Characterization of the Noncovalent Complex of Human Immunodeficiency Virus Glycoprotein 120 with Its Cellular Receptor CD4 by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry†

Christoph Borchers and Kenneth B. Tomer*

Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

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ABSTRACT: The initial event in infection by the human immunodeficiency virus type 1 (HIV-1) is the interaction of the viral envelope glycoprotein (HIV-gp120) with its primary cellular receptor, the glycoprotein CD4. Molecular structure information about the HIV-gp120/CD4 complex can provide information relevant to an understanding of the basic processes occurring in HIV infection and to development of therapies that can inhibit AIDS. Previous studies by sugar gradient sedimentation of the interaction of HIV-gp120 with a cytoplasmic domain truncated soluble CD4 (sCD4) suggested that a one-to-one complex was formed. The stoichiometry, however, of the sCD4/HIV-gp120 complex remained to be confirmed by an independent method because (i) recent X-ray examination revealed dimerization of sCD4 and (ii) the low resolution and low accuracy of molecular weight determination by sugar gradient sedimentation can lead to artifactual data. Therefore, in this study matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was used to determine the molecular mass of the complex of fully glycosylated HIV-gp120 and sCD4. A mass of 145 kDa was measured, which is exactly the sum of the molecular masses of one HIV-gp120 and one sCD4 molecule. Complexes of higher order of stoichiometry were not detected. Identical results were obtained by chemically cross-linking the HIV-gp120/sCD4 complex with subsequent analysis by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and MALDI-MS. This study confirms the earlier suggestions of the stoichiometry of the sCD4/HIV-gp120 complex in solution and also demonstrates the potential of MALDI-MS in investigations of specific noncovalent complexes of glycoproteins.

The surface glycoprotein of human immunodeficiency virus type 1 (HIV-1),[1] HIV-gp120, is synthesized as an HIV-gp160 precursor that is subsequently cleaved intracellularly into HIV-gp120 and HIV-gp41 (*1*). HIV-gp120 constitutes the exterior envelope of the virus and contains about 480 amino acids (*2*). Approximately half of its molecular weight is attributed to N-linked oligosaccharides of highly heterogeneous structures (*3*).

The interaction of HIV-gp120 with its primary cellular receptor, CD4, is a key event in AIDS infection (*4*). CD4, a glycoprotein of approximately 60 kDa, is expressed on the cell membrane of mature helper/inducer T lymphocytes, and its binding to HIV-gp120 dictates the trophism of the virus (*5, 6*). This high-affinity interaction not only initiates entry of HIV into host immune cells but also occurs in several other steps in HIV-1 infection (*7*). The HIV-gp120/CD4 interaction, therefore, is a pharmacological target of major interest. Molecular structure information about the HIV-gp120/CD4 complex can provide critical information relevant to an understanding of the basic processes occurring during HIV infection and to development of therapies that can inhibit AIDS.

Numerous studies have been performed on the stoichiometry of the HIV-gp120/CD4 complex and the molecularity of the binding proteins (*8*−*12*). Sugar gradient sedimentation and chemical cross-linking indicated that CD4 (*8*) and virion-associated gp120 (*9*) are in dimeric and dimeric/trimeric and in mainly dimeric states, respectively, and HIV-gp120/CD4 complexes of higher order are generated (*8, 10*). Another sugar gradient sedimentation study showed that HIV-gp120 was secreted into the medium in a monomeric state (*11*). The results of other investigations, however, suggested that HIV-gp120 is secreted in an oligomeric form (*12*) and cross-linking of recombinant HIV-gp120 in solution with a homobifunctional amino-reactive reagents revealed the presence of homodimers and -trimers (*9*). Studies by sugar gradient sedimentation with recombinant soluble CD4 (sCD4), which represents the extracellular glycosylated part of native CD4 (*13*), suggested a monomeric state with formation of one-to-one complex with recombinant HIV-gp120 (*8*). Additionally, recent X-ray studies revealed dimerization of sCD4 (*14*), but a 1:1:1 stoichiometry for a complex consisting

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* Corresponding author: National Institute of Environmental Health Sciences, P.O. Box 12233, MD F0-03, Research Triangle Park, NC 27709. Phone (919) 541-1966; fax (919) 541-0220; email tomer@niehs.nih.gov.

