Deamidation of Soluble CD4 at Asparagine-52 Results in Reduced Binding Capacity for the HIV-1 Envelope Glycoprotein gp120

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ABSTRACT: High-performance cation-exchange chromatography of recombinant soluble CD4 (rCD4) allowed the resolution of four charge variants. This charge heterogeneity could be eliminated by neuraminidase treatment of rCD4 and therefore can be attributed to different degrees of sialylation of the carbohydrate portion of this glycoprotein. A single acidic variant was observed upon cation-exchange chromatography of neuraminidase-treated rCD4 that had been stored in liquid solution, pH 7.2, at 25 °C for 6 months. This acidic variant was isolated by semipreparative cation-exchange chromatography and subjected to tryptic mapping analysis. Tryptic peptides were characterized by fast atom bombardment mass spectrometry (FABMS). The results of this analysis demonstrated that the acidic variant of neuraminidase-treated rCD4 is generated from deamidation at Asn-52. Digestion of the deamidated rCD4 with endoproteinase Asp-N confirmed Asn-52 as the primary site of deamidation. The ability of the deamidated rCD4 variant to bind gp120 was assessed by use of an ELISA-based binding assay. The binding capacity of the deamidated variant was 24% of the binding capacity of unmodified rCD4. The overall structure of the V1 domain in the deamidated variant was not markedly different from that of the native protein as probed with eight conformationally dependent anti-V1 monoclonal antibodies. Therefore, it appears that Asn-52 is directly involved in binding to gp120.

Acquired immunodeficiency syndrome (AIDS)[1] is caused by a retrovirus referred to as human immunodeficiency virus (HIV-1) (Barre-Sinoussi et al., 1983; Curran et al., 1985; Weiss et al., 1986). In HIV-infected patients, the levels of a subset of peripheral T cells expressing the cell surface glycoprotein CD4 are severely diminished, leading to the onset of AIDS-related symptoms (Curran et al., 1985; Weiss et al., 1986).

In an attempt to design an AIDS therapeutic, a truncated version of the HIV-1 receptor CD4 lacking the transmembrane and cytoplasmic domains was produced by transfecting mammalian cell lines with an expression vector containing cDNA coding for a soluble form of CD4 truncated after Pro-368 (Smith et al., 1987). This soluble form of CD4 and others produced by similar methods blocked HIV infection in vitro (Smith et al., 1987; Traunecker et al., 1988; Fisher et al., 1988; Deen et al., 1988; Hussey et al., 1988) and inhibited cell-to-cell fusion (syncytium formation) (Hussey et al., 1988), a possible precursor to cell death.

CD4 is composed of an extracellular domain containing four regions with homology to immunoglobulin G, a transmembrane domain, and a cytoplasmic domain (Maddon et al., 1985). The soluble version of CD4 has a molecular mass of 45 000 Da. Soluble CD4 contains three disulfide bonds whose arrangements have been characterized in recombinant soluble CD4 (Carr et al., 1989; Harris et al., 1990). The two potential sites for asparagine-linked glycosylation have been found to be glycosylated and to carry sialylated structures (Harris et al., 1990).

The initial event in the infection process involves the interaction of HIV-1 with the CD4 receptor, thereby allowing entry of the virus into the host cell. Evidence for the role of CD4 in mediating HIV-1 infectivity comes from studies in which antibodies to CD4 blocked HIV infectivity (Dalgleish et al., 1984; Klatzman et al., 1984). McDougal et al. (1986a) showed that the major envelope glycoprotein of HIV-1, gp120, forms a specific complex with CD4. The gp120 binding region of CD4 has been localized to the one or two amino-terminal immunoglobulin-like domains of CD4 (Richardson et al., 1988; McDougal et al., 1986b; Traunecker et al., 1988; Chao et al., 1989), since constructs containing just the amino-terminal region were shown to be effective at blocking HIV infectivity in vitro. Using mutational analysis, several groups have shown that a region encompassing residues 40–55 is important in gp120 binding (Peterson & Seed, 1988; Clayton et al., 1988; Arthos et al., 1989; Brodsky et al., 1990; Ashkenazi et al., 1990). In this paper, we report on a charge variant of rCD4 attributable to deamidation at Asn-52. Since Asn-52 is located within the putative binding region, it was of interest to evaluate the capacity of the CD4 variant to bind gp120.

