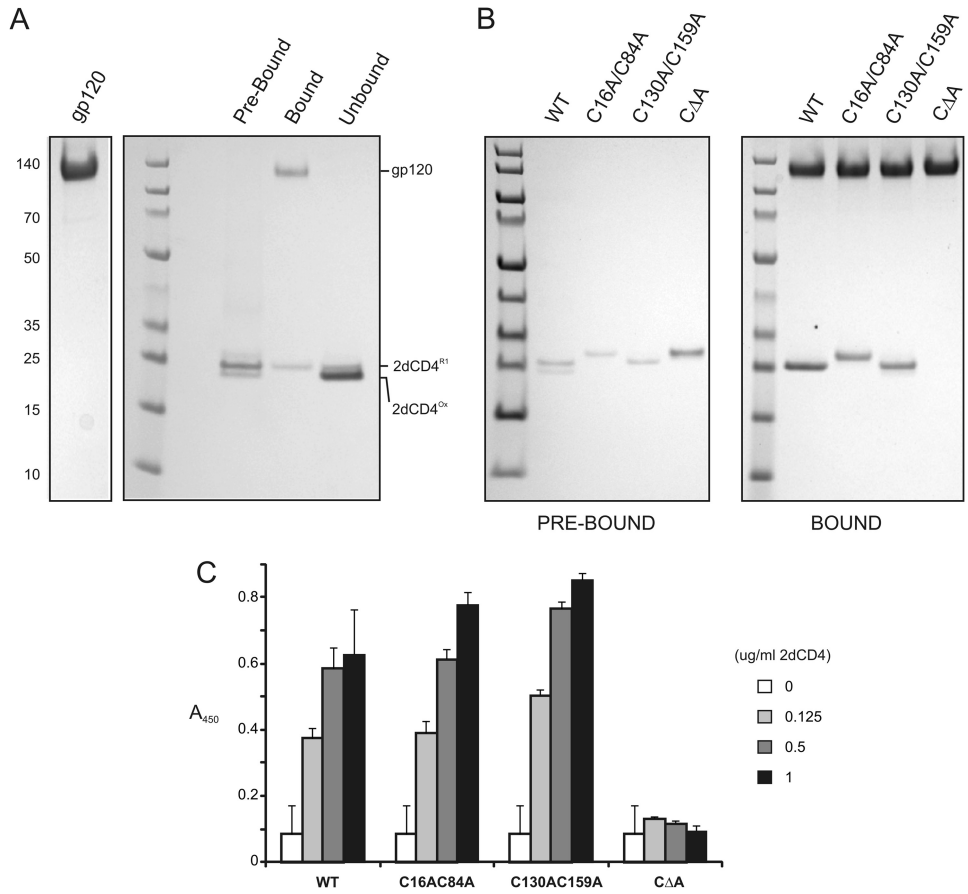

FIGURE 4. **2dCD4 isomer-specific gp120 interaction.** *A* and *B*, recombinant 2dCD4-WT was incubated with purified recombinant HIV-1 $gp120_{BaL}$ (*left panel*) for 1 h at room temperature, and the gp120-bound and unbound isomers were fractionated by FPLC. The samples were treated with IAM (50 mM), concentrated, and resolved by non-reducing SDS-PAGE. *B*, 2dCD4-WT and the indicated Cys/Ala variants were incubated with $gp120_{BaL}$, and the gp120-bound proteins purified and analyzed by non-reducing SDS-PAGE. *C*, gp120–2dCD4 variant complexes were captured on an anti-gp120 antibody-coated microtiter plate and then probed with the monoclonal antibody 17b to detect induction of the CD4-induced epitope (CD4i) on gp120. The assays were performed in triplicate in three independent experiments, and representative data are shown. *Error bars* represent S.D. from average A_{450} levels in one experiment.

ing the functionality of its interactions with the 2dCD4 isomers. Taken together, these data are consistent with previous reports demonstrating the preference of gp120 for reduced forms of CD4 and expand on these with two intriguing observations: that CD4 requires reduction of either the domain 1 or domain 2 disulfide (but not both) to bind gp120 and is impaired in doing so when both domain disulfides are intact.

To gain further insights into the impact of disulfide ablation in D1 or D2 on 2dCD4 structure, we then performed far-UV CD spectroscopy on 2dCD4-WT, -C16A/C84A, and -C130A/C159A, and compared these with the CD spectrum predicted for the 2dCD4 crystal structure (Protein Data Bank code 1CDH) using the DichroCalc CD spectra prediction algorithm (Fig. 5) (35). Relative to the curve predicted for 1CDH, which is typical of classical β-sheeted secondary structure with an ellipticity minimum of approximately 40×10^{-3} deg.cm^2/dmol at 220 nm, the curve for 2dCD4-WT, which is a composite of fully and partially oxidized isoforms, is leftward-shifted (min $[\theta_{MRW} \times 10^{-3}]$ of $\sim -60 \times 10^{-3}$ deg·cm^2/dmol at 212 nm). The magnitude of the ellipticity minimum, and the wavelength at which this occurs for 2dCD4-C16A/C84A and 2dCD4-C130A/C159A, are further decreased to -75 and -79×10^{-3} deg·cm^2/dmol at 206 and 204 nm, respectively. This progressive decrease in wavelength and magnitude of the ellipticity minimum correlates inversely with total disulfide content and is consistent with a relaxation in the ordered β-sheeted structure (Fig. 5*A*) that disulfide reduction in each 2dCD4 domain is likely to effect.

gp120 Inhibits Oxidoreductase-mediated Dimerization of 2dCD4—Oligomerization of CD4 is now known to be a critical component of establishing functional CD4/TCR-MHCII complexes (36), which are required for efficient transduction of activation signals transmitted to T cells by MHCII-associated antigenic peptides. Failure to achieve such antigen presentation complexes results in T cell anergy and apoptosis (2). By virtue of the critical role that $CD4^+$ T cell activation plays in mediating effective humoral immune responses, corruption of normal CD4 function, potentially mediated by virus-associated gp120 or soluble gp120 known to be shed from the viral envelope into the blood of HIV-infected individuals, may make a significant contribution to the pathogenic consequences of HIV infection. Considering this, we investigated whether one of the corruptive effects of gp120 binding could involve inhibition of oxidoreductase-driven dimerization of CD4. To check this, we treated equal amounts of gp120-bound and unbound 2dCD4 with Trx and analyzed the reaction products by non-reducing SDS-PAGE (Fig. 6*A*) and densitometry (Fig. 6*B*). Although unliganded 2dCD4 undergoes measurable Trx-mediated reduction