[1] Abbreviations: HIV, human immunodeficiency virus; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; SAED, sulfosuccinimdo-2-(7-azido-4-methylcoumarin-3-acetamido)-ethyl-1,3′-dithiopropionate; PBS, phosphate-buffered saline.

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of HIV-gp120, CD4, and an antigen-binding fragment of a neutralizing antibody (*15*). The CD4 and HIV-gp120 that were used for the X-ray study, however, were both truncated protein derivatives. Furthermore, the HIV-gp120 was extensively deglycosylated, and the V-3 loop was deleted. Because the sugar gradient sedimentation analyses were not accurate enough to determine unambiguously the molecular weight, and because of the conflicting results of sugar gradient sedimentation, cross-linking of HIV-gp120, and crystallography, the stoichiometry of the sCD4/HIV-gp120 complex remains in doubt.

Electrospray ionization mass spectrometry (ESI-MS) has recently been introduced as a highly precise, novel method for the determination of molecularity/stoichiometry of homo/heterooligomers (*16, 17*). The use of ESI-MS, however, is usually limited to homogeneous components of molecular masses less than 150 kDa due to the difficulty of resolving the complex envelope of multiply charged ions observed. Due to the high heterogeneity of the oligosaccharides of HIV-gp120 and the high MW of the protein complex, ESI-MS, therefore, is not suitable for this determination. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), an alternative mass spectrometric technique, seems to have limited application to the study of noncovalent interactions and only a few examples of the characterization of noncovalent complexes have been published (*18*−*20*). The major limitation of MALDI-MS is due to sample preparation, during which denaturation and dissociation of the protein complex usually occurs as the analyte is incorporated into crystals of the strongly acidic benzoic and cinnamic acid derivatives (e.g., α-cyano-4-hydroxycinnamic acid, CHCA) used as matrixes. Recently, new sample matrixes and new procedures for sample preparation at neutral pH were developed to overcome dissociation of specific complexes (*21*). In this paper, we report the results of our investigation of the use of MALDI-MS to probe the noncovalent interaction between HIV-gp120 and sCD4, and we compare our results with those obtained by chemical cross-linking.

MATERIALS AND METHODS

Materials. The following reagent was obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH: Recombinant soluble CD4 from R. Sweet, SmithKline Beecham. Soluble CD4 (sCD4) comprises amino acids 1−369 of the mature CD4 protein (*13*). HIV-gp120 was purchased from Austral Biologicals (San Ramon, CA). The protein containing the entire gene sequence from amino acid Glu39−Arg517 of the HIV-1 (SF2 isolate) was expressed in CHO cells (*22*). The protein molecular weight markers for gel electrophoresis were purchased from Pharmacia Biotech (Piscataway, NJ). α-Cyano-4-hydroxycinnamic acid (CHCA) and 2′,4′,6′-trihydroxyacetophenone were from Aldrich Chemical Co. (Milwaukee, WI). CHCA was recrystallized from methanol before use as a matrix. Sulfosuccinimido-2-(7-azido-4-methylcoumarin-3-acetamido)ethyl-1,3′-dithiopropionate (SAED) was purchased from Pierce (Rockford, IL). Bovine serum albumin (BSA), 6-aza-2-thiothymine (ATT), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Noncovalent HIV-gp120/sCD4 Complex. The solution of sCD4, which is originally dissolved in a histidine (10 mM) buffer was purified by gel filtration and simultaneously exchanged against 20 mM phosphate buffer, pH 7.5. For gel filtration, porous polyacrylamide gel with a molecular weight size exclusion limit of 1800 (Bio-Gel P-2) from Bio-Rad (Hercules, CA) was used. Gel filtration was performed in spin chromatography columns from Bio-Rad according to the manufacturer's instructions. The concentration of purified sCD4 was determined by HPLC. The complex was formed by incubation of 10 pmol/μL HIV-gp120 in 2-fold concentrated PBS [where PBS is 0.15 M NaCl and 0.01 M phosphate (Na/K) to obtain pH 7.4] and purified sCD4 in different concentrations at room temperature under rotation for 1 h. Control experiments with BSA were performed under the same conditions.