Experimental Procedures (Materials and Methods)

Recombinant soluble human CD4 (rCD4) was purified from cell culture supernatants of CHO cells transfected with an expression vector containing cDNA coding for a form of CD4 truncated after Pro-368 (Smith et al., 1987). Degraded samples were generated by incubating rCD4 in PBS buffer

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[1] Abbreviations: AIDS, acquired immunodeficiency syndrome; BSA, bovine serum albumin; CHO, chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; FABMS, fast atom bombardment mass spectrometry; gp, glycoprotein; HPIEC, high-performance ion-exchange chromatography; HPLC, high-performance liquid chromatography; HIV-1, human immunodeficiency virus; mAbs, monoclonal antibodies; PBS, phosphate-buffered saline; PCMT, protein carboxyl methyltransferase; rCD4, recombinant CD4; rgp, recombinant glycoprotein; SAM, *S*-adenosyl-L-methionine; SP, sulfopropyl; TFA, trifluoroacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; UV, ultraviolet.

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(0.13 M sodium phosphate, pH 7.2, with 0.1 M sodium chloride) at 25 °C for 6 months. Samples were vialed under sterile conditions and not opened until analyzed. Neuraminidase (type X) was purchased from Sigma. Trypsin (TPCK treated) was obtained from Worthington. Endoproteinase Asp-N (sequencing grade) came from Boehringer-Mannheim.

Analytical high-performance ion-exchange chromatography (HPIEC) of rCD4 was carried out on a Hewlett-Packard 1090M system. Recombinant CD4 (100 μg) was loaded onto a Tosohaas SP-5PW column (7.5 mm × 75 mm) at ambient temperature and eluted with a salt gradient of ammonium acetate, pH 8.5, at a flow rate of 0.5 mL/min. The peak response was monitored with a UV photodiode array detector at 280 nm.

Neuraminidase Digestion. Sialic acid was removed from rCD4 by digestion with neuraminidase (0.2 unit/mg of protein) at 37 °C for 16 h. The neuraminidase digest buffer consisted of 0.2 M sodium acetate buffer at pH 5.6 containing 2 mM calcium chloride and 0.02% sodium azide.

Semipreparative scale chromatography was carried out on a Tosohaas 6021 semipreparative HPLC system. Neuraminidase-treated rCD4 (3 mg) was loaded onto a Tosohaas SP-5PW column (21 mm × 150 mm) and eluted with a salt gradient of ammonium acetate, pH 8.5, at a flow rate of 4.0 mL/min.

Reduction, S-Carboxymethylation, and Digestion with Trypsin. Fractions from the semipreparative scale isolation of rCD4 were concentrated to a volume of less than 0.5 mL with Centri-prep 10 concentrators (Amicon). The isolated fractions were exchanged into 8 M urea/0.35 M Tris, pH 8.6, with use of a microdialysis unit (Bethesda Research Labs). Samples were treated with 10 mM dithiothreitol for 30 min at ambient temperature followed by S-carboxymethylation with 35 mM iodoacetic acid for 20 min at ambient temperature. The reduced and S-carboxymethylated protein was dialyzed into 10 mM Tris buffer, pH 8.2, containing 100 mM sodium acetate and 0.1 mM calcium chloride. Fractions were treated with TPCK-treated trypsin (1:50 enzyme to substrate weight ratio) at 37 °C for 4 h. The trypsin-digested protein (40 μg) was loaded onto a Vydac C18 column (4.6 mm × 250 mm) at 40 °C equilibrated with 0.15% aqueous TFA and eluted with a linear gradient from 0 to 50% acetonitrile in 100 min at a flow rate of 1 mL/min. Ultraviolet absorbance was monitored at 214 nm. Collected peptides were identified by amino acid analysis and FABMS.

Amino Acid Analysis. Dried samples were treated with 6 N HCl at 110 °C for 20 h. Amino acid analysis was performed by use of a Beckman 6300 amino acid analyzer.

Fast Atom Bombardment Mass Spectrometry (FABMS). Peptides were dissolved in 1-thioglycerol acidified with formic acid. Spectra were obtained on a JEOL HX110HF/HX110HF instrument with a cesium ion LSIMS source. The primary beam energy was 15 eV with an acceleration of 10 kV.

Endoproteinase Asp-N Digestion. Fractions from the semipreparative scale isolation of rCD4 were concentrated to a volume of less than 0.5 mL and exchanged into 0.13 M sodium phosphate buffer, pH 7.2, containing 0.1 M sodium chloride with use of a Centri-prep 10 concentrator. Fractions were treated with endoproteinase Asp-N (1:50 enzyme to substrate weight ratio) at 37 °C for 4 h. The Asp-N-digested protein (40 μg) was loaded onto a Vydac C18 column (4.6 mm × 250 mm) at 40 °C equilibrated with 0.15% aqueous TFA and eluted with a linear gradient from 0 to 40% acetonitrile in 120 min at a flow rate of 1 mL/min. Ultraviolet absorbance was monitored at 214 nm. The peptides were collected, dried down, and subjected to amino acid analysis.