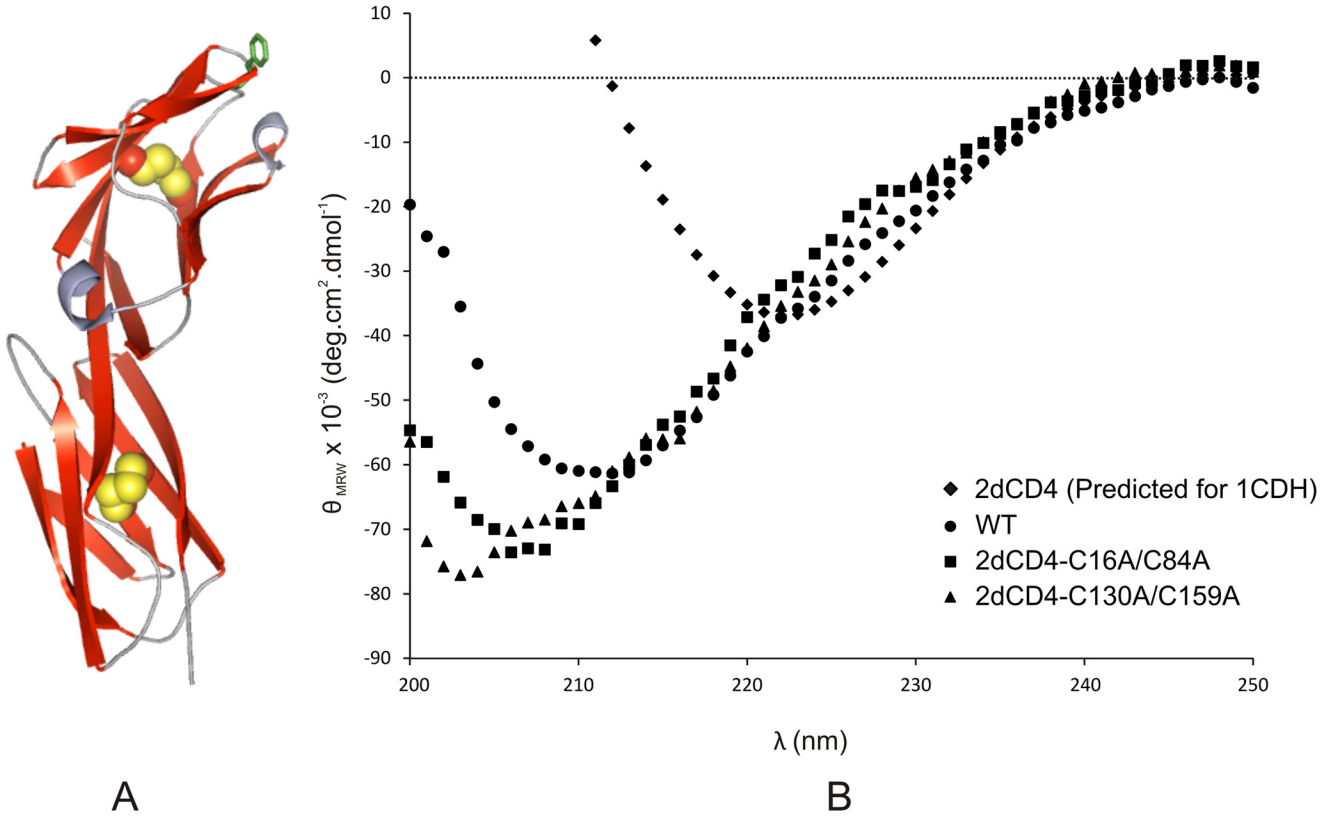

FIGURE 5. **Secondary structure analysis of 2dCD4-WT, -C16A/C84A and -C130A/C159A.** *A*, ribbon representation of two-domain CD4 (2dCD4, Protein Data Bank code 1CDH), showing the extensive β-sheeted secondary structures (*red*) within domains 1 and 2, with respective inter- and intra-sheet disulfide bonds (Cys^{16}–Cys^{84} and Cys^{130}–Cys^{159}) shown in space-filling mode (*yellow*). Also shown is Phe^{43} of D1 (*green*), a major constituent of the gp120-binding site on CD4. *B*, far-UV CD spectra of 2dCD4-WT, -C16A/C84A, and -C130A/C159A (10 μM PBS, pH 7.4) were collected using a Jasco J-810 spectropolarimeter with a 1-mm path length cuvette. Five scans were made at a scan rate of 100 nm/min, a bandwidth of 1 nm, and a response time of 2 s. All spectra were buffer-corrected, and mean residue ellipticities ($deg \cdot cm^2 \cdot dmol^{-1}$) were calculated and plotted against wavelength (nm). The pronounced leftward shift in ellipticity minima of both 2dCD4-C16A/C84A and 2dCD4-C130A/C159A suggests disruption of ordered β-sheeted structure in these proteins.

and dimerization, we found that gp120-bound 2dCD4 has significantly compromised capacity for isomerization effected by Trx. In summary, the data reported in this experiment allude to inhibitory effects mediated by gp120 on oxidoreductase-mediated isomerization of CD4, and this may represent one of the mechanisms by which gp120 corrupts important CD4-dependent signal transduction events.

DISCUSSION

An abundance of data has now demonstrated the deleterious effect of inhibiting cell surface oxidoreductase activity on HIV infection, implicating redox exchange reactions in the mechanism of HIV entry. For example, inhibition of cell surface redox activity with the membrane-impermeant sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) impairs HIV-1 infection in cell culture, as do specific inhibitors of PDI such as bacitracin, anti-PDI antibodies (20), and PDI siRNA (22), and similar effects have now been shown for Trx (11) and other important cellular reductases such as glutaredoxin-1 (24). Considerable debate remains on the physiological relevance of these observations because, in addition to facilitating HIV replication in cell culture, all three enzymes have been shown to be expressed on the surface of mammalian cells (31, 32) and to reduce disulfide bonds of gp120 *in vitro* (11, 24, 25). Contributing to the controversy are questions on how these enzymes would source the reducing equivalents required to charge their active sites in the oxidizing environment of the extracellular milieu. Indeed, in the PDI assays applied in this study, enzyme activation was achieved with 100 μM DTT, a more potent reductant at substantially higher molar concentration than what reasonable estimates suggest would typically be present on the cell surface (roughly 10 μM GSH equivalent) (24). The Trx relay system we employed, used to charge Trx with TR and NADPH, reconstitutes the components of a physiologically relevant redox system and mediates significantly more efficient reduction of CD4, providing further support to previous proposals that Trx is a good candidate for CD4 reduction in the cellular context. However, for the time being at least, the exact oxidoreductases involved and the mechanism(s) by which such systems secure adequate supplies of reduced co-factors (*e.g.* NADPH in the case of Trx/TR) to carry out their catalytic functions efficiently at the cell surface remain incompletely understood. Studies on the existence of membrane-embedded reduction systems should provide further insights in this regard.

Given an appropriate means of activation, however, our data show unequivocally that Trx is capable of robust reduction of CD4. In general, its exertion results in the rapid depletion of an oxidized monomeric isomer of the protein ($2dCD4^{Ox}$) and the progressive, commensurate formation of reduced counterparts, a process that leads either directly or via several intermediate steps to the production of a disulfide-linked 2dCD4