Cross-linking of HIV-gp120/sCD4 Complex. The HIV-gp120/sCD4 complex formed (molar ratio of HIV-gp120:sCD4 was 1:1.4) was allowed to react in the dark with a 10-fold molar excess of freshly prepared SAED, 1 mM in 20 mM phosphate buffer, pH 7.5, for 15 min at room temperature. The reaction was quenched by adding 100 mM lysine (30-fold excess compared to SAED) in 20 mM phosphate buffer, pH 7.5, for another 15 min at room temperature. The derivatized complex was separated from free reagent by gel filtration through spin chromatography columns (Bio-Rad) filled with porous polyacrylamide gel (Bio-Gel P-2 from Bio-Rad) and equilibrated in 20 mM phosphate buffer, pH 7.5. Photoactivation was carried out for 15 min by exposing the sample to a black-ray, long-wave, 100-W ultraviolet lamp (Ultra Violet Products Inc., San Gabriel, CA). The filter used transmits wavelengths between 300 and 400 nm, and at approximately 50% transmittance, the wavelength is around 360 nm.

Gel Electrophoresis. Native polyacrylamide gel electrophoresis (native PAGE) and SDS−PAGE were performed by use of the PhastSystem from Pharmacia LKB Biotechnology (Uppsala, Sweden) according to the manufacturer's instructions. For native PAGE, approximately 1 μL of each protein solution (5−14.6 pmol/μL) was applied to a continuous 8−25% gradient polyacrylamide gel (PhastGel gradient 8−25 from Pharmacia). Electrophoresis was run at 15 °C for 268 V·h at 10 mA with buffer strips that contain 0.88 M L-alanine and 0.25 M Tris. The protein samples for SDS−PAGE were lyophilized, redissolved in 6 μL of 10 mM Tris-HCl, 1 mM EDTA, 2.5% SDS, and 0.01% bromophenol blue, pH 8.0, with or without 1% 1,4-dithio-DL-threitol and 1% β-mercaptoethanol, and incubated for 1 h at 56 °C. Approximately 4 μL of each redissolved sample was loaded on a homogeneous 7.5% polyacrylamide gel (PhastGel homogeneous 7.5 from Pharmacia) and electrophoresis was run at 15 °C for 60 V·h at 10 mA with buffer strips containing 0.2 M tricine, 0.2 M Tris, and 0.55% SDS, pH 8.1. The gels were stained with Coomassie blue according to the instruction for the PhastSystem.

Calculation of the Isoelectric Point. The isoelectric points (p*I*) of HIV-gp120 and sCD4 were calculated by using the program Biolynx, which is a part of the program package MassLynx (Micromass Ltd., Manchester, U.K.). The calculation is based on the amino acid sequences, using the algorithm generated from Dr. Sergei Levin (Albert Einstein College of Medicine, New York).

MALDI-MS. The MALDI analyses were performed on a Voyager-RP time-of-flight mass spectrometer (Perseptive

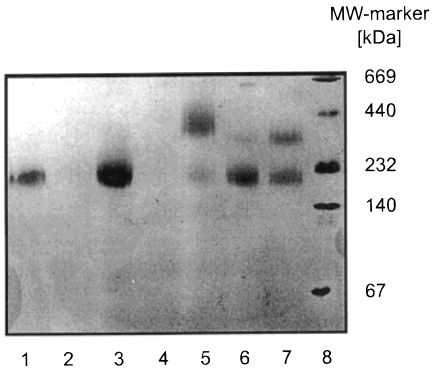

FIGURE 1: Detection of the formation of HIV-gp120/sCD4 complex by native polyacrylamide gel electrophoresis. HIV-gp120 and sCD4 were incubated for 1 h at room temperature at different molar ratios. Approximately 1 μL of each protein solution (5−14.6 pmol/μL) was applied to a continuous 8−25% gradient polyacrylamide gel. Lanes 1−4, controls: lane 1, HIV-gp120 (0.5 μg/μL); lane 2, sCD4 (0.33 μg/μL); lane 3, HIV-gp120 (1 μg/μL); and lane 4, sCD4 (0.66 μg/μL). Lanes 5−7, samples containing HIV-gp120 (0.5 μg/μL) and sCD4 at different molar ratios: lane 5, 1:1.32; lane 6, 1:0.33; and lane 7, 1:0.66. Lane 8, molecular weight reference proteins.