Methylation Assay. Methylation of rCD4 by protein carboxyl methyltransferase (PCMT) using radiolabeled methyl donor *S*-adenosyl-L-methionine (SAM) was carried out according to the method described by Johnson et al. (1989).

rCD4 Direct-Binding ELISA. Microtiter plates (Nunc) were coated for 12 h at 4–8 °C with 100 μL/well of a 2 μg/mL rgp120 solution in 50 mM carbonate/bicarbonate, pH 9.5. The remaining solution was aspirated, and each well was blocked for 1 h at room temperature with 250 μL of 1% BSA in phosphate-buffered saline (PBS), pH 7.4. The plates were washed three times with PBS containing 0.05% Tween-20 and used immediately.

Test samples of rCD4 were diluted in assay diluent (0.5% BSA–PBS, 0.05% Tween-20) from 800 to 10 ng/mL ($n = 8$), in a separate 96-well tissue culture plate (Falcon). Aliquots (100 μL/well) were transferred to the rgp120-coated plate and incubated at room temperature with agitation for 2 h. The plates were aspirated and washed five times as before. The anti-CD4 monoclonal antibody conjugate (463-HRP) diluted to 0.5 μg/mL in assay diluent was added at 100 μL/well. The plates were incubated for 1 h at room temperature with agitation and washed five times. TMB Microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Inc.), prepared according to manufacturer's protocol, was added (50 μL/well). The color was developed for 20 min, and the substrate reaction was then stopped with 50 μL of 1 N HCl. Absorbance was read at 450 nm on a Molecular Devices UV max automatic plate reader. The data was reduced by use of a four-parameter nonlinear-fit program generating binding concentrations compared to rCD4 stored at −60 °C.

RESULTS

High-Performance Cation-Exchange Chromatography of rCD4. A sample of rCD4 (100 μg) that had been stored at 25 °C for 6 months (see Experimental Procedures) and a sample of rCD4 (100 μg) that had been stored at −60 °C were analyzed by high-performance cation-exchange chromatography with a Tosohaas SP-5PW column (Figure 1). Four peaks were resolved in the rCD4 sample stored at −60 °C (Figure 1a) while eight peaks were resolved in the sample stored at 25 °C for 6 months (Figure 1b). To determine whether some of these peaks were due to sialic acid heterogeneity, samples were treated with neuraminidase. Following neuraminidase treatment, the samples of rCD4 (40 μg) were again analyzed by high-performance cation-exchange chromatography (Figure 2). In the −60 °C rCD4 sample (Figure 2a), one major peak was observed, suggesting that all of the heterogeneity could be attributed to differences in sialic acid content. In the 25 °C rCD4 sample (Figure 2b), one major peak and one acidic minor peak were resolved. The minor peak was approximately 10% of the total by peak area integration. In order to isolate sufficient quantities of the acidic component for characterization studies, a Tosohaas 6021 semipreparative HPLC system and a Tosohaas SP-5PW (21 mm × 150 mm) column were used.

Tryptic Mapping of Isolated Fractions. Tryptic maps of the reduced and S-carboxymethylated ion-exchange fractions are shown in Figure 3. The maps appear similar except for the presence of peak 1 in the digest of the main peak (Figure 3a) and its absence in the digest of the acidic variant (Figure 3b). Peak 1 was identified as Leu-Asn-Asp-Arg (51–54) with an atomic mass of 517.2 (Figure 4, upper panel). The tryptic peptides Asn-Ile-Gln-Gly-Gly-Lys (137–142) and Val-Thr-

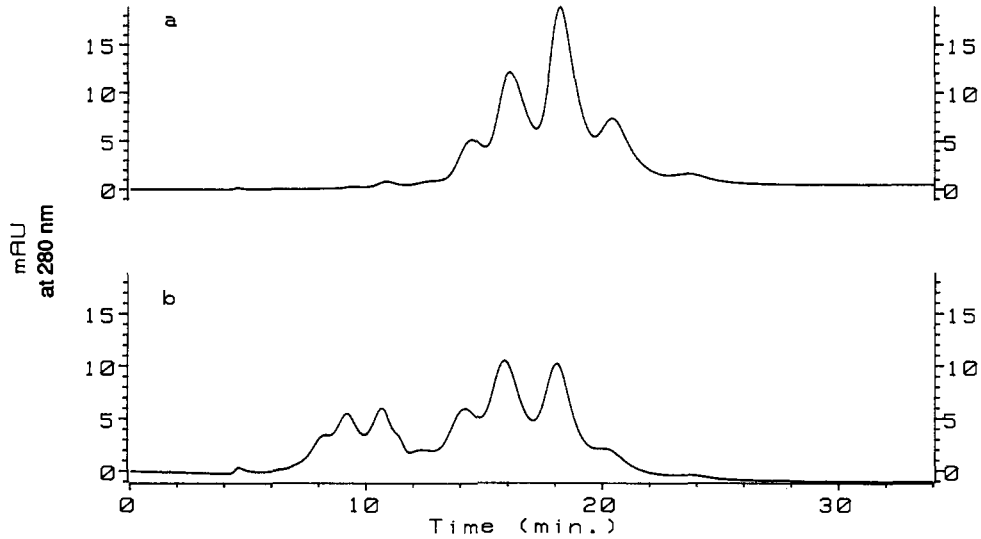

FIGURE 1: High-performance ion-exchange chromatography (HPIEC) of rCD4 stored at −60 °C (a) and 25 °C (b). Samples were analyzed by HPIEC as described in Experimental Procedures. Recombinant CD4 was eluted with a linear gradient from 75 to 280 mM ammonium acetate, pH 8.5, in 40 min.

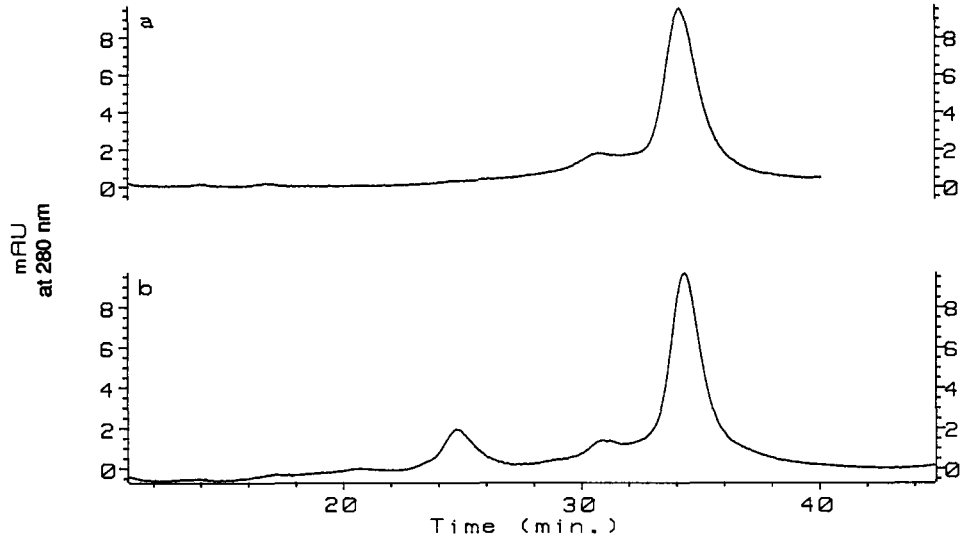

FIGURE 2: High-performance ion-exchange chromatography of neuraminidase-treated rCD4. Samples were treated with neuraminidase prior to analysis by HPIEC. Chromatographic conditions were as described in Experimental Procedures with a linear gradient from 60 to 190 mM ammonium acetate, pH 8.5, in 50 min: (a) rCD4 stored at −60 °C; (b) rCD4 stored at 25 °C for 6 months.

Table I: Amino Acid Composition of Peptides from Endoproteinase Asp-N Maps

residue	84-min peptide	86-min peptide	(10–52)[a]–(80–87)	(10–51)[a]–(80–87)
Asx	5.7	4.7	6	5
Glx	6.7	6.6	7	7
Ser	4.6	4.6	5	5
Gly	2.9	2.9	3	3
His	1.0	1.0	1	1
Arg	0	0	0	0
Thr	4.6	4.6	5	5
Ala	1	1	1	1
Pro	0.9	1.0	1	1
Tyr	0.9	0.8	1	1
Val	1.3	1.4	2	2
Met	0	0	0	0
Cys	1.0	1.0	2	2
Ile	3.2	3.2	4	4
Leu	3.4	3.3	4	4
Phe	2.0	1.9	2	2
Lys	6.0	6.0	6	6

[a] Theoretical values; experimental error ± 5%.

Gln-Asp-Pro-Lys (241–246), from the equivalent of peak 2 (Figure 3a), were previously identified by amino acid analysis in the tryptic map of native rCD4 (Harris et al., 1990). Peak 2, in the tryptic map of the acidic variant, contained Asn-Ile-Gln-Gly-Gly-Lys (137–142) with an atomic mass of 616.3, Val-Thr-Gln-Asp-Pro-Lys (241–246) with an atomic mass of 687.3, and the deamidated peptide Leu-Asp-Asp-Arg (51–54) with an atomic mass of 518.2 (Figure 4, lower panel). The increase of one mass unit (517.2–518.2) is consistent with the conversion of asparagine to aspartic acid at position 52.

Endoproteinase Asp-N Mapping. Endoproteinase Asp-N selectively cleaves at the N-terminal side of aspartate residues (Drapeau, 1980). Digestion of the intact protein with endoproteinase Asp-N was used to confirm Asn-52 as the major site of deamidation in rCD4. Digestion was done without reduction and S-carboxymethylation so that deamidation induced by the reaction conditions would be minimized. The Asp-N maps of the main peak (Figure 5a) and the acidic variant (Figure 5b) appear similar except for the disappearance of a peptide eluting at 84 min in trace a and the appearance of a later eluting peptide at 86 min in trace b. These two peptides were collected and subjected to amino acid analysis. The amino acid compositions (Table I) are consistent with the disulfide-linked peptides shown in Figure 6. The compositions

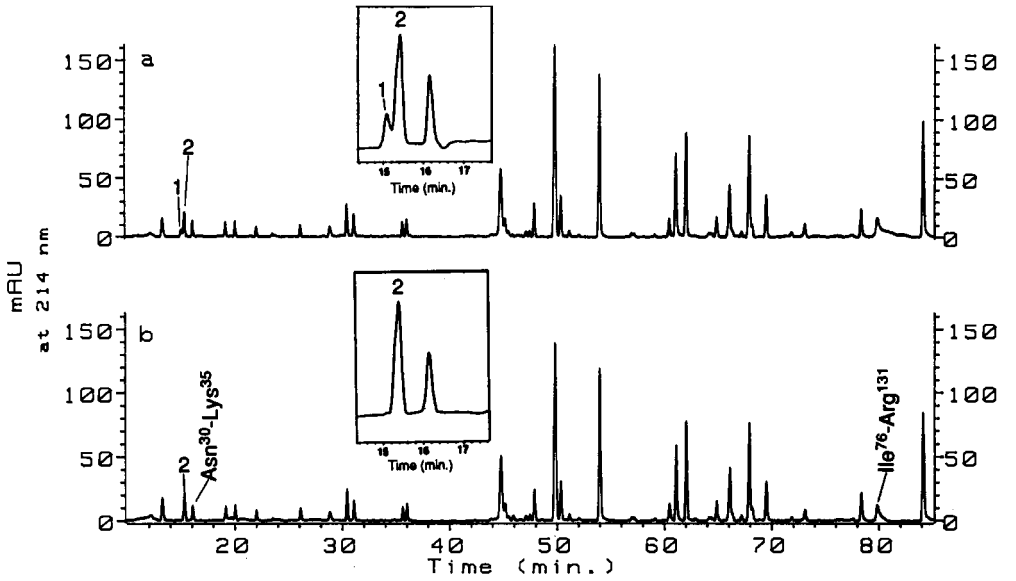

FIGURE 3: Reversed-phase HPLC tryptic maps of reduced and S-carboxymethylated ion-exchange fractions. The main peak and the acidic variant isolated by semipreparative ion-exchange chromatography of a neuraminidase-treated rCD4 sample stored at 25 °C were reduced and carboxymethylated prior to tryptic mapping. The tryptic mapping procedure and reversed-phase HPLC are as described in Experimental Procedures: (a) tryptic map of main peak; (b) tryptic map of acidic variant.

of the 84-min and 86-min peptides were identical except for one less Asx (Asn + Asp) in the 86-min peptide. The 84-min peptide in trace a (main peak map) was identified as 10–52 disulfide linked to 80–87. The 86-min peptide in trace b (acidic variant map) was identified as 10–51 disulfide linked to 80–87. The observation is consistent with the conversion of Asn-52 to Asp-52 in the deamidated variant and the removal of Asp-52 by endoproteinase Asp-N.

Methyl Incorporation of rCD4. A methylation assay was used for assessing the isoaspartyl content of rCD4 samples stored at 25 and −60 °C (Di Donato et al., 1986; Ota et al., 1987; Johnson et al., 1989; Ota & Clarke, 1989). Recombinant CD4 was methylated by protein carboxyl methyltransferase (PCMT) with use of the radiolabeled methyl donor *S*-adenosyl-L-methionine (SAM) as described in Experimental Procedures. The enzyme (PCMT) has been shown to specifically methylate isoaspartyl linkages (Aswad, 1984; Murray & Clarke, 1984). For the −60 °C rCD4, 0.017 mol of isoaspartyl methylation sites per mol of protein was detected. For the 25 °C sample, 0.103 mol of isoaspartyl methylation sites/mol of protein was detected. Assuming a single site of deamidation, 0.103 mol/mol of methyl incorporation for the rCD4 sample stored at 25 °C would correspond to 10.3% deamidation at Asn-52. By comparison, the acidic minor peak (monodesamido at Asn-52) from the HPIEC of the 25 °C sample was 10% of the total by peak area integration.

rCD4 Binding Capacity to gp120. The acidic variant, the main peak fraction, and the unfractionated rCD4 sample stored at −60 °C from HPIEC were tested in an ELISA-based binding assay to determine their binding capacities to rgp120. Figure 7 shows the binding profiles for each sample. There was no significant difference in binding capacity between the main peak and the unfractionated rCD4 sample stored at −60 °C. The main peak was desialylated and the unfractionated rCD4 sample was not suggesting that desialylating rCD4 does not result in diminished binding capacity. There was, however, a 76% reduction in binding capacity for the acidic variant over the linear range of the assay when compared to the main peak.

Discussion

Proteins can be exposed to conditions during the purification process or to improper storage conditions that accelerate the rate of deamidation of asparagine side chains. Deamidation of asparagine residues has been shown to be involved in the inactivation of lysozyme (Ahern & Klibanov, 1985), ribonuclease A (Zale & Klibanov, 1986), and triosephosphate isomerase (Ahern et al., 1987). It has, therefore, become important to develop methods for monitoring levels of deamidation in proteins and to assess the effect of deamidation on biological function.

High-performance ion-exchange chromatography was utilized to detect rCD4 charge variants. The sample was incubated at pH 7.2 and 25 °C to mimic conditions whereby the protein might be deamidated in the field, hence of clinical significance. Except for an acidic variant, all of the charge variants separated by HPIEC could be attributed to sialic acid heterogeneity. The acidic variant was characterized as monodesamido (Asn-52 to Asp-52) by tryptic and endoproteinase Asp-N mapping. It is unlikely that deamidation occurs to any significant extent at secondary sites since no other major differences in the tryptic or endoproteinase Asp-N maps were detected. More extreme conditions such as high pH and elevated temperature would be expected to cause deamidation at secondary sites.

We determined that Asn-52 with a neighboring aspartate is the primary site of deamidation in rCD4. The greatest rates of deamidation in small peptides have been observed when the neighboring amino acid is glycine, serine, or threonine (Bodanszky & Kwei, 1978). There are no Asn-Gly or Asn-Thr sequences in rCD4. There are two Asn-Ser sequences, one occurs at Asn-30 in tryptic peptide residues 30–35 and the other at Asn-103 in tryptic peptide residues 76–131. Both peptides appear in the tryptic maps of the main peak and acidic variant, suggesting that neither of these sites is deamidated (Figure 3). Kossiakoff (1988) observed that certain Asn-Ser sequences in trypsin were more prone to deamidation than others and explained this by the differing bond angles controlling access of the α nitrogen of serine to the β carboxyl of asparagine. Asn-52 is located in a highly exposed and flexible region of the molecule on the basis of the crystal structure of the V1 and V2 domains (Wang et al., 1990; Ryu et al., 1990). The two Asn-Ser sequences may lie in more constrained regions that inhibit cyclic imide formation. Our observation that Asn-52 with a neighboring aspartate de-

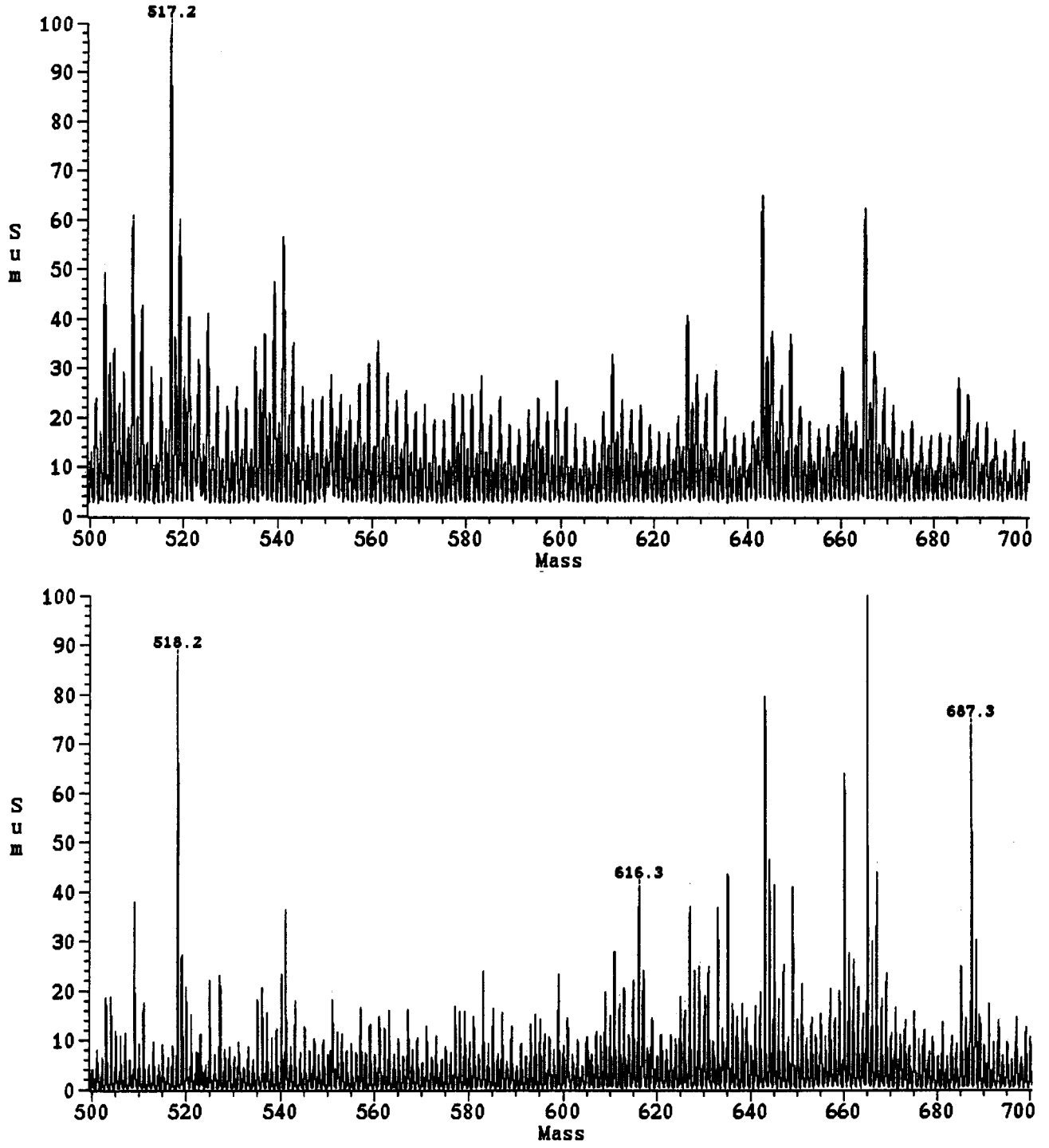

FIGURE 4: FABMS analysis of peak 1 from the tryptic digest of the main peak (upper panel) and peak 2 from the tryptic digest of the acidic variant (lower panel).

amidates more rapidly than either of the Asn-Ser sequences suggests that in rCD4 structure has a major influence on the rate of deamidation.

The generally accepted mechanism of deamidation involves two steps (Geiger & Clarke, 1987). In the first step, regarded as the rate-limiting step, an intramolecular cyclic imide intermediate is formed. The imide is then readily hydrolyzed (fast step), resulting in the substitution of a carboxyl group for the amide group in an asparagine residue. Either an α or β (iso)aspartate will be formed depending upon which bond on either side of the nitrogen atom in the imide ring is broken. In the α form, the β-carboxyl group of aspartate is part of a two-carbon carboxylic acid side chain, whereas in the β form, the β-carboxyl group is part of the polypeptide backbone and the α-carboxyl group is present as an atypical one-carbon carboxylic acid side chain.

We were not able to experimentally determine the ratio of α to β aspartate formed at position 52 since their corresponding tryptic peptides were not resolved. However, we observed a 1:1 correlation between levels of deamidation as determined from HPIEC and by a methylation assay developed for assessing the isoaspartyl content of proteins (Di Donato et al., 1986; Ota et al., 1987; Johnson et al., 1989; Ota & Clarke, 1989). These results suggest that most of the aspartate is in the isoaspartyl form and are consistent with previous studies that have reported the ratio of isoaspartate to aspartate formed during deamidation to be about 4:1 (Murray & Clarke, 1984; Johnson et al., 1989).

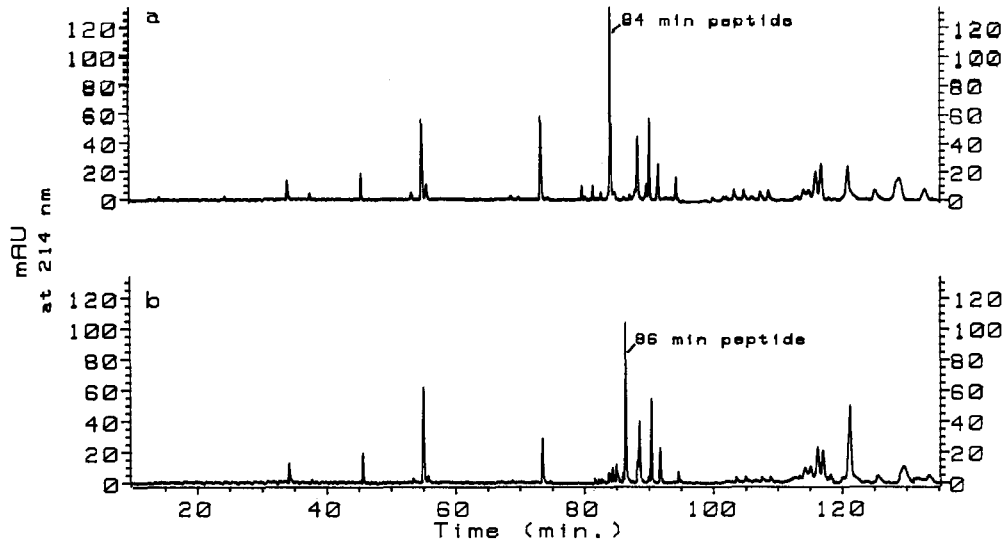

FIGURE 5: Reversed-phase HPLC endoproteinase Asp-N maps of ion-exchange fractions. The main peak and an acidic variant isolated by semipreparative ion-exchange chromatography of a neuraminidase-treated rCD4 sample stored at 25 °C were digested with endoproteinase Asp-N and analyzed by reversed-phase HPLC as described in Experimental Procedures: (a) endoproteinase Asp-N map of main peak; (b) endoproteinase Asp-N map of acidic variant.

84 min peptide: (10-52)-(80-87)

DTVELTCTASQKKSIQFHWKNSNQIKILGNQGSFLTKGPSKLN

DTYICEVE

86 min peptide: (10-51)-(80-87)

DTVELTCTASQKKSIQFHWKNSNQIKILGNQGSFLTKGPSKL

DTYICEVE

FIGURE 6: Primary sequence of 84-min and 86-min peptides from endoproteinase Asp-N maps showing endoproteinase Asp-N cleavage sites. Asp-N peptide sequences were inferred from amino acid composition analysis.

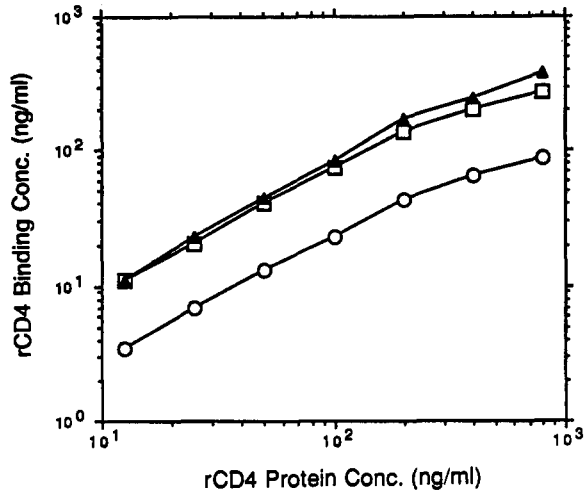

FIGURE 7: rCD4 binding capacity to gp120. Samples were tested in the rCD4/rgp120-binding ELISA as described in Experimental Procedures. Each sample was assayed (n = 4) with seven dilution points in the linear range of the binding curve: (○) acidic variant, (□) main peak, and (▲) rCD4 stored at −60 °C.

Binding of gp120 to the Deamidated Variant of rCD4. Binding of gp120 to the CD4 receptor is the key event in allowing HIV to enter the host cell. The effectiveness of CD4 as a therapeutic agent depends on its ability to bind gp120. Several groups have attempted to delineate the gp120 binding site of CD4. Peterson et al. (1988) identified point mutants with reduced affinity for gp120 over an eight-residue segment (residues 40–47) of V1. Clayton et al. (1988) used site-directed mutagenesis to change one to three amino acid residues in 16 different CD4 regions and determined that as few as three residues (Pro-48, Lys-50, and Leu-51) were critical for binding to gp120. Arthos et al. (1989) identified two CD4 regions as being involved in binding gp120: residues 40–43 and residues 51–55. Substitutions at residues 43 and 55 and insertion of Phe between residues 52 and 53 severely inhibited binding. These studies generally indicate that a region between residues 40–55 of the V1 domain is important for binding to gp120. Recently, it has been reported that the binding site on CD4 is not confined to residues 40–55; several other segments in the V1 domain also appear to be involved in gp120 binding (Brodsky et al., 1990; Ashkenazi et al., 1990).

In this study, we have demonstrated that deamidation at Asn-52 in soluble CD4 leads to a reduction in binding capacity. Asn-52 may be directly involved in gp120 binding or indirectly involved via a larger conformational change in the V1 domain. To distinguish between these possibilities, eight conformationally dependent anti-V1 monoclonal antibodies (mAbs) were used to probe the overall structure of the V1 domain in the deamidated variant and native protein (data not shown). The deamidated variant bound most of the mAbs as well as native rCD4, suggesting that the overall structure was not significantly affected. These results support the direct involvement of Asn-52 in the interaction of CD4 with gp120.

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