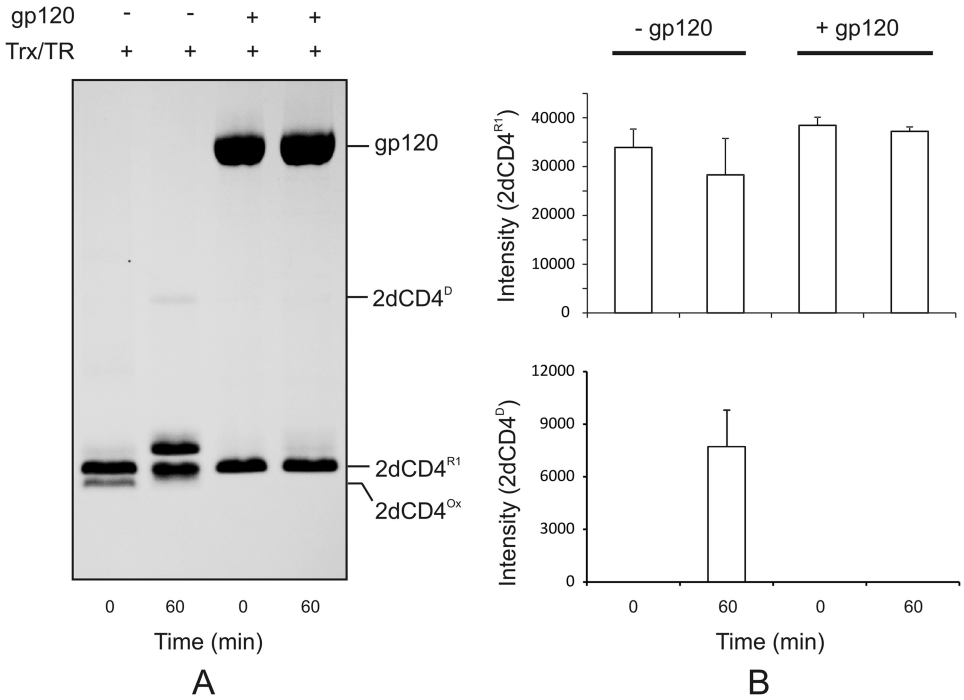

FIGURE 6. **gp120-mediated inhibition of Trx-driven CD4 dimerization.** *A*, gp120-bound and unbound 2dCD4-WT was treated with Trx/TR/NADPH at 37 °C, and reactions were stopped at 0 and 60 min by the addition of IAM (50 mM). The samples were then resolved by non-reducing SDS-PAGE. *B*, the relative quantities of the indicated 2dCD4 isomers were measured by densitometry. The peak intensities of the 2dCD4 isomers are indicated in *parentheses* above the corresponding bars. Representative data are shown, with *error bars* representing the S.D. of peak intensities determined for the corresponding bands on three independent gels derived from three independent isomerization experiments.

dimer. That both D1 and D2 disulfides evidently participate in these transactions is somewhat surprising, considering the documented structural characteristics of each disulfide and the preeminence given to the D2 disulfide bond as the likely mediator of CD4 allostericity in several recent reports (11, 14). However, other studies have shown that under certain conditions, CD4, in which the "non-allosteric" disulfides of D1 or D4 are reduced exists in relative abundance on the surface of cells (30), and support the notion that reduction of multiple CD4 disulfides may be functionally significant.

In this regard, there is now persuasive evidence to suggest that redox exchange reactions in the disulfides of CD4 mediate, or are at least an important component of, the structural rearrangements required for effective assembly of CD4 into TCR-MHCII antigen presentation complexes. Domain 2 Cys/Ala CD4 variants do not dimerize efficiently (11), and T cells expressing dimerization-defective CD4 cannot be activated through canonical MHCII-based presentation mechanisms (16). These data propose a rationale for the biological utility of oxidoreductive isomerization of CD4, and a direct implication of these findings, together with the results of previous reports describing the preference of gp120 for reduced isoforms of CD4, now confirmed in this study, is that HIV particles may capitalize on this during their association with target cells.

The molecular basis of the specific affinity HIV-1 gp120 has for reduced isoforms of CD4 is unclear. gp120 has been shown to induce conformational changes in CD4 (37), and reduction of the D2 disulfide bond has been suggested to participate in this process. Implied in the observation that gp120 is incapable of binding fully oxidized 2dCD4 is that prereduction of CD4 may be required for effective gp120 engagement, and this may prime CD4 for the structural rearrangements that occur post binding. Such a model would be congruent with existing observations that the levels of surface-active oxidoreductases such as Trx are elevated on activated T cells (38), that the abundance of reduced forms of CD4 increases on the surfaces of such cells (11), and that HIV replicates more efficiently in proliferating T cells (39). Although we note that the cysteines of CD4 crystallized in complex with gp120, presumably inferred from three-dimensional atomic proximity calculations, are presented in disulfide-bonded form (40), we submit that oxidation state in this context is essentially indeterminable and that crystallographic data may not completely describe the dynamic redox events at play before, during, and after interaction of the two binding partners.

The conformational distinctions between the oxidized and reduced isoforms of CD4 that dictate their respective capacities for gp120 binding have not been characterized in detail, and the ability to separate and stabilize each isomer by approaches that are conventionally applied to such endeavors obviously present fundamental technical challenges. A salient feature of both the 2dCD4 D1 and D2 disulfide mutants revealed through spectroscopic analysis is the characteristic circular dichroic spectra they generate, which are consistent with the presence of extensive irregular β-sheeted secondary structure (Fig. 5*B*). In contrast, 2dCD4-WT preparations (30–40% of the content of which is oxidized in D1 and D2) show far more regular and ordered β-pleating patterns (27, 41–43). This suggests that a degree of structural flexibility in CD4, potentially conferred through CD4 disulfide reduction, may be required for the initial engagement with gp120. That reduction of either domain disulfide is sufficient to enable this is an intriguing observation, the

basis for which is currently unclear. However, the finding that 2dCD4-CΔA (in which both domain disulfides are ablated) cannot bind gp120 at all, suggests that cooperative contributions to stabilizing the overall architecture of the protein may be made by each domain and that independent reductions of their resident disulfides have related global consequences. Ongoing work in our laboratory will hopefully provide further insights in this regard. Finally, by binding reduced isomers that are ostensibly the transient precursors of CD4 dimer formation, we intuited that gp120 may corrupt redox-driven CD4 dimer assembly, which evidence now compellingly suggests is essential for effective T cell activation (15–17). We confirmed this effect using a reconstituted oxidoreduction assay, demonstrating that gp120-bound 2dCD4 experiences inefficient Trx-mediated dimerization *in vitro*.

In summary, we have shown that gp120 binds exclusively to recombinant forms of two-domain CD4 in which either the domain 1 (Cys^{16}–Cys^{84}) or domain 2 (Cys^{130}–Cys^{159}) disulfides are reduced. Conversely, gp120 is completely unable to bind the same protein in which both disulfides are intact. When appropriately activated, Trx reduces 2dCD4 efficiently, which leads to a rapid increase in the concentration of reduced 2dCD4 intermediates and the formation of disulfide-linked 2dCD4 dimers. Thus is proposed another potential link between the physiological requirement for redox exchange in CD4 and oxidoreductase-dependent HIV entry of host $CD4^+$ cells. Further studies will hopefully reveal details on the structural characteristics of the CD4 redox isomers that explain their specificities for gp120, the kinetics and thermodynamics of these interactions, and establish to what extent the corruptive effects of gp120 on oxidoreductive dimerization of CD4 observed *in vitro* are relevant physiologically.

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* **See the addendum to Chapter 3 for further supporting evidence for the assignment of oxidation states using Mass Spectrometry**

3.1 Addendum to Chapter 3: Redox isomer analysis using LC-MS/MS

In order to confirm the assignment of the oxidation states of the cysteine pairs within the two redox isomers of 2dCD4-WT ($2dCD4^{ox}$ and $2dCD4^{R1}$), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was performed.