Biosystems, Framingham, MA) in the linear mode with an accelerating voltage of 30 kV and a 1.3 m flight path. A 337 nm nitrogen laser was used to desorb/ionize the samples. For the sample preparation, 0.5 μL of the protein solution (10 pmol/μL) was spotted on the target followed by 0.5 μL of matrix solution and allowed to dry at room temperature (dried-drop method). The following matrix solutions were used for the dried-drop method: ATT (5 mg/mL in 10 mM ammonium acetate, pH 5.5, and in 20 mM diammonium citrate, pH 5.5), CHCA (saturated solution in 45:45:10 ethanol/water/formic acid) and 2′,4′,6′-trihydroxyacetophenone, which was dissolved in ethanol (0.5 M) and mixed with either 0.1 M diammonium citrate, pH 5.0, or 0.1 M ammonium bicarbonate, pH 8.0, at 2:1 (v/v). An alternative to this sample preparation procedure, the thin-layer method, was performed by using saturated CHCA solution in acetone as matrix (*23*). After deposition of the matrix solution (0.5 μL) on the MALDI target, the solution rapidly spread by itself, the acetone evaporated in a few seconds, and a homogeneous surface of very small crystals was formed. Protein solution was then placed onto this matrix surface and the solvent was allowed to dry at room temperature.

RESULTS

Generation of HIV-gp120/sCD4 Complex. The concentration of sCD4 after gel filtration was determined by quantification from its HPLC UV chromatogram. Knowledge of the exact protein concentration allows generation of protein complexes at specific molar ratios of the components, which can provide stoichiometric information. To detect formation of the HIV-gp120 and sCD4 complex under the buffer conditions, the proteins were incubated for 1 h and analyzed by native gel electrophoresis. The polyacrylamide gel after electrophoretic analysis of HIV-gp120, sCD4, and the complex formation generated at different molar ratios of the proteins is shown in Figure 1.

In contrast to sCD4 (lanes 2 and 4), HIV-gp120 (lanes 1 and 3) possesses electrophoretic mobility, which, however, corresponds to an apparent molecular mass of approximately 210 kDa. The electrophoretic analyses of HIV-gp120 after incubation with sCD4 show an additional broad band at approximately 350−400 kDa (lanes 5−7), which demonstrates the formation of the HIV-gp120/sCD4 complex under the conditions used. The intensity of this band increased with higher amounts of sCD4, concomitant with decreasing intensity of the HIV-gp120 band and at a molar ratio of HIV-gp120:sCD4 1:1.3 (lane 5) the HIV-gp120 band had almost disappeared. Native PAGE analyses of HIV-gp120/sCD4 complexes generated with a higher molar excess of sCD4 (performed up to HIV-gp120:sCD4 = 1:8) did not show an additional band of the complex, nor did this affect the shape and position of the complex band at 350−400 kDa (data not shown).

MALDI-MS Analysis of HIV-gp120/sCD4 Complex. For the successful determination of the stoichiometry/molecularity of molecular aggregates by MALDI-MS, disruption of the complex during (a) the incorporation of the analyte into the matrix, (b) transmission from the solid to the gas state, and (c) transfer to the detector should be avoided or minimized. The first studies were carried out, therefore, with 6-aza-2-thiothymine (ATT) in aqueous buffer as the matrix. This matrix has been recently shown to maintain intact protein tertiary structure and to allow successful MALDI-MS analysis of complexes in some cases (*21*). The dried droplet sample procedure and ATT as matrix with either ammonium acetate (pH 5.5) and diammonium citrate (pH 5.5) buffer, however, yielded spectra containing only molecular ions of the isolated HIV-gp120 and sCD4 at very low intensity and signal-to-noise ratio (data not shown). No ion signals from the HIV-gp120/sCD4 complex could be detected by MALDI-MS. In further experiments, 2′,4′,6′-trihydroxyacetophenone was employed as a matrix under physiological buffer conditions. This material has been used successfully for the mass spectrometric analysis of oligonucleotides (*24*). An alternative sample preparation procedure, the thin-layer method, was also investigated. In the thin-layer method, the samples were added to a crystallized matrix surface, which had been laid down by deposition of an acetone solution of CHCA. Neither the MALDI-MS spectra obtained with 2′,4′,6′-trihydroxyacetophenone as matrix nor the thin-layer method contained any molecular signals of HIV-gp120, sCD4, and the complex.

In contrast to these investigations, the MALDI-MS analysis obtained by using the conventional MALDI matrix (CHCA in organic and strongly acid solution) and MALDI sample procedure (dried-droplet) revealed for the first time ion signals that could be assigned to the HIV-gp120/sCD4 complex with a 1:1 stoichiometry (Figure 2). A broad molecular ion $[M + H]^+$ and doubly charged ion $[M + 2H]^{2+}$ were observed in the mass spectrum of HIV-gp120/sCD4 complex with the molecular mass centered at 145 410 Da. The determined molecular mass is in a good agreement with the calculated sum of the molecular masses of one sCD4 molecule (44 650 Da; Figure 3a) and one HIV-gp120 molecule (100 340 Da; Figure 3b) obtained by MALDI-MS as determined from the individual spectra of sCD4 and HIV-gp120. Molecular ions indicating complex formation of higher order could not be observed in the mass spectrum of HIV-gp120/sCD4. Ion signals corresponding to HIV-gp120 and sCD4 are detected, however, showing that partial dissociation of the noncovalent complex of HIV-gp120 and sCD4 has occurred.

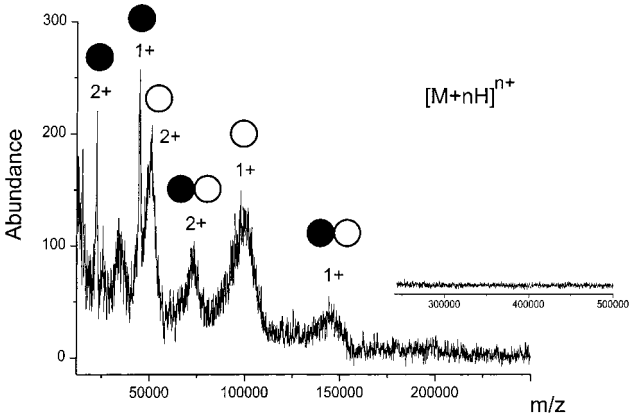

FIGURE 2: MALDI-MS analysis of noncovalent HIV-gp120/sCD4 complex. The spectrum was obtained with a CHCA matrix and shows the singly and doubly charged ions of sCD4 (●), HIV-gp120 (○), and HIV-gp120/sCD4 (joined solid and open circles).

Because it is well-known that during the MALDI procedure unspecific aggregation of simultaneously desorbed molecules can be generated in the gas phase (*25*), control experiments with BSA were performed. The mass spectra of sCD4 and of HIV-gp120 in the presence of BSA, obtained by MALDI-MS analysis under the same conditions and in the same manner as in the analysis of the HIV-gp120/sCD4 complex, are shown in Figure 3, panels c and d, respectively. Both BSA and sCD4 and BSA and HIV-gp120 were detected, respectively, by their singly- and doubly-charged ions. No ions of significant intensity due to nonspecific aggregation of BSA and sCD4, or BSA and HIV-gp120, were observed. Identical results were obtained from a mixture of either HIV-gp120 or sCD4 with carbonic anhydrase (data not shown), indicating that, under the conditions used, no nonspecific complex formation occurs during the MALDI analysis.

Analysis of Cross-Linked HIV-gp120/sCD4 Complex. The SDS−PAGE analysis of the HIV-gp120/sCD4 complex after cross-linking by a 10-fold molar excess of SAED and photolysis is shown in Figure 4 (lane 5). Coomassie staining of the gel visualized a band migrating at 150−160 kDa in addition to the sCD4 band at 45 kDa and the HIV-gp120 band at 120 kDa. Considering the apparent molecular mass of 150−160 kDa as well as the fact that the SDS−PAGE analyses of sCD4 (lane 1), HIV-gp120 (lane 2), the noncovalent HIV-gp120/sCD4 complex (lane 3), and the SAED-derivatized noncovalent HIV-gp120/sCD4 before photoactivitation (data not shown) did not exhibit this band, the 150−160 kDa protein was assigned to the covalent complex of HIV-gp120/sCD4 with a stoichiometry of 1:1. Further confirmation of the assignment was obtained by SDS−PAGE analyses of the SAED-derivatized HIV-gp120/sCD4 complex after photolysis under reducing conditions, which resulted in the cleavage of the disulfide bond of the SAED cross-linker and led to release of the individual proteins (lane 4).