3.1.1 Materials and Methods

Wild-type 2dCD4 (10 µg) was treated with 50 mM iodoacetamide (IAM) for 1 hour at room temperature prior to denaturing, non-reducing PAGE analysis. Following separation on precast 4-12 % bis/tris gels, bands corresponding to each 2dCD4 redox isomer were excised and in-gel trypsin digested as per the protocol described by Shevchenko *et al.* [271]. Briefly, excised gel bands were destained using 50 mM ammonium bicarbonate/50% methanol followed by in-gel protein reduction (50 mM DTT in 25 mM ammonium bicarbonate) and alkylation (4 M acrylamide). Proteins were digested over-night at 37 °C using 5 – 50 µl, 10 ng/µl trypsin depending on the excised gel size. Digests were resuspended in 35 µl, 2% acetonitrile/0.2% formic acid and analysed using a Dionex Ultimate 3000 RSLC system coupled to a QSTAR ELITE mass spectrometer. Peptides were first de-salted on an Acclaim PepMap C18 trap column (75 µm × 2 cm) for 8 min at 5 µl/min using 2% acetonitrile/0.2% formic acid, followed by separation on an Acclain PepMap C18 RSLC column (75 µm × 15 cm, 2 µm particle size). Peptide elution was achieved using a flow-rate of 500 nl/min with a gradient of 4-60% B in 30 min (A: 0.1% formic acid; B: 80% acetonitrile/0.1% formic acid). Nano-spray was achieved using a MicroIonSpray head assembled with a New Objective, PicoTip emitter. An electrospray voltage of 2.0-2.8 kV was applied to the emitter. The QSTAR ELITE mass spectrometer was operated in Information Dependant Acquisition using an Exit Factor of 7.0 and Maximum Accumulation Time of 2.5 seconds. MS scans were acquired from *m/z* 400-1500 and the three most intense ions were automatically fragmented in Q2 collision cells using nitrogen as the collision gas. Collision energies were chosen automatically as a function of *m/z* and charge. Protein pilot v4.0.8085 using Paragon search engine (AB Sciex) was used for comparison of the obtained MS/MS spectra with protein sequences in a Uniswiss 2011 database supplemented with the sequence of wild-

type 2dCD4. Proteins with a threshold above ≥ 99.9% confidence were reported. Only peptides with ≥ 99% confidence were used to monitor the cysteine modification status. The LC-MS/MS experiment was performed in triplicate and representative data from one experiment is shown.

3.1.2 Results and Discussion

Wild-type 2dCD4 was initially treated with IAM to alkylate any thiol groups present in the native form (i.e. reduced cysteines). Following separation using non-reducing SDS-PAGE, excised protein bands were treated with reducing agent followed by alkylation with acrylamide. Using this protocol, unpaired, solvent exposed cysteine residues in the native state would be labelled with carbamidomethyl (CAM) whereas paired residues (oxidised cysteines) would be modified with propionamide (PAM). The differentially labelled CD4 was then subjected to in-gel trypsin digestion. The detected peptides, modification of their resident cysteines and the percentage modification are presented in Appendix B (Table B1). For the 22 kDa band of 2dCD4 (oxidised CD4, $2dCD4^{ox}$), results shown in Figure 3.7 indicate that none of the C16 residues were reduced whereas 1.0% of the C84 residues, 4.3% of the C130 residues and 4.6% of the C159 residues contained exposed thiol groups. Since CAM labels would only be observed in those isomers containing reduced cysteines, these MS results suggest that both the D1 and D2 disulphides in $2dCD4^{ox}$ are oxidised since no CAM modification was observed in >95% of peptides.

For $2dCD4^{R1}$ (25 kDa band, oxidation in either D1 or D2) the percentage of CAM modification increased to 11.0% (from 0%), 4.5% (from 1.0%), 11.9% (from 4.3%) and 12.2% (from 4.5%) for C16, C84, C130 and C159, respectively. Since the gel mobility data clearly show that this particular 2dCD4 isoform is partially reduced (in either D1 or D2) these results were unexpected as the differences between the oxidised and partially reduced 2dCD4 were predicted to be more substantial. Since the cysteine residues of CD4 are buried in the protein interior and maintained in a highly hydrophobic core, low solvent accessibility of these residues could potentially explain the inefficient labelling of thiols present in $2dCD4^{R1}$ with IAM. In addition, complete blocking of thiols with carbamidomethyl may require higher concentrations of IAM, as shown in a study of the alkylation of myofibril preparations where 65 mM of IAM was unable to completely block thiols after 1 hour [272]. 2dCD4 in

this study was treated with 50 mM IAM for 1 hour. In another study measuring the redox potential of human peroxiredoxin 3, it was found that alkylation of two out of three cysteine residues of the protein with IAM was rate limiting and the third cysteine could only be labelled at a concentration of 100 mM IAM [273].

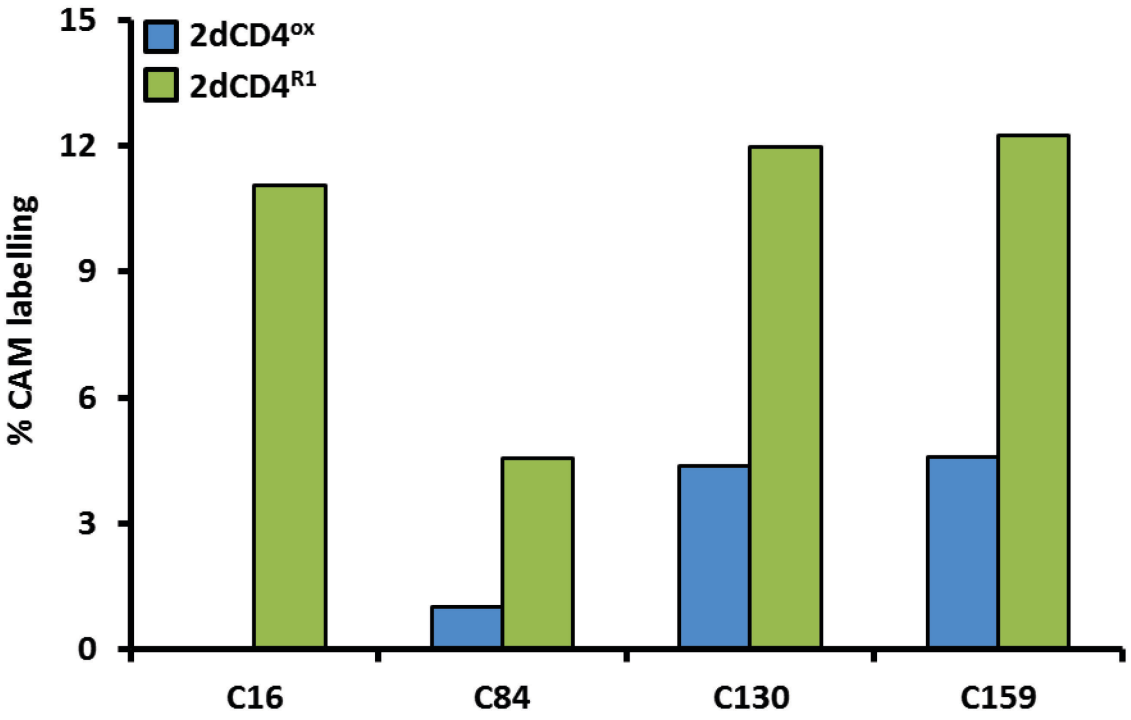

Figure 3.7: CAM labelling of 2dCD4^{ox} and 2dCD4^{R1}
The histogram plot shows the percentage CAM labelling in the two redox isomeric species of 2dCD4. The amount of CAM labelling increases upon partial reduction of disulphides in either D1 or D2 of 2dCD4.