The MALDI mass spectrum of the SAED-derivatized HIV-gp120/sCD4 complex after cross-linking showed ion signals corresponding to SAED-derivatized HIV-gp120 and SAED-derivatized sCD4 (Figure 5). The determined molecular weight of the SAED-HIV-gp120 derivative (104 300 Da) and the SAED-sCD4 derivative (45 410 Da) revealed an average incorporation rate of approximately 10.5 molecules of SAED/HIV-gp120 and 2 molecules of SAED/sCD4 (ΔM 378 amu/SAED). In addition to these ion signals, singly- and doubly-charged molecular ions of a protein with a molecular mass centered at 150 500 Da were observed. The molecular weight of this protein is in a good agreement with the sum of the molecular weights of SAED-derivatized HIV-gp120 and SAED-derivatized sCD4 and was, therefore, assigned to the protein complex with a stoichiometry of 1:1. As in the studies of both covalently bound and noncovalently bound HIV-gp120/sCD4 complexes by SDS−PAGE and MALDI-MS analyses, formation of complexes of higher order than 1:1 were not observed in the MS spectrum of the cross-linked HIV-gp120/sCD4 complex.

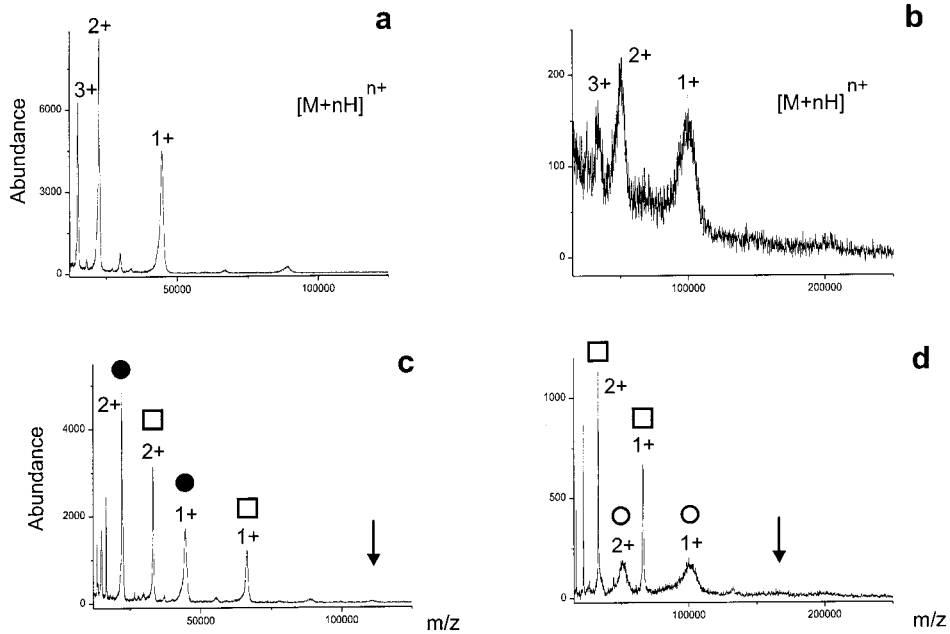

FIGURE 3: MALDI-MS analysis of sCD4 and HIV-gp120 in the presence and absence of BSA. Spectra were obtained with a CHCA matrix, showing singly and multiply charged ions. The arrows indicate the calculated MW of a 1:1 complex of BSA with sCD4 (panel c) or HIV-gp120 (panel d). (a) CD4; (b) HIV-gp120; (c) CD4 (ions are marked with ●) and BSA (□); (d) HIV-gp120 (○) and BSA (□).

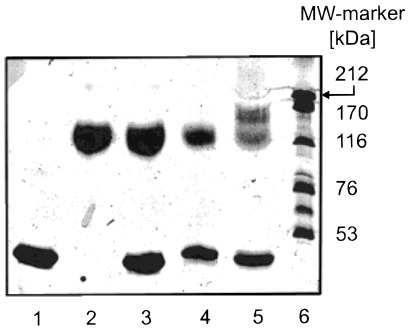

FIGURE 4: SDS−polyacrylamide gel electrophoresis analysis of SAED cross-linked HIV-gp120/sCD4. After incubation of HIV-gp120 and sCD4 at a molar ratio of 1:1.4, the generated complex was cross-linked with a 10-fold molar excess of SAED. The protein samples were lyophilized and redissolved in nonreducing (lanes 1−3, 5, and 6) or reducing SDS sample buffer (lane 4) and loaded on a 7.5% polyacrylamide gel. The samples contained; Lanes 1−3, controls: lane 1, sCD4 (46 pmol); lane 2, HIV-gp120 (33 pmol); lane 3, sCD4 (46 pmol) and HIV-gp120 (33 pmol). Lanes 4 and 5: SAED cross-linked HIV-gp120/sCD4 complex (approximately 50 pmol of total protein) after incubation with reducing (lane 4) or nonreducing sample buffer (lane 5). Lane 6, molecular weight reference proteins.