A complication of using increased IAM concentrations however, is that at neutral or alkaline pH, IAM may also react with the hydroxyl group of tyrosine, the ε-amino group of lysine, the imidazole group of histidine and the N-terminus of proteins (reviewed in [274]). This would lead to difficulty in the interpretation of results obtained from MS. Taking into consideration the inefficiencies of IAM as well as the difficulties of labelling due to disulphide bond inaccessibility in CD4, the results observed are explicable as similar results have been reported by others [275]. The trends observed, however, fit well with the assigned oxidation states observed in Chapter 3 with 2dCD4^{ox} representing a fully oxidised isomer and 2dCD4^{R1} representing an isomer where the disulphide in either D1 or D2 is

reduced. When comparing the thiol modifications in both species there is clearly an enrichment of CAM-modified thiols in the 2dCD4^{R1} isomer indicative of an increase in the number of free thiols when either one of the disulphide bonds are reduced.

Chapter 4: CD4 metastability is a function of the allosteric disulphide in domain 2

4.1 Introduction

Together with previous studies performed by others, the results outlined in Chapters 2 and 3 provide further confirmation of the importance of disulphide bond rearrangement for the functions carried out by both HIV-1 gp120 and CD4. In the latter case, reductions of disulphides in domain 1 and 2 of CD4 are now known to be critical for potentiating the structural changes required for effective engagement of CD4 with TCR-MHCII complexes and HIV-1 gp120. However, the biophysical consequences of CD4 disulphide reduction that are likely to underpin the requirement for this remain poorly understood. The crystal structures of CD4 as defined in the unliganded form [144, 145] and in complex with gp120- [62], MHCII- [134] and MHCII-TCR are readily superimposable (RMS of 0.98 Å) inferring that no major structural reordering of CD4 is required for binding gp120, and that CD4 acts as rigid docking site for its cognate receptors. However, several authors have suggested significant structural rearrangements in specific amino acid stretches in the first two domains of CD4 post-gp120 binding by using CD4 specific mAbs [276], molecular simulation studies of entropic changes in D1 [277] as well as normal mode analysis of 2dCD4 [278]. In addition, upon refinement of the initial 2dCD4 structure, Ryu *et al.* [279] demonstrated moderate flexibility in the region of CD4 involved in gp120 binding as well as a detectable motion between domain 1 and domain 2. As mentioned in Chapter 3, although Cys in the crystal structure of unliganded CD4 as well as CD4 in complex with the above mentioned receptors have been assigned as oxidised (due to atomic proximity calculations), these assignments are unable to reflect the dynamic redox events which occur in the unliganded and liganded state.

This chapter describes preliminary, low-resolution biochemical data on the secondary structure of two recombinant 1dCD4 proteins (1dCD4-D1 and 1dCD4-D2), as part of our laboratory's ongoing efforts to gain further insights into the structural consequences of

disulphide bond oxidoreduction, and why the distinctive CD4 redox isomers have such specific receptor-binding profiles.

Here, the basic analysis of the 2dCD4 proteins by non-reducing SDS-PAGE and circular dichroism (CD) spectroscopy described in Chapter 3 was expanded by performing similar experiments on individual CD4 domain 1 or 2 polypeptides. Experimental results suggest that while the highly ordered β-sheeted secondary structures typical of the Ig-family of proteins are able to form in 1dCD4-D1, those observed for 1dCD4-D2 are far less ordered. This preliminary study highlights the importance of creating a reliable platform for the elucidation of the structural effects of disulphide bonds present in the different and distinct CD4 isomers. Structural knowledge of this nature, will not only provide the information required to better understand the specificities of CD4 ligand binding but a more general appreciation of the structural importance of allosteric disulphides in other biological systems.

4.2 Materials and Methods

4.2.1 Expression Plasmids

The generation of the 2dCD4-WT pET15b plasmid has been described in Chapter 2 under experimental procedures. In addition to this, two single domain CD4 cassettes were synthesised by GENEART (Regensburg, Germany): domain 1 (1dCD4-D1, residues 1-98 of CD4) containing L5I, A55V, I76P, L96I and F98L [280] substitutions, which have been documented to maintain secondary structural content, increase protein solubility, affinity for HIV-1 gp120 and neutralise pseudoviruses to a similar degree; and domain 2 (1dCD4-D2, residues 99-178 of CD4, wild-type sequence). Both 1dCD4-D1 and 1dCD4-D2 pET15b plasmids were generated as described in Chapter 2 (under recombinant proteins and expression plasmids).

4.2.2 2dCD4 Expression, Purification and Refolding

The recombinant 2dCD4-WT, 1dCD4-D1 and 1dCD4-D2 variants were expressed and purified by standard denaturing metal-chelate affinity chromatography and refolded using oxidative refolding protocols according to methods described in Chapter 2 and 3. Both 1dCD4

variants were expressed, purified, refolded and analysed by SDS-PAGE under reducing and non-reducing conditions, and stained with Coomassie Blue R250 as described in Chapter 2 (under recombinant 2dCD4 folding and functionality analysis) and Chapter 3 (under vectors and proteins).

4.2.3 Circular Dichroism Analysis of Recombinant CD4s

CD spectra of 10 µM 2dCD4-WT, 1dCD4-D1 and 1dCD4-D2 in 10 mM Na-phosphate (pH 7.4), 140 mM NaCl (Sigma-Aldrich, Germany) were collected using a Jasco J-810 spectropolarimeter with a 1 mm pathlength cuvette. Five scans were made at a scan rate of 100 nm/min, a bandwidth of 1 nm, and a response time of 2 seconds. All spectra were buffer-corrected and normalised mean residue ellipticities (degrees.cm^2/dmol) were calculated as described in Chapter 2 (under recombinant protein folding and functionality analysis). In addition, predicted spectra for the 1dCD4 variants were obtained using the DichroCalc spectra prediction algorithm [281]. Amino acid crystal structure co-ordinates for each domain were extracted from PDB ID 1CDH and submitted for analysis.

4.3 Results

4.3.1 Single Domain CD4 Electrophoretic Mobility Analysis

Chapter 3 reported the assignment of oxidation states of purified 2dCD4 isomers resolved by non-reducing SDS-PAGE (Chapter 3, Figure 1): 2dCD4^{R2} (where both the domain 1 and 2 disulphide bonds are fully reduced), 2dCD4^{R1} (where only one of the two disulphides is reduced) and 2dCD4^{ox} (where both domain 1 and domain 2 disulphides are oxidised). Similarly, reducing and non-reducing SDS-PAGE analysis was performed on 1dCD4-D1 and 1dCD4-D2 (Figure 4.1). As discussed in Chapters 2 and 3, by far the most common effect of reduction of disulphide-bonded bonded proteins analysed by SDS-PAGE is to decrease relative electrophoretic mobility [282]. This is essentially attributable to an increase in hydrodynamic volume caused by relaxation of disulphide-linked secondary structural elements. Consistent with this, reduction of 1dCD4-D1 resulted in an apparent increase in molecular mass by approximately 4 kDa (Figure 4.1, Lane 2). Remarkably and in contrast, the effect of reduction on 1dCD4-D2 was to reduce apparent molecular mass by approximately 2 kDa (Figure 4.1, Lane 3), suggesting that disulphide bond reduction in CD4

domain 2 decreases the hydrodynamic volume of this protein. A possible implication of this is that reduction of the allosteric domain 2 disulphide in CD4 may be associated with a release of the high energy contained in the disulphide bond with an inward motion of the β-strands (collapse). It is speculated that this may in turn lead to a more stable, hydrodynamically compact and energy-minimised structural intermediate.