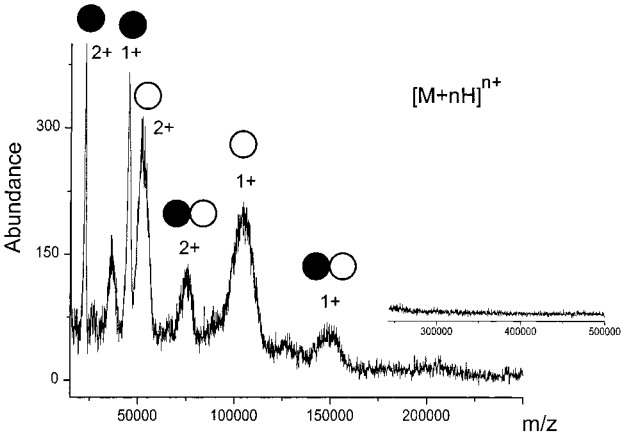

FIGURE 5: MALDI-MS analysis of SAED cross-linked HIV-gp120/sCD4 complex. The spectrum shows the singly and doubly charged ions of SAED-labeled CD4 (●), SAED-labeled HIV-gp120 (○), and SAED cross-linked HIV-gp120/sCD4 (joined solid and open circles).

DISCUSSION

The present study is being performed in order to determine the stoichiometry of the complex between recombinant HIV-gp120 and the complete extracellular part of its primary cellular receptor CD4 (sCD4) by mass spectrometric methods. In contrast to sugar gradient sedimentation, which had been used in a previous investigation to study the stoichiometry of the HIV-gp120/sCD4 complex, the exact molecular weight measurement by mass spectrometry allows the unambiguous determination of the stoichiometry.

To determine the stoichiometry of the HIV-gp120/sCD4 complex by MALDI-MS analysis, it is necessary to choose buffer conditions that maintain the native structure of the protein complex and are compatible with mass spectrometry. Furthermore, to compare the mass spectrometric results with those obtained by cross-linking of the protein complex, the buffer conditions should not interfere with the cross-linker. In this study a cross-linker (SAED) is used that conjugates to the noncovalently bound proteins by reaction with their amino groups. To avoid loss and side reactions of SAED, the buffer should be free of amines. The solution of recombinant, water-soluble CD4 (sCD4), which is originally dissolved in histidine buffer (10 mM), was therefore purified by gel filtration and simultaneously exchanged against a buffer compatible with both SAED cross-linking and mass spectrometry (20 mM phosphate, pH 7.5).

The formation of the complex under these buffer conditions was confirmed by native PAGE, which showed a broad band at approximately 350−400 kDa for the HIV-gp120/sCD4 complex. The electrophoretic mobility of the HIV-gp120 corresponds to an apparent molecular mass of 210 kDa, while sCD4 does not possess any mobility. The slower and nonexistent mobility of HIV-gp120 and sCD4, respectively, can be explained as resulting from their high positive charge, as indicated by their calculated isoelectric points (p*I*) of 9.8 and 9.9, respectively. The p*I*, however, was calculated from the amino acid sequences without any consideration of the tertiary structures and steric shielding effects on the charge state. The apparent p*I* of HIV-gp120 is obviously lower than that calculated, probably caused by shielding of the positive charge by the high content of oligosaccharides. The fact that the HIV-gp120 band almost disappeared at ratio of 1:1.3 for HIV-gp120/sCD4 and the fact that the complex band is not affected by higher molar excess of sCD4 indicate formation of a single class binding complex with equal numbers of HIV-gp120 and sCD4 molecules. Because, however, the electrophoretic mobility does not directly correspond to the molecular mass, but also depends on the charge state and shape of the molecule, the stoichiometry of the complex cannot be unambiguously determined by native PAGE.

To determine the stoichiometry of the HIV-gp120/sCD4 complex by measuring its exact molecular weight by MALDI-MS, several matrixes and sample preparations were investigated. This included the matrixes ATT and 2′,4′,6′-trihydroxyacetophenone in physiological buffer as well as CHCA redissolved in organic and strongly acid solution using the thin-layer and dried-droplet sample procedure. Surprisingly, our study showed that complex formation could only be detected with CHCA by the dried-droplet sample preparation procedure. The failure of the MALDI-MS analysis when the matrixes are used under physiological buffer conditions and the failure of the thin-layer method could be attributed to the intrinsic lower detection sensitivity of very high-mass ions (*20*, *26*). The MALDI-MS analysis with CHCA, however, revealed complex formation between HIV-gp120 and sCD4 at a stoichiometry of 1:1; higher order complex formation could not be detected. The control experiments with BSA and carbonic anhydrase indicated that the determined complex formation is not a result of an unspecific gas-phase aggregation of molecular ions during the MALDI desorption (*25*). Unfortunately, experiments to ascertain the specifity of the complex formation by studying the concentration dependence of the abundance of the complex could not be performed, because the sensitivity of the MALDI-MS analysis is not sufficient to detect molecular ions at concentrations below the dissociation constant of the HIV-gp120/sCD4 complex (1×10^{-9} M) (*27*).

The fact that the MS spectra of the noncovalent HIV-gp120/sCD4 complex showed no significant dependence on the laser penetration depth is further evidence for the

remarkable stability of the complex under the conditions involved in the sample preparation with the acidic CHCA matrix. As the laser is fired repetitively at a given spot, the surface of the matrix is ablated. Thus, as the laser is continuously focused on a spot, later shots penetrate deeper into the crystals. For more acid-sensitive complexes, it has been reported that successful MALDI-MS analysis can be achieved only by using the first few laser shots (*20*, *28*) since it seems that the denaturating conditions at the surface of the matrix crystals are less pronounced than those deeper in the crystals (*19*). The remarkable stability of the HIV-gp120/sCD4 complex may be attributed to the high oligosaccharide content of the carbohydrate, especially from the HIV-gp120, which can protect the complex from dissociation by blocking access of the matrix and solvent to the sites of interaction between the proteins that form the complex.

Even if the noncovalent HIV-gp120/sCD4 higher order complexes could not be detected by MALDI-MS, these complexes may still exist. The well-known suppression effects of the MALDI-MS technique could account for the absence of the noncovalent HIV-gp120/sCD4 higher order complexes. In addition, the higher order forms of the complexes may not have the acidic stability that the 1:1 complex has demonstrated.

Because of the potential limitations of MALDI-MS the mass spectrometrically determined stoichiometry of the noncovalent HIV-gp120/sCD4 complex was compared with that of the covalent bound complex. Therefore, the noncovalent complex was cross-linked and analyzed by SDS−PAGE and MALDI-MS. For cross-linking, the heterobifunctional, cleavable fluorescent cross-linker sulfosuccinimido--2-(7-azido-4-methylcoumarin-3-actamido)ethyl-1,3′-dithiopropinate (SAED) was used. SAED possesses two different reactive groups that allow for sequential conjugation of proteins, minimizing undesirable polymerization or self-conjugation (*29*). The terminal sulfo-*N*-hydroxysuccinimido ester reacts first with amines on the proteins of the noncovalent protein complex, resulting in SAED coupled to the proteins. The photoreactive azide points outward and is available for interaction with the affinity-bound protein. In the second step, UV exposure leads to the conversion of the azido group into a highly reactive nitrene that reacts nonspecifically with the noncovalently bound protein. The noncovalent complex is converted to a covalently bound complex when a nitrene bound to one of the interacting proteins reacts with the second protein. The studies of HIV-gp120/sCD4 complexes after cross-linking by SDS−PAGE and MALDI-MS analysis detected only complex formation of HIV-gp120/sCD4 at a stoichiometry of 1:1. Both techniques do not show any indication of the existence of higher-order complexes. These results are identical to those obtained by MALDI-MS analyses of the noncovalent HIV-gp120/sCD4 complex.

Our studies confirm the previous suggestion of the 1:1 stoichiometry of HIV-gp120/CD4 and indicate that this is more than likely the only complex formed. Furthermore, this study demonstrates that, in suitable circumstances, MALDI-MS can be used to characterize noncovalent complexes containing highly glycosylated proteins.

In addition, region-specific cross-linking of the HIV-gp120/sCD4 complex combined with mass spectrometric analysis of the complex after specific proteolytic digestion could potentially be used for further studies to identify the interacting amino acids of the proteins. By use of these techniques, information on the binding site can be obtained on full-length glycosylated, native proteins, in contrast to the recently reported X-ray crystallographic structure of the complex, which was obtained from a highly deglycosylated and truncated HIV-gp120 derivative (*30*, *31*).

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