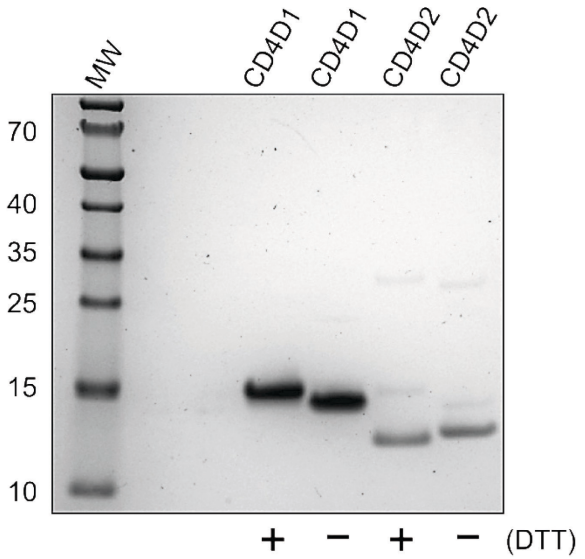

Figure 4.1: Reducing and non-reducing SDS-PAGE analysis of 1dCD4-D1 and 1dCD4-D2
Purified recombinant 1dCD4-D1 and 1dCD4-D2 were treated with LDS loading buffer with or without DTT (50 mM), then with iodoacetamide (50 mM) and resolved by SDS-PAGE. Gels were stained with Coomassie Blue R250. Molecular mass, in kDa, is shown on the left.

4.3.2 Analysis of CD4 Domains 1 and 2 by CD Spectroscopy

In order to gain more insights into the contributions made by each domain in defining the overall secondary structure content of 2dCD4, and the unusual effect that reduction of the 1dCD4-D2 disulphide had on the hydrodynamic volume of this protein, the secondary structure content of the individual CD4 domains (1dCD4-D1 and 1dCD4-D2) was characterised by CD spectroscopy. As described in Chapter 3, the CD spectra of the 2-domain proteins are indicative of an extensive β-sheeted structure, which is partially disrupted by disulphide ablation in either 2dCD4 D1 or D2 (Chapter 3, Figure 1). The spectra of the individual CD4 domains are shown in Figure 4.2. As can be seen, domain 1 has typical

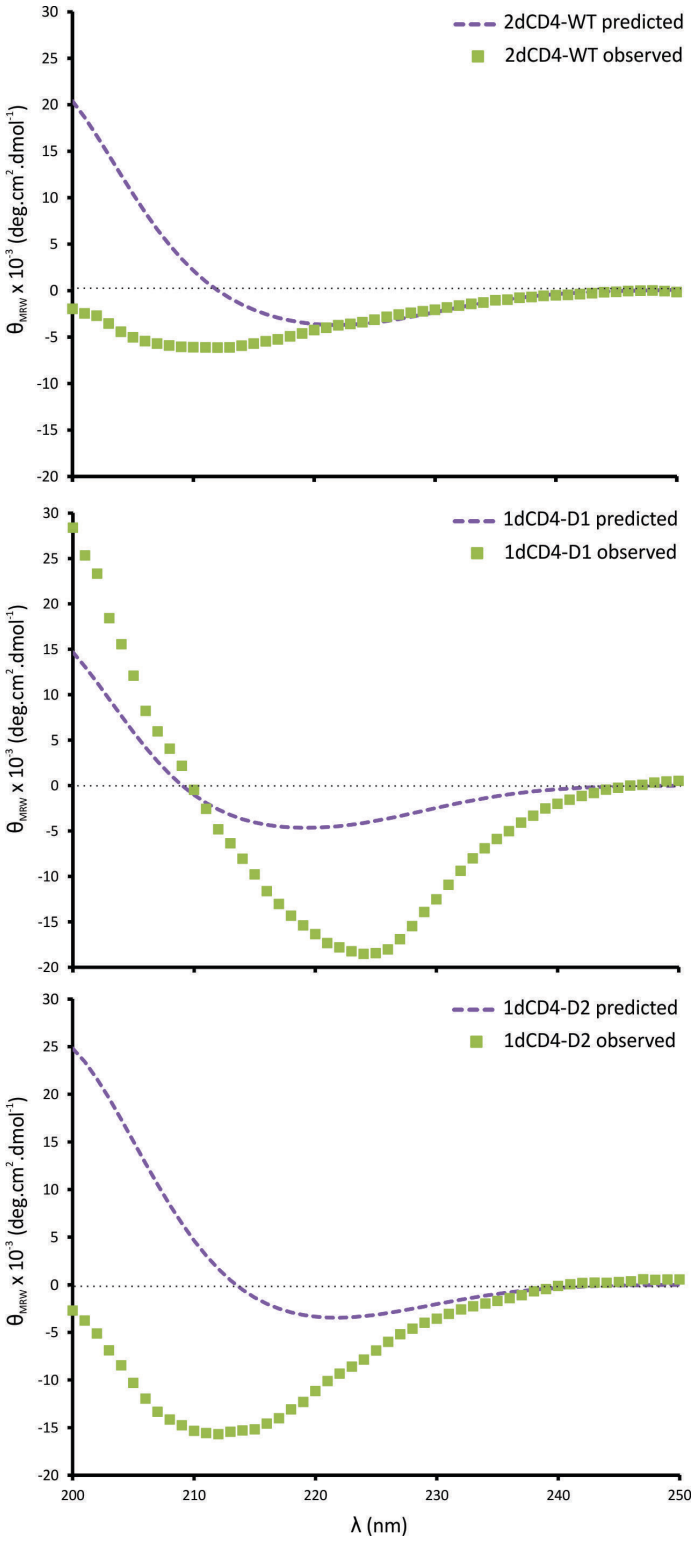

Figure 4.2: Far-UV CD analysis of 1dCD4 variants

Far-UV spectra of 10 µM 2dCD4-WT (top panel), 1dCD4-D1 (middle panel) and 1dCD4-D2 (bottom panel) were collected using a Jasco J-810 Spectrapolarimeter with a 0.1 cm path-length cuvette. Five scans were made at a scan rate of 100 nm/min, a bandwidth of 1 nm, and a response time of 2 seconds. All spectra were buffer-corrected, and mean residue ellipticities (deg.cm^2.dmol^{-1}) were calculated and plotted against wavelength (nm). Predicted CD spectra for all CD4 variants are shown as purple lines.

highly ordered β-sheet fold topology, with an ellipticity minimum at approximately 222 nm and positive ellipticity below 210 nm. Interestingly, this spectrum follows the trend of the spectrum predicted by the DicroCalc CD spectra prediction algorithm [281] for domain 1 extracted from PDB ID 1CDH (amino acid co-ordinates 1 – 98). In contrast, 1dCD4-D2 generated a CD spectrum more consistent with β-stranding or irregular β-sheeted structure, with a significant decrease in both the magnitude and position of the ellipticity minimum, which occurs between 208 – 214 nm. The characteristically large magnitude of the ellipticity minimum possibly indicates that this domain bares a more open/flexible fold topology capable of more efficient rotation of plane-polarised light. Moreover, the predicted (ellipticity minimum at approximately 220 nm) and experimental (ellipticity minimum at approximately 212 nm) spectra for domain 2 are substantially different, indicating that the structure observed in PDB ID 1CDH reflects only a single specific isomer and suggests that in solution, this molecule is more flexible. Taken together, these data are consistent with a model in which the highly ordered, inflexible domain 1 architecture is contrasted with the metastability of domain 2, biophysical characteristics that are shared with their respective structural and allosteric disulphide bonds. Thus, the increase in hydrodynamic volume of 1dCD4-D1 upon treatment with DTT (Figure 4.1) is in keeping with the structural perturbation that reduction of 16C-C84 is likely to effect. However, ablation of the domain 2 disulphide serves to decrease the distance between secondary structural elements in 1dCD4-D2 resulting in a more energetically stable and compacted structure with lower hydrodynamic volume.

4.4 Discussion

The structural role of the D2 disulphide bond in CD4 has until now remained unclear. It has been suggested that the allosteric D2 disulphide is not an essential part of the fold since the homologous D4 domain of CD4 has a conventional inter-sheet bridge and the D1/D2 and D3/D4 domains are structurally comparable [279]. Since the data presented in this thesis and that previously by others has demonstrated a vital role of disulphide reduction for the receptor function of CD4, it follows that this reduction is likely to effect significant structural change in the latter. In addition, presentation of the disulphide bonds for reduction by cell-surface oxidoreductases would possibly be dependent upon their relative position in the

receptor. Herein, it has been demonstrated that the D2 disulphide does indeed have a significant effect on the secondary structural elements of the protein. The specific observation that the 1dCD4-D2 CD spectrum does not match the predicted spectrum implies that this domain does not conform to the classic β-sheeted topology observed for 1dCD4-D1. It is well accepted that X-ray crystal structures need to be examined with caution as these structures are obtained using a combination of experimental observation as well as modelling. Experimental evidence may not always match X-ray crystal structure data, a phenomenon which is most commonly attributable to the solid phase nature of protein crystals. In addition, buffer compositions that may be favourable for crystallography may not reflect the physiological environment of the protein. The dynamic nature of the disulphide bonds in CD4 and the specific effects that oxidoreduction may have on its structural topology, are thus unlikely to be fully illustrated by the crystal structure reported.

The well-accepted stability inferred by disulphide bonds in the native 3D structure of a protein is often off-set by the instability caused by enthalpic and strain effects. Factors such as intramolecular strain and conformational space due to the protein chain are also suggested to influence the disulphide/thiol redox potential [283]. Highly strained disulphide conformations impart high conformational energy with a consequential ease of reduction. These unstable, “forbidden” disulphides (such as the D2 disulphide in CD4) introduce metastable areas within the native protein and allow the protein to sample a number of alternative isomeric forms [283]. This implies that once formed, disulphide bonds are not chemically inert and can be reduced back to their constituent Cys residues [284]. Highly strained disulphide bonds have been shown to be readily reduced although there is a limited amount of experimental data to suggest that disulphides adopting strained conformations have an enhanced likelihood for reduction to dithiol [285]. Recent computational studies by Roos *et al* [286] show that disulphide redox potential is linked to the conformation of the disulphide (both in model systems and in examples from PDB). The driving force behind this facile reduction is the release of intramolecular strain (high conformational energy) as the thiol is formed [286].

In summary, the data presented here allude to the metastable nature of the D2 disulphide bond, a characteristic that presumably assists in the presentation of the correct isomeric

form of CD4 at the cell surface. Current efforts in our laboratory are focused on obtaining more detailed descriptions of the structural changes that reduction of the metastable disulphide in D2 is likely to effect, using more detailed CD analysis as well as fluorescence spectrophotometry and dynamic light scattering. It is anticipated that these data will provide new insights into the biological utility of disulphide reduction for CD4-dependent entry of HIV into host cells.

Chapter 5: Concluding Remarks

This study has provided new insights into the mechanistic importance of redox biology in the process of HIV-1 entry. The importance of allosteric disulphides during biological processes and the effects these bonds have on protein structure has also been highlighted. In addition to assisting the folding of proteins into native conformations, the classical disulphide is commonly known to stabilise the native state. Over the last decade, dynamic redox regulation of these bonds has been demonstrated to play a vital part in protein function. In particular, the functional utility of the unusual allosteric disulphide bond has become clinically significant in areas of therapeutic molecule and vaccine development [287]. Although the importance of these bonds is becoming more accepted, the effects they have on protein structure and ultimately function, remains unclear in many examples.

HIV-1 entry is dependent upon the interaction of the Env glycoprotein with receptor CD4 and oxidoreduction of disulphides in both receptors is essential for this process. With this in mind, two broad areas concerning the redox biology of the CD4 receptor were investigated. In the first study, the main aim was to engineer a CD4 molecule containing a free thiol which would target an allosteric disulphide in the CD4bs of gp120 and competitively inhibit HIV entry. In the second study, the aim was to investigate the effects of cellular oxidoreductases on CD4 isomerisation and uncover possible biological utilities of these effects.

Chapter 2 describes a recombinant 2dCD4 molecule containing a Ser to Cys mutation (2dCD4-S60C) in the first domain that strategically placed a thiol in the vicinity of the V1/V2 loop disulphide (126C-C196) of the CD4bs of gp120. Under conditions that promoted thiol/disulphide exchange, this molecule showed a significant increase in affinity for HIV-1 gp120 as compared to wild-type protein. This increase in affinity was attributed to the formation of a disulphide bond between 2dCD4-S60C and gp120. The observed increase in affinity could be translated into an increase in the efficiency of CD4-mediated inhibition of viral entry. In this chapter, it was suggested that incorporation of nucleophiles into the

designs of new or existing CD4bs-targeted gp120-binding compounds may act to enhance the affinities of such compounds for their target. After the publication of this article, two important studies have further demonstrated the value of this strategy.

Martin *et al* [288] engineered a small CD4 mimetic (M64U1-SH) containing a thiol modified lysine residue (Lys4) in the gp120-binding site of CD4. This thiol was strategically positioned in order to potentiate the reduction of the C126-196C disulphide in the CD4bs of gp120 with a resultant disulphide formed between the thiol of M64U1-SH and Cys196 of gp120. These authors were able to demonstrate efficient cross-linking of M64U1-SH to gp120 *in vitro*, following which, these complexes were used to immunise rabbits in an attempt to effect the production of CD4i antibodies. In addition to neutralisation of a homologous vaccine strain (SF162), these authors were able to neutralise heterologous subtype B viruses. Du *et al* [289] engineered CD4-linker-DC-SIGN fusion proteins in an attempt to block the binding sites on gp120 that recognise these two molecules. In addition to a wild-type CD4-DC-SIGN bi-functional protein, a CD4-DC-SIGN conjugate containing the S60C mutation described in Chapter 2 was also produced. This S60C construct showed enhanced HIV-1 inhibition of infection and dissemination in cell lines, primary dendritic cells and mucosal cervical tissues.

Although disulphide bond targeting was investigated in the context of HIV-1 therapy and vaccine development, the general potential value of this approach in manipulating therapeutically targetable receptor-ligand interactions has recently been expounded by others. In particular, Hogg [287] reviewed the importance of targeting allosteric disulphide bonds in cancer where oxidoreduction of allosteric disulphides has emerged as one of the many important themes in cancer-related proteins.

Chapter 3 showed how the oxidoreductive power of Trx leads to reduction of the disulphides in CD4 thereby supporting the notion that Trx is the most likely physiological reductant of CD4. Furthermore, one of the isomeric products of CD4 reduction (a disulphide reduced in either domain 1 or 2 of 2dCD4) is specifically bound by HIV gp120. Another profound finding of this study was that Trx-mediated dimerisation of CD4 is impaired when CD4 is bound to gp120. These CD4 dimers play an essential role in the assembly of CD4-

MHCII-TCR complexes and by binding the partially reduced isomer of CD4, HIV-1 particles inhibit this immunologically essential event by preventing Trx-mediated dimerisation of CD4.

The basis for the exclusive binding of gp120 to specific CD4 isomers, which are, as evidence suggests, only transiently present on the cell surface under a limited number of physiological conditions, is poorly understood. However, the findings of this study are likely to have important implications for understanding the architecture of the CD4bs on gp120, and the role its evolution played as a facilitator of viral entry and mediator of immune avoidance. Further, the demonstration of active oxidoreductase-dependent CD4 isomerisation, and the likely importance of this process physiologically and in the context of HIV infection, suggests another potential mechanism by which HIV mediates pathogenic effects and may provide a platform for the development of novel antiviral approaches.

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Appendices

Appendix A: Recombinant CD4 Constructs used in this Study



Figure A1: CD4 construct design

The 2dCD4 wild-type sequence is shown at the top of the figure. All constructs used in this study are schematically represented. Cys residues are shown in yellow in both the sequence and the schematic diagram with Cys to Ala mutations shown in white. D1 of 2dCD4 is coloured pink and D2 coloured orange. The C-terminal 6xHis tag is underlined in the amino acid sequence and shown on each schematic. Specific amino acid changes with regards to $2dCD4^{FD}$ and the 1dCD4-D1 variant have been detailed in the Materials and Methods section of Chapter 2 and 4, respectively.

Appendix B: LC-MS/MS Peptide Summary

Table B1: 2dCD4-WT isomer LC/MS/MS peptide summary

	Peptides Detected	*Modification*	*%Contribution*	*%PAM[1]*	*%CAM[2]*
2dCD4[R1]					
C16	KGDTVELTCTASQK	PAM	45.60	88.93	11.06
	GDTVELTCTASQK	PAM	38.15		
	KGDTVELTCTASQK	CAM	5.61		
	GDTVELTCTASQK	CAM	5.44		
	GDTVELTCTASQK	PAM	3.35		
	KGDTVELTCTASQK	PAM	1.82		
C84	IEDSDTYICEVEDQKEEVQLLVF	PAM	23.46	95.44	4.56
	IEDSDTYICEVEDQKEEVQLLVFGLTAN	PAM	16.93		
	IEDSDTYICEVEDQKEEVQLLVFGLTAN	CAM	1.72		
	IEDSDTYICEVEDQK	PAM	14.51		
	IEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	14.51		
	IEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQGQ	PAM	13.90		
	IEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQGQ	CAM	0.90		
	IEDSDTYICEVEDQK	PAM	5.39		
	IEDSDTYICEVEDQKEEVQLLVF	PAM	3.72		
	IEDSDTYICEVEDQK	PAM	2.57		
	IEDSDTYICEVEDQK	CAM	1.23		
	IEDSDTYICEVEDQK	CAM	0.69		
	IEDSDTYICEVEDQKEEVQLLVFGLTAN	PAM	0.26		
	IEDSDTYICEVEDQK	PAM	0.14		
C130	SDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	44.84	88.04	11.96
	SDTHLLQGQSLTLTLESPPGSSPSVKCR	CAM	4.91		
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	12.93		
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	CAM	1.50		
	SDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	8.11		
	SDTHLLQGQSLTLTLESPPGSSPSVQCR	CAM	1.11		
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	6.20		
	SDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	4.52		
	SDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	1.77		
	TANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	3.72		
	TANSDTHLLQGQSLTLTLESPPGSSPSVQCR	CAM	0.42		
	TANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	1.92		
	SLTLTLESPPGSSPSVQCR	CAM	1.75		
	SLTLTLESPPGSSPSVQCR	CAM	0.94		
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	0.88		
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	CAM	1.29		
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCRS	PAM	0.20		
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	2.90		
C159	TLSVSQLELQDSGTWTCTVLQNQK	PAM	50.86	87.76	12.24
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	11.49		
	TLSVSQLELQDSGTWTCTVLQNQK	CAM	0.30		
	TLSVSQLELQDSGTWTCTVLQNQK	CAM	5.37		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	4.35		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	3.55		
	TLSVSQLELQDSGTWTCTVLQNQK	CAM	3.36		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	1.16		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	3.00		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	1.43		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	2.71		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	2.67		
	TLSVSQLELQDSGTWTCTVLQNQK	CAM	2.64		
	TLSVSQLELQDSGTWTCTVLQNQKK	PAM	1.03		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	1.01		
	TLSVSQLELQDSGTWTCTVLQNQKK	PAM	0.77		
	NIQGGKTLSVSQLELQDSGTWTCTVLQNQK	PAM	0.69		
	VSQLELQDSGTWTCTVLQNQK	PAM	0.52		
	TLSVSQLELQDSGTWTC	PAM	0.45		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	0.44		
	TLSVSQLELQDSGTWTCTVLQN	PAM	0.42		
	TLSVSQLELQDSGTWTCTVLQN	CAM	0.55		
	NIQGGKTLSVSQLELQDSGTWTCTVLQNQK	PAM	0.40		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	0.33		

	QLELQDSGTWTCTVLQNQK	PAM	0.32		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	0.05		
2dCD4^{ox}					
C16	KGDTVELTCTASQK	PAM	60.46	100	0
	GDTVELTCTASQK	PAM	39.53		
C84	IEDSDTYICEVEDQKEEVQLLVF	PAM	42.03	98.97	1.03
	IEDSDTYICEVEDQK	PAM	16.68		
	IEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQGQ	PAM	11.72		
	IEDSDTYICEVEDQKEEVQLLVFGL	PAM	10.75		
	IEDSDTYICEVEDQKEEVQLLVFGLTAN	PAM	8.99		
	IEDSDTYICEVEDQK	PAM	5.35		
	IEDSDTYICEVEDQK	PAM	2.26		
	IEDSDTYICEVEDQKEEVQLL	PAM	1.05		
	IEDSDTYICEVEDQK	CAM	1.03		
	NLKIEDSDTYICEVEDQK	PAM	0.11		
C130	SLTLTLESPPGSSPSVQCR	PAM	24.9	95.64	4.36
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	19.08		
	TANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	8.82		
	SDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	8.70		
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	8.64		
	SDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	6.10		
	TLTLESPPGSSPSVQCR	PAM	4.64		
	SDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	4.32		
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	4.10		
	TANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	3.80		
	SDTHLLQGQSLTLTLESPPGSSPSVQCR	CAM	2.92		
	SLTLTLESPPGSSPSVQCR	CAM	1.43		
	GLTANSDTHLLQGQSLTLTLESPPGSSPSVQCR	PAM	1.32		
	LLQGQSLTLTLESPPGSSPSVQCR	PAM	1.09		
C159	TLSVSQLELQDSGTWTCTVLQNQK	PAM	49.99	95.42	4.58
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	18.22		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	12.32		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	6.23		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	4.66		
	TLSVSQLELQDSGTWTCTVLQNQK	CAM	4.10		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	2.82		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	0.80		
	TLSVSQLELQDSGTWTCTVLQNQK	CAM	0.47		
	TLSVSQLELQDSGTWTCTVLQNQK	PAM	0.34		

[1] *PAM*: Propionamide (indicates disulphide is oxidised, shaded orange)

[2] *CAM*: Carbamidomethyl (indicates disulphide is reduced, shaded blue)

***Appendix C**: Human Research Ethics Committee Clearance Certificate*

R14/49 Ms Nicole Cerutti

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130384

NAME: (Principal Investigator)	Ms Nicole Cerutti
DEPARTMENT:	Molecular Medicine & Haematology Medical School
PROJECT TITLE:	On the Redox Biology of of the Immuno-Virological Receptor CD4: Biological Function and Applications in HIV-1 Drug and Vaccine Development
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof M Papathanasopoulos
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 08/04/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES