Chemical Modification of Recombinant HIV-1 Capsid Protein p24 Leads to the Release of a Hidden Epitope Prior to Changes of the Overall Folding of the Protein†

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ABSTRACT: It was found that the affinity of a monoclonal antibody directed against a recombinantly expressed HIV-1 capsid protein p24 (rp24) strongly increased after chemical modification of the lysine residues of rp24 with different amounts of maleic anhydride. The extent and the sites of modification were analyzed by MALDI-TOF mass spectrometry. Unmodified rp24 and the differently modified rp24 samples were tested for binding the murine monoclonal antibody CB4-1 which recognizes the epitope GATPQDLNTML comprising residues 46−56 of rp24. An increase in the number of modified lysine residues led to enhanced binding affinity of CB4-1. Most pronounced effects were observed after substitution of the first amino groups: an average number of three modified residues per protein molecule increases the binding affinity by a factor of 23, but the substitution of the remaining nine residues increases the binding affinity only by a factor of 11. Fully modified rp24 variant proteins were bound by CB4-1 with K_d values comparable to that of the peptide epitope. Conformation and stability of the unmodified rp24, highly (rp24F, 9 residues; rp24G, 11 residues) modified, and fully modified protein (rp24I, 11 lysine residues and N-terminus) were analyzed by circular dichroism (CD) and fluorescence spectroscopy under different solvent conditions. Little difference in conformation and unfolding behavior was observed between the unmodified and highly modified rp24, which differ drastically in the antibody binding behavior. The fully modified sample, however, displayed a significant decrease in α-helical content. Thus, the epitope seems to be hidden (cryptotope) in the unmodified rp24 in a low-affinity binding conformation and becomes displayed at low levels of chemical modification which obviously induce subtle structural changes prior to changes of the overall folding observable by spectroscopic means.

The inner core of the human immunodeficiency virus is composed of a ribonucleoprotein complex surrounded by a protein shell consisting of the capsid protein p24 (Gelderblom et al., 1987). Up to now, no information about the three-dimensional structure of HIV-1 p24[1] has been available. Argos (1989) proposed an eight-stranded β-barrel model structure of p24 on the basis of its sequence homology to other viral capsid proteins of known structure, whereas a high α-helical content was predicted by Coates et al. (1987) from the amino acid sequence. Recently published CD data of wild-type and recombinant p24 (Ehrlich et al., 1994; Hausdorf et al., 1994; Misselwitz et al., 1995) confirm the prediction results rather than the proposed model. We observed that rp24 can adopt a new conformation at low pH. This acid-induced form is characterized by a reduced but still relatively high α-helical content, a strongly decreased CD in the aromatic region, a red shift of the fluorescence spectrum, and an increased binding of ANS (Misselwitz et al., 1995).

The recombinant p24 molecule used in this study consists of 234 amino acids and differs in the N-terminal region in five positions from the wild-type sequence (Hausdorf et al., 1994). The monoclonal antibody CB4-1 used for the antigen binding studies was raised against recombinant p24−β-galactosidase fusion protein (Grunow et al., 1990). CB4-1 is biologically active, since it is able to inhibit virus spread in cell culture (Franke et al., 1992). Peptide scanning revealed that CB4-1 recognizes the linear peptide epitope

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[1] Abbreviations: HIV-1 rp24, human immunodeficiency virus type 1 recombinant capsid protein p24; LD-TOF mass spectrometry, laser desorption time-of-flight mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MA, maleic anhydride; ELISA, enzyme-linked immunosorbent assay; GdnHCl, guanidine hydrochloride; ANS, 8-anilino-1-naphthalenesulfonate; CD, circular dichroism; $[\Theta]_{222}$, molar mean residue ellipticity at 222 nm; FPLC, fast protein liquid chromatography; K_u, unfolding constant; f_u, fraction of unfolded protein; ΔG_u, unfolding free energy (kJ/mol); $\Delta G_u^{H_2O}$, unfolding free energy in denaturant free buffer (kJ/mol); K_d, dissociation constant; *m*/*z*, mass per charge; PBS, phosphate-buffered saline; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; NHS, *N*-hydroxysuccinimide.

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GATPQDLNTML (Höhne et al., 1993). The antibody binds to rp24 with 2 orders of magnitude less affinity compared to the epitope peptide. On the other hand, the CB4-1 binding affinities to microtiter plate-coated or heat-denatured rp24 were similar to the peptide epitope. A structural model of the interaction of CB4-1 with its peptide epitope was proposed in which the peptide binds in a loop conformation (Höhne et al., 1993).

Association of CB4-1 Fab fragment to unmodified rp24 proceeded in two phases, while dissociation was monoexponential. During the fast phase CB4-1 Fab bound to less than 10% of the antigen. The fast association followed a first-order rate equation, while the slow phase could not be described by simple kinetic models. These unusual kinetics were studied on the surface of biosensor chips as well as in solution (Glaser & Hausdorf, 1996). It was assumed that at least two conformational forms of rp24 might exist, only one of them binding CB4-1 with high affinity. The slow phase of binding was interpreted as a conformational change of rp24 to the high-affinity form, in which it was trapped by binding to the antibody.

Here we investigate in detail the observation that a high affinity of rp24 to antibody CB4-1 can be induced by covalent modification of this protein by maleic anhydride. Chemical modification of rp24 was originally initiated as an attempt to map the CB4-1/rp24 interaction site by reaction of rp24 lysine residues with maleic anhydride in the presence and absence of antibody. It turned out that maleylation is connected with a strong increase of antibody CB4-1 binding. Thus we became interested in the effects of maleylation on the structure of rp24 and have therefore studied the antibody binding behavior, conformation, and stability of rp24 samples which were chemically modified to different extents by reaction of maleic anhydride with the lysine residues of rp24. LD-TOF mass spectrometry was used to identify the modification sites. Binding affinities of the modified rp24 variants were analyzed by competitive ELISA experiments. The conformation and stability of modified rp24 samples were investigated by circular dichroism and fluorescence measurements and compared to unmodified rp24.

MATERIALS AND METHODS

Chemicals. Ultrapure guanidine hydrochloride (GdnHCl) was from ICN Biomedicals (Cleveland, OH) and was used without further purification. Sodium acetate and sodium phosphate were purchased from E. Merck (Darmstadt, Germany). 8-Anilino-1-naphthalenesulfonate (ANS) was from Serva (Heidelberg, Germany).

Protein Preparation. Recombinant HIV-1 rp24 was expressed in *Escherichia coli* LE392 and formed inclusion bodies. Isolation and purification of HIV-1 rp24 were described recently (Hausdorf et al., 1994).

Modification Procedure. Maleylation of lysine and N-terminal amino groups was essentially performed as described (Habeeb & Atassi, 1970). Maleic anhydride (MA), analytical grade (Fluka, Buchs, Switzerland) was added at various concentrations (0.1−50 mol/mol of amino groups) to 100 μL of 40 μM (1.0 mg/mL) rp24 in 50 mM phosphate buffer. Reactions were carried out at pH 8.3 for 30 min at 25 °C.

Mass Spectrometry. Mass spectra were obtained with a laser desorption time-of-flight spectrometer (LaserTec Bench-Top II System, PerSeptive Biosystems, Vestec, Houston, TX) at an accelerating voltage of 25 kV. For calibration insulin (MW 5733.5 $[M + H]^+$, $m/z = 5734.5$, and $[M + 2H]^{2+}$, $m/z = 2867.75$) was used as an external standard. Samples to be analyzed (complete modified rp24 and tryptic digests) were mixed 1:1 (v:v) with a saturated solution of α-cyano-4-hydroxycinnamic acid (Aldrich, Gillingham, England) in 50% acetonitrile/0.05% trifluoroacetic acid in water. 2 μL of this mixture was applied to the 2 mm diameter stainless steel probe tip and air-dried at room temperature. The peptide matrix deposit was washed with deionized water to remove residual buffer salts and redried (Beavis & Chait, 1990).

Tryptic Peptide Mapping Analysis. Disulfide bridges of rp24 derivatives (10 μg each) were cleaved for 30 min at 25 °C by addition of 3 μL of dithiothreitol at 10 mg/mL in 50 mM phosphate buffer, pH 8.3. Proteolytic digestion was subsequently performed for 2 h at 37 °C by addition of 2 μL of L-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, Diesenhofen, Germany; 0.5 mg/mL) in 50 mM phosphate buffer, pH 8.3. The digestion was stopped by 1.5 μL of 5% trifluoroacetic acid prior to mass spectrometric analysis.

Competitive ELISA. The modified proteins were tested for their ability to compete with the CB4-1/rp24 interaction using competitive ELISA. The 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 0.1 μg/mL HIV-1 recombinant p24 (Hausdorf et al., 1994) in 0.1 M sodium carbonate buffer, pH 9.6, and incubated for 20 h at 4 °C. After being washed three times with PBS/0.1% Tween-20, 0.05 μg of horseradish peroxidase-labeled anti-p24 antibody CB4-1/mL was added, with the modified protein in decreasing quantities, starting with 40 μM in PBS/0.1% Tween-20 containing 6% Gelifundol S (Biotest, Dreieich, Germany) in a total volume of 50 μL for 2 h at 25 °C. After being washed three times with PBS, the bound enzymatic activity was determined by adding 5.5 mM *o*-phenylenediamine hydrochloride (Fluka, Buchs, Switzerland) and 8.5 mM H_2O_2 in 0.1 M citrate buffer, pH 5.0. The reaction was terminated after 10 min by adding 1 M sulfuric acid containing 0.05 M sodium sulfite. The absorbance was measured at 492 and 620 nm, using an ELISA reader (Anthos, Köln, Germany). Dissociation constants (K_d) were determined according to Friguet et al. (1985).

Biomolecular Interaction Analysis. Real-time interaction measurements were performed with a BIAlite (Pharmacia Biosensor AB, Uppsala, Sweden). Modified and unmodified rp24 were coupled to CM5 sensor chips (Pharmacia Biosensor AB) after EDC/NHS chemical modification as described by Johnsson et al. (1991). Measurements were done in 10 mM HEPES buffer, pH 7.4, with 150 mM NaCl, 3.4 mM EDTA, and 0.005% v/v surfactant P20 (HBS). To resolve the fast phase of binding, measurements were made at flow rates up to 50 μL/min and with a sampling rate of 10 s^{-1}. Binding and dissociation curves were analyzed with the BIAevalution 2.0 software package (Pharmacia Biosensor AB).

Protein Concentration Determination. Before spectroscopic measurements the modified proteins were exhaustively dialyzed against the appropriate buffers. The buffer solutions used were 50 mM sodium phosphate, pH 8.3; 20 mM and 5 mM sodium phosphate, pH 2.0; and 20 mM sodium acetate, pH 5.8. It is difficult to determine protein concentrations of modified proteins because of the strong absorbance of the substituent in the aromatic region. Therefore, the

concentrations were determined from amino acid analyses after hydrolysis in the gas phase with 6 N HCl for 24 h according to Meltzer et al. (1987).

FPLC Measurements. Gel exclusion chromatography measurements were performed by FPLC (Pharmacia) using a calibrated Superose 12 HR 10/30 column equilibrated with 50 mM sodium phosphate buffer, pH 8.3 at 25 °C. The concentration of the injected samples was about 1 mg/mL.

Circular Dichroism. CD measurements were performed with a Jasco J-720 spectropolarimeter at 25 °C as described recently (Misselwitz et al., 1995). Mean residue ellipticities [Θ] were calculated using a mean residue weight of 110.5 for each of the studied HIV-1 rp24 samples. Far-UV spectra were measured with a cell of 0.010 cm path length and a protein concentration of 0.90 mg/mL or with a 0.10 cm cell and a protein concentration of 0.080 mg/mL in the case of GdnHCl-induced unfolding experiments. CD spectra in the near-UV region were registered in a 1.0 cm cell at a protein concentration of 0.90 mg/mL.

Fluorescence Measurements. Fluorescence spectra were measured at 25 °C with Shimadzu fluorimeter RF-5001PC. Tryptophan fluorescence spectra and ANS fluorescence spectra were measured with excitation wavelengths of 295 and 400 nm, respectively. All measurements were performed at 5 nm bandwidths for both the excitation and emission monochromator. The absorbance of all samples was adjusted to be less than 0.1 absorbance units at the excitation wavelength. Fluorescence spectra were buffer and denaturant corrected and normalized to an identical protein concentration and to the Raman band of water, which was used as an internal intensity standard.

ANS Binding. Aliquots of modified HIV-1 rp24 stock solutions in 50 mM sodium phosphate buffer, pH 8.3, or 5 mM sodium phosphate, pH 2.0, were mixed with aliquots of an ANS stock solution, yielding samples of 1 μM protein and 20 μM ANS. The concentration of ANS stock solution in methanol was determined using a molar extinction coefficient of 6.8×10^3 M^{-1} cm^{-1} at 370 nm (Mann & Matthews, 1993). The mixtures were incubated 2 h before the registration of fluorescence spectra.

Equilibrium Unfolding and Refolding Studies. GdnHCl-dependent transitions of the conformation were monitored measuring changes of the circular dichroism at 222 nm. Concentrations of GdnHCl stock solutions in the appropriate buffer were determined from refractive index measurements (Pace et al., 1989). For unfolding experiments aliquots of protein stock solution were mixed with aliquots of denaturant stock solution and buffer. The protein concentration was adjusted to 0.080 mg/mL. All samples were incubated overnight at room temperature and were measured at 25 °C.

Analysis of Unfolding Curves. The fraction of the unfolded protein f_u was calculated as described by Pace (1986). Assuming a "two-state" transition of unfolding, we calculate the equilibrium constant K_u between the folded and unfolded states and the free energy change ΔG_u from the following equations:

$$K_u = f_u/(1 - f_u) = (F_n - F)/(F - F_u) \quad (1)$$

$$\Delta G_u = -RT \ln K_u \quad (2)$$

where F is the observed ellipticity at 222 nm, F_n and F_u are the corresponding values of the folded (n) and unfolded (u) forms, R is the gas constant, and T is the absolute temperature. The base lines in the pre- and post-transition regions were corrected by linear extrapolation to obtain values of F_n and F_u in the transition region (Pace, 1986). The free energy change of unfolding $\Delta G_u^{H_2O}$ in the absence of denaturant was determined by a linear least-squares analysis according to the following equation:

$$\Delta G_u = \Delta G_u^{H_2O} - m[\text{denaturant}] \quad (3)$$

where ΔG_u is the free energy change of unfolding and m is the slope of the plot of ΔG_u versus denaturant concentration. Only ΔG_u values from the linear range of the transition region were taken for the extrapolation to $\Delta G_u^{H_2O}$.

RESULTS

Maleylation of rp24. After modification reactions of rp24 with up to 50-fold molar excess of maleic anhydride per mole of amino groups the extent of maleylation was determined by direct MALDI-TOF-MS analysis. A gradual molecular mass increase (98 atomic mass units per MA residue) at increasing reagent concentrations was found. A maximum uptake of roughly 12 maleic groups (11 lysine residues and the N-terminus) was observed at 16-fold and higher molar ratios of reagent per mole of amino groups (Figure 1A). Unmodified and all modified rp24 samples were then tested for antibody binding by competitive ELISA studies with solid phase-adsorbed rp24. Dissociation constants for each variant were determined as described (Friguet et al., 1985; Volkmer-Engert et al., 1994). A continuous decrease in the dissociation constants depending on reagent concentration was observed (Figure 1B). Thus, the chemical modification of the lysine residues led to enhanced binding to the monoclonal antibody CB4-1. A low degree of modification (1−2 out of 12 amino groups) already caused a decrease of the K_d value by 1 order of magnitude. The uptake of more than seven molecules of maleic acid per protein molecule (modification extent > 70%) led to a K_d value comparable to that of the free linear epitope peptide GATPQDLNTML ($K_d = 1.5 \times 10^{-8}$ M) (Höhne et al., 1993).

MALDI-TOF Analysis of Maleylated rp24 Samples. In order to identify the maleylation sites of the differently modified rp24 samples we subjected the probes to proteolysis by trypsin with subsequent analysis of the fragments by MALDI-TOF mass spectrometry (Figure 2 and summarized in Table 1). As rp24 contains 11 Lys and 11 Arg residues, a complete tryptic digest should yield 23 peptide fragments of different molecular weight. However, up to 276 theoretical fragments from 174 (Arg 132) to 25 864 dalton (uncleaved protein) are possible in the case of the limited tryptic digest. The unmodified and modified derivatives of rp24 were subjected to peptide mapping analysis as shown in Figure 2 for protein samples containing zero (rp24), one (rp24A), four (rp24D), nine (rp24F), and twelve (rp24I) maleyl groups on average. Molecular ions of most relevant tryptic peptides were found, which enabled the direct identification of several maleylation sites (see Table 1).

In comparison to the spectrum of the unmodified rp24 (Figure 2, rp24) the spectrum of monomaleylated rp24A (Figure 2, rp24A) showed an additional $[M + H]^+$ ion at m/z 2439 due to initial maleylation at the N-terminus, which can be considered the most reactive amino group present (Suckau et al., 1992). In the further course of maleylation the small signal of the $[M + H]^+$ ion at m/z 1423 became a main peak. This indicates that the lysine 140 in fragment

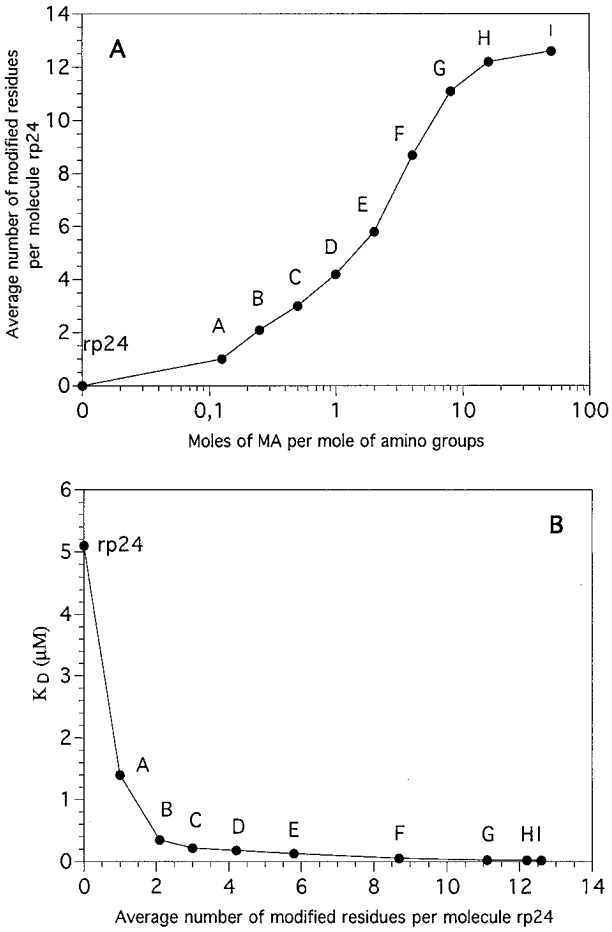

FIGURE 1: (A) Average number of modified lysine residues in rp24 depending on the molar ratio of maleic anhydride (MA) to amino groups as determined by MALDI-TOF mass spectrometry. One mole of amino groups (11 Lys residues and one N-terminal amino group) corresponds to 1/12 mol of rp24. The average number of modified residues is calculated from the mass increase of the molecular ion peak. (B) CB4-1/rp24 dissociation constants of unmodified and modified rp24 determined by competitive ELISA experiments with solid-phase-adsorbed rp24 (rp24, 5.1×10^{-6} M; rp24A, 1.4×10^{-6} M; rp24B, 3.5×10^{-7} M; rp24C, 2.2×10^{-7} M; rp24D, 1.8×10^{-7} M; rp24E, 1.3×10^{-7} M; rp24F, 5.5×10^{-8} M; rp24G, 3.0×10^{-8} M; rp24H, 2.5×10^{-8} M; rp24I, 2.0×10^{-8} M).

133−143 was modified next. In the spectrum of unmodified rp24 the corresponding signal at *m*/*z* 1321 could not be detected since this fragment is easily cleaved into two smaller peptides at *m*/*z* 956 and *m*/*z* 386 due to the unmasked cleavage site at lysine 140. The signal at *m*/*z* 831 (fragment 19−25) could only be detected for the unmodified rp24 (Figure 2). Low concentrations of modifier already made it disappear, indicating modification of lysine 25 at the C-terminus of this fragment masking this potential cleavage site.

With proceeding maleylation (Figure 2, rp24D and rp24F), the intensity of the $[M + H]^+$ ion at *m*/*z* 4235 diminished. Since the peptide fragment 31−70 is formed by cleavage at lysines 30 and 70, maleylation of one or both of these residues caused the decrease in signal intensity. It was not possible to distinguish between modification of lysines 30 and 70. The partial maleylation of lysine 158 in the rp24D sample (Figure 2) is evident from the appearance of the modified peptide ion at *m*/*z* 2143 as well as from the abundant unmodified peptide 155−173 at *m*/*z* 2045. Molar ratios of at least 1 equiv of maleic anhydride per amino function were needed for the modification of Lys 158. At a 4-fold excess of maleic anhydride (rp24F) the ion intensity of the lysine 131-maleylated peptide at *m*/*z* 3573 increased while the ions of the unmodified peptide at *m*/*z* 3475 diminished. Maleylation at Lys 170 was identified by the ions at *m*/*z* 925 and *m*/*z* 1574 (rp24F, I). At high degrees of modification (rp24I), even 2-fold maleylation of rp24 (155−182) could be observed at *m*/*z* 2612, indicating that both Lys 158 and Lys 170 were modified.

The results of mass spectrometric peptide mapping describing the relative chemical reactivities of the lysine residues in rp24 are summarized in Table 1. The complete evaluation of all mass spectra revealed that lysine 203 and lysine 227 appeared to be the least reactive. No information could be obtained for Lys 182 and Lys 199 as they were only detected within very large fragments also containing other lysine residues.

BIAlite Measurements. Sample rp24F with an average of nine modified lysine residues was coupled to a sensor chip, and the association of CB4-1 Fab fragment at concentrations between 0.01 and 2 mg/mL as well as the dissociation in buffer flow was studied with real-time interaction analysis. The kinetics were compared with those for unmodified rp24. The total binding capacity of the antigen on the sensor chips was estimated by association of the 1 mg/mL CB4-1 Fab solution for 20 min (flow: 1 μL/min). It was found that about half of the rp24 molecules could bind CB4-1 Fab. This value is in the expected range; the covalent coupling procedure always destroys the epitope in a part of the ligand molecules.

For both modified and unmodified rp24 two association phases could be distinguished at CB4-1 Fab concentrations above 100 μg/mL. However, in the modified rp24F sample 52% of the total available epitopes bound CB4-1 Fab during the fast phase compared to 8% in the unmodified rp24 sample (Glaser & Hausdorf, 1996). The association rate constant for the fast phase was about 4×10^5 M^{-1} s^{-1} at 33 °C, and it was not significantly different in both samples. Dissociation was monoexponential with less than 5% offset and approximately the same (0.026 s^{-1} at 33 °C) rate in both samples. A dissociation constant of 6×10^{-8} M for the high-affinity form of rp24 results from these kinetic constants. The apparent association rate constant of the slow phase of binding was slightly increased in modified rp24F.

Analytical Gel Chromatography. HIV-1 rp24 samples have a pronounced tendency to form associates (Rosé et al., 1992; Ehrlich et al., 1992, 1994; Hausdorf et al., unpublished results). To prove the effect of maleylation on the association behavior we have analyzed unmodified rp24 and two modified HIV-1 rp24 preparations (rp24F and rp24I) by analytical gel chromatography (data not shown). Each of the three protein samples elutes in a single peak. The elution volumes are between those expected for dimers and monomers. The modified samples elute earlier than the unmodified rp24, and the smallest elution volume was found for the completely modified rp24I. The shift to smaller elution volumes observed with increasing extent of modification may correspond to an increase of the hydrodynamic radius due to an increase of the molar mass, to a partial unfolding of the protein as a consequence of the modification, to a larger water shell, or to changes in the interaction with the matrix of the column. Whatever the observed differences might explain, there are two relevant facts, namely, at first the flat base line of the elution curves in front of the peaks shows that the samples of modified and unmodified rp24 are free

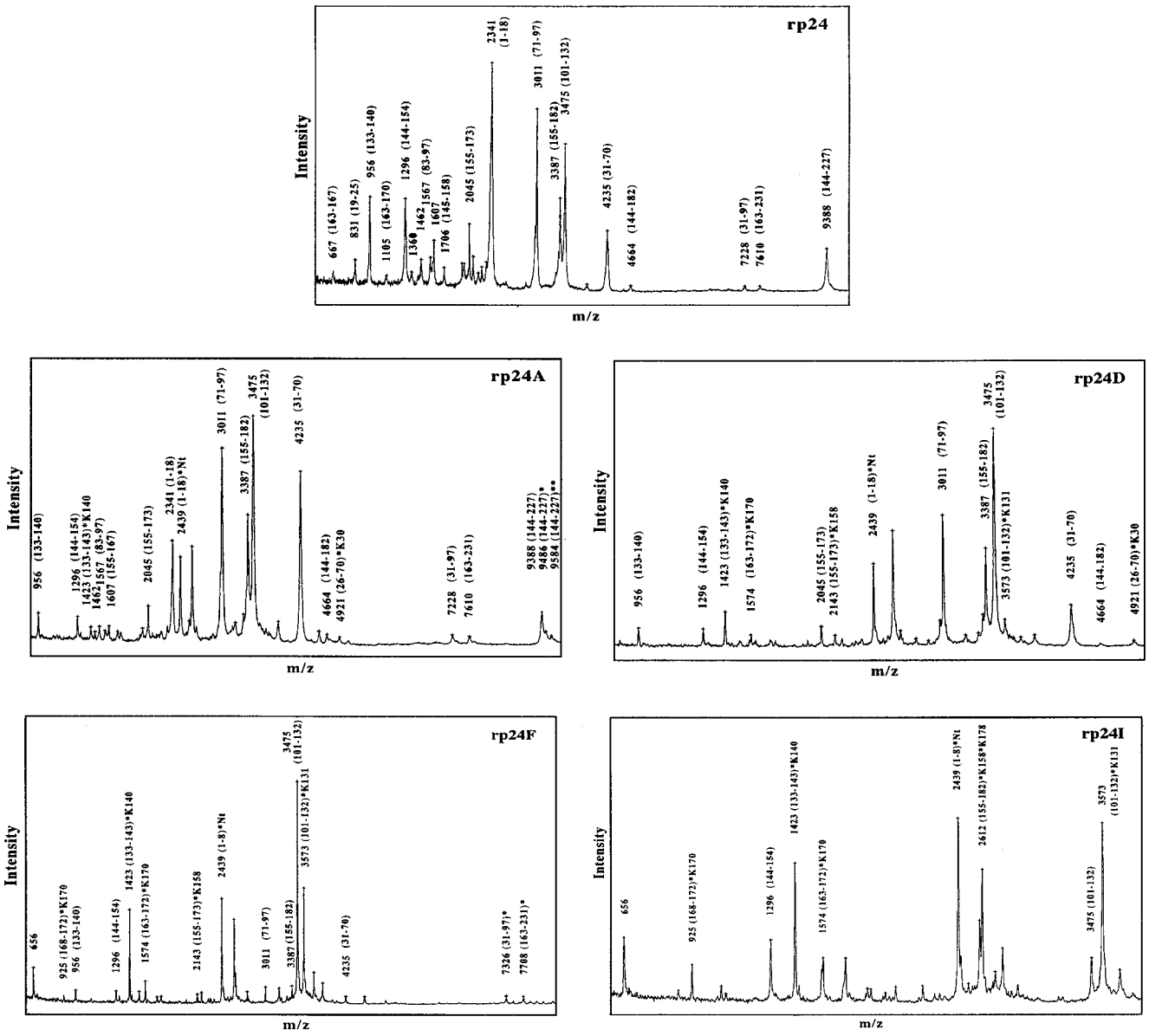

FIGURE 2: MALDI-MS peptide mapping analysis of unmodified rp24 and modified rp24 samples rp24A, rp24D, rp24F, and rp24I. Numbers denote molecular weights ($[M + H]^+$ ions) and sequences (in parentheses) of tryptic peptides. Maleylated lysine (K) residues are marked with asterisks.

Table 1: Relative Reactivities of Lysine Residues in rp24 Derived from MALDI-MS Mapping Data

identified peptide fragment	*m/z*	residue	relative reactivity
[MA-Met[1]] rp24-(1−18)	2439	N-terminus	1
[MA-Lys[140]] rp24-(133−143)	1423	Lys 140	2
rp24-(19−25)	831	Lys 25	2
rp24-(31−70)	4235	Lys 30/70	3
[MA-Lys[158]] rp24-(155−173)	2143	Lys 158	4
[MA-Lys[131]] rp24-(101−132)	3573	Lys 131	5
[MA-Lys[170]] rp24-(168−172)	925	Lys 170	6
[MA-Lys[170]] rp24-(163−172)	1574	Lys 170	6
[MA_2-Lys[158]-Lys[170]] rp24-(155−182)	2612	Lys 158, Lys 170	7

of larger aggregates, and second, there are no indications for a shift from dimers to monomers in the elution behavior of the modified samples.

Circular Dichroic and Fluorescence Measurements of Maleylated rp24. Circular dichroism and fluorescence spectroscopy are sensitive tools to monitor conformation and conformational changes of proteins. In order to characterize the effects of maleic acid modifications of the ϵ-amino groups of Lys residues and of the N-terminus on the secondary structure of HIV-1 rp24, CD spectra of unmodified rp24 and of rp24F, rp24G, and rp24I with 9, 11, and all 12 amino groups substituted, respectively, are registered in the far-UV region (Figure 3A). The spectrum of rp24 in the presence of 5 M GdnHCl (spectrum 4) is shown as a reference. This spectrum clearly demonstrates unfolding of the protein under these conditions, while the spectra of native rp24 and of the modified rp24 forms are typical for α-helical structures. At an extent of modification as high as 9 substituted amino groups out of 12 per molecule of rp24 the CD spectrum (spectrum 2) is practically identical with that

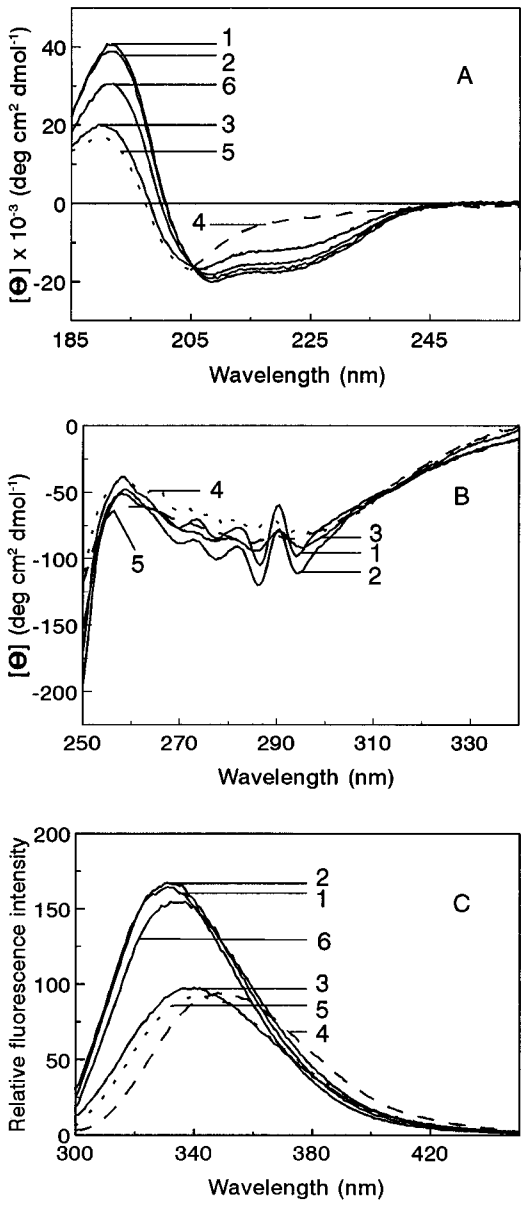

FIGURE 3: Spectroscopic characterization of unmodified and modified HIV-1 rp24. Far-UV CD spectra (A), near-UV CD spectra (B), and fluorescence spectra (C) were recorded in 50 mM sodium phosphate, pH 8.3 at 25 °C. rp24 (1); rp24F (2); rp24G (6); rp24I (3). Additionally are shown the spectra of rp24 in the presence of 5 M GdnHCl (4) and the spectra of the acid form of rp24 in 20 mM sodium phosphate, pH 2.0, (5). CD and fluorescence measurements were performed at protein concentrations of 0.90 and of 0.090 mg/mL, respectively. The excitation wavelength was 295 nm.

of the unmodified protein (spectrum 1). Only when 11 or 12 amino groups are modified are the ellipticities at 222 nm and 190 nm reduced and the zero cross points shifted to shorter wavelengths (spectra 6 and 3, respectively). Thus the spectra of rp24G and rp24I indicate a decrease of the α-helical content in comparison to rp24 and rp24F. This is quantified by a calculation of the content of secondary structures using the program VARSCL1 (Table 2). The calculated secondary structure content of rp24 at pH 8.3 (54% α-helix, 5% β-sheet, and 20% turn) corresponds satisfactorily with recently published experimental data (Hausdorf et al., 1994; Misselwitz et al., 1995) and with results of secondary structure predictions calculated from the amino acid sequence (Coates et al., 1987; Hausdorf et al., 1994).

The CD spectra in the near-UV region of the native rp24, partially modified rp24F, and fully modified rp24I are shown in Figure 3B. The peaks above 270 nm can be attributed to Trp and Tyr residues (Strickland et al., 1972). There are small quantitative differences between the spectra of the native rp24 and the low modified form, but both spectra are characterized by a pronounced and comparable fine structure in the Trp and Tyr region. These features are consistent with similar surroundings of the aromatic amino acids in unmodified rp24 and in partially modified rp24F. The spectrum of the fully modified rp24I has a reduced negative ellipticity and a less pronounced fine structure. These spectral differences show for rp24I changes in the aromatic amino acids surroundings and a loss of asymmetry of their electronic configurations. As references are shown the CD spectra of the unmodified rp24 in the presence of a high GdnHCl concentration of 5.0 M and at a low pH value of 2.0. The broad and featureless spectra observed in 5 M GdnHCl and at pH 2.0 are typical for proteins with a flexible and disordered tertiary structure (Strickland, 1974). The remaining spectral features may partly be attributed to effects of the disulfide bond present in HIV-1 rp24 (Strickland, 1974).

HIV-1 rp24 contains five tryptophan residues at positions 26, 83, 120, 136, and 187. Figure 3C shows the fluorescence spectra of rp24, rp24F, rp24G, and rp24I excited at 295 nm and pH 8.3. The position of the fluorescence maximum (330 nm) at pH 8.3 in the spectra of rp24 and rp24F (spectra 1 and 2) indicates a predominantly hydrophobic environment of the tryptophan residues in these protein forms (Burstein et al., 1973). Also the fluorescence intensities of unmodified rp24 and of rp24F are very similar. The fluorescence spectrum of rp24G, however, shows a red shift of about 5 nm and a reduced fluorescence intensity (spectrum 6). Complete modification of the amino groups results in a further decrease of the fluorescence quantum yield and in a red shift of the fluorescence maximum to 341 nm as shown by the spectrum of rp24I (spectrum 3). This red shift reflects an increased accessibility of the tryptophan residues to the solvent (Burstein et al., 1973). The spectral features are similar to those of rp24 under acidic conditions (spectrum 5), but in the highly modified form at pH 8.3 and in the acid-induced form of unmodified HIV-1 rp24 the tryptophan side chains are still partially shielded in comparison to the GdnHCl-unfolded state (Table 2).

Acid-Induced Conformation of rp24. Recently, we described for the unmodified rp24 a conformational change at pH values below 2.5, which is characterized by a decrease of the ellipticities at 222 and 190 nm and an isodichroitic point at 206 nm (Misselwitz et al., 1995). Very similar acid-induced conformational changes were found for the modified protein rp24F. At pH 2.0, the spectral features of the partially modified form rp24F are similar to those of the unmodified protein whereas the spectrum of the fully modified rp24I differs to a certain extent (data not shown). According to the CD spectra at pH 2, rp24I assumes a conformation with a significantly increased β-structure content in comparison to unmodified rp24 (see Table 2).

Raising the pH again to 8.3, we note that the observed spectral changes are completely reversible both for the partially and for the completely modified samples. The CD spectrum of rp24I at pH 8.3 resembles that of the acid-induced form of unmodified rp24 measured at pH 2.0 (compare spectra 3 and 5 in Figure 3A). Nevertheless, the conformations of these two protein forms are not identical as shown by small spectral differences and different ANS binding (see below).

Table 2: Conformational Properties of rp24 and Modified rp24 Forms

		CD spectra[a]		tryptophan fluorescence[b]		
protein	conditions	α-helix (%)	β-sheet (%)	emission maximum (nm)	relative intensity (at 330 nm)	ANS binding[c]
rp24	pH 8.3	54 (±1)	5 (±1)	330	100	100
rp24F	pH 8.3	50 (±1)	12 (±1)	330	102	115
rp24G	pH 8.3	47 (±1)	6 (±1)	335	94	nd[d]
rp24I	pH 8.3	33 (±1)	9 (±1)	341	55	100
rp24	pH 2.0	33 (±1)	10 (±1)	342	50	1350
rp24F	pH 2.0	31 (±1)	11 (±1)	342	50	1080
rp24I	pH 2.0	33 (±1)	19 (±1)	342	10	470
rp24	pH 2.0, 5 M GdnHCl	–	–	349	40	105
rp24F	pH 2.0, 5 M GdnHCl	–	–	349	44	nd
rp24I	pH 2.0, 5 M GdnHCl	–	–	349	25	nd

[a] Calculated using the program VARSCL1 (Manavalan & Johnson, 1987). [b] Excitation at 295 nm. [c] Excitation at 400 nm, fluorescence intensity of 1 μm protein and 20 μm ANS; relative fluorescence intensity at maximum. [d] Not determined.

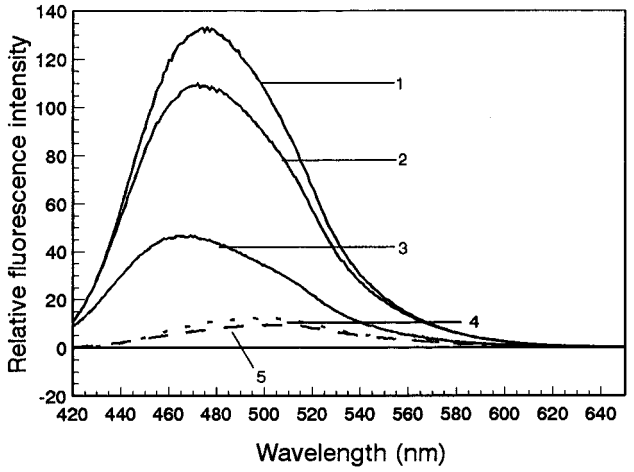

FIGURE 4: ANS binding of unmodified and modified HIV-1 rp24. Fluorescence spectra of 20 μM ANS in the presence of 1 μM protein were measured in 5 mM sodium phosphate, pH 2.0 at 25 °C. rp24 (1), rp24F (2), rp24I (3), and buffer solution (4). As reference is shown the spectrum of ANS in the presence of rp24 in 50 mM sodium phosphate buffer, pH 8.3 (spectrum 5). The excitation wavelength was 400 nm.

ANS binding studies were performed to characterize the hydrophobicity of the protein surface and were used to study protein folding intermediates such as the molten globule state (Semisotnov et al., 1991; Goldberg et al., 1990). The binding of ANS to hydrophobic clusters of a protein is connected with an increase of the fluorescence intensity (Stryer, 1965) and a blue shift of the fluorescence emission maximum from about 540 to 470 nm (Turner & Brand, 1968). In our experiments the fluorescence maxima of ANS were found at 510 and 480 nm in protein-free buffer and at pH 2.0 in the presence of unmodified rp24, respectively (Figure 4).

In Figure 4 ANS fluorescence spectra measured in the presence and absence of protein and at different pH values are shown. The ANS fluorescence intensity is very low in protein-free buffer (spectrum 4, dotted line) and in the presence of rp24 at pH 8.3 where the protein is folded and the hydrophobic clusters are inaccessible to the dye (spectrum 5, dashed line). At pH 8.3, the same observations were made for rp24F and rp24I (spectra not shown). At pH 2.0, however, ANS binding is indicated to unmodified rp24 and to both modified variants of rp24 by a 5−10-fold increase of the ANS fluorescence intensity (spectra 1−3). The highest extent of ANS binding is observed for the unmodified rp24, and the binding decreases gradually with increasing modification of the protein. Interestingly, a concomitant small blue shift of the fluorescence maxima from about 480 nm in the presence of unmodified protein to 470 nm in the presence of highly modified protein was observed. From the available evidence it is not possible to decide if this is accomplished by a smaller number but higher hydrophobicity of the ANS binding sites or by effects of the increasing number of negative charges on the ANS fluorescence. The increase of the ANS fluorescence intensity at pH 2.0 provides strong evidence for the formation of new, acid-induced conformations in unmodified rp24 and in the modified samples. Depending on the degree of modification by maleic anhydride these new conformations seem to differ with respect to the number, accessibility, or properties of their hydrophobic clusters.

GdnHCl-Induced Unfolding. The effect of maleic anhydride modification on the stability of rp24 was studied by monitoring the changes in the ellipticity [Θ] at 222 nm with increasing GdnHCl concentrations. Unfolding of unmodified rp24, of the highly modified form rp24G, and of the completely modified rp24I was studied. The recovery of the ellipticity upon dilution with buffer indicates that unfolding of rp24, rp24G, and rp24I is reversible.

GdnHCl-dependent unfolding transition curves of rp24, rp24G, and rp24I are sigmoidal and monophasic, suggesting that the protein achieves the unfolded state through a single-phase transition. The experimental curves were normalized and the fractions, f_u, of unfolded rp24, rp24G, and rp24I were determined for each GdnHCl concentration as shown in Figure 5. Despite of some deviations in the experimental values, the normalized curves show that the unfolding behavior of unmodified and modified samples is surprisingly similar. Unfolding of unmodified rp24 starts at denaturant concentrations of about 0.8−0.9 M GdnHCl and reaches plateau values near 2 M GdnHCl. The half-transition value, $c_{1/2}$, was found at 1.07 M GdnHCl (Misselwitz et al., 1995). Unfolding of rp24G and rp24I starts at somewhat lower GdnHCl concentrations of about 0.5−0.6 M GdnHCl and reaches the plateau at denaturant concentrations of about 2 M. The half-transition concentrations, $c_{1/2}$, of rp24G and rp24I have values of 0.97 and 0.99 M GdnHCl, respectively. The cooperativity of unfolding is reduced (lower *m* values) in comparison to unmodified rp24 (Table 3).

ΔG_u values were calculated using the data shown in Figure 5 assuming a "two-state" mechanism of unfolding. The free energy change, $\Delta G_u^{H_2O}$, the slopes, *m*, of the plots, and the half-transition concentrations, $c_{1/2}$, of GdnHCl for denaturant-induced unfolding studies measured with CD are summarized in Table 3. The $\Delta G_u^{H_2O}$ values of rp24, rp24G, and rp24I are in the range of about 18, 15, and 12 kJ/mol, respectively.

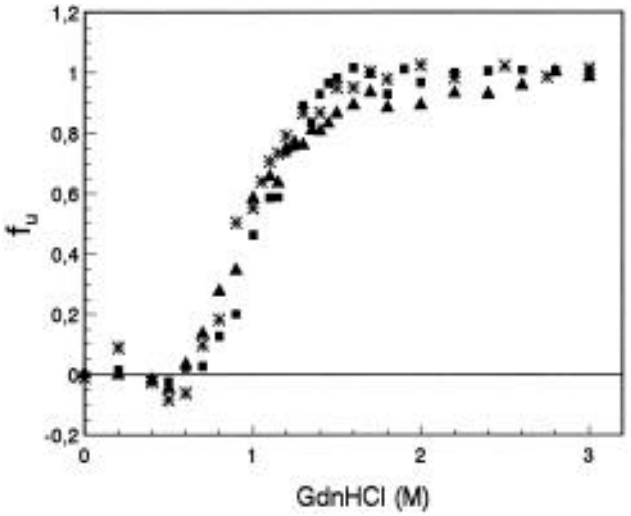

FIGURE 5: Normalized unfolding curves of rp24 (■), rp24G (*), and rp24I (▲) determined from the measured changes of $[\Theta]_{222}$ at 25 °C. The fractions, f_u, of unfolded protein are plotted versus the GdnHCl concentration. The proteins were in 20 mM sodium acetate buffer, pH 5.8; the protein concentration was 0.080 mg/mL.

Table 3: Free Energy of Unfolding of Modified rp24 forms at 25 °C[a]

protein	$\Delta G_u^{H_2O}$ (kJ/mol)	m (kJ/mol/M)	$c_{1/2}$ (M)
rp24[b]	18.1	16.9	1.07
rp24F	15.4	15.9	0.97
rp24I	12.1	12.2	0.99

[a] CD data were obtained in 20 mm sodium acetate buffer, pH 5.8, with the molar mean residue ellipticity [Θ] at 222 nm. Denaturant was GdnHCl. [b] Values were taken from Misselwitz et al. (1995).

Thus, at high degrees of modification the free energy of unfolding becomes reduced in comparison with the unmodified protein.

DISCUSSION

All amino groups of 11 lysine residues and the N-terminal amino group of recombinant HIV-1 capsid protein p24 potentially can react with maleic anhydride. The reactivity of these groups differs over a wide range and is reflected in the molar ratio of MA to amino groups necessary to achieve modification. At a ratio of 0.125 mol of MA to 1 mol of amino groups (corresponding to 1.5 mol of MA per 1 mol of rp24) an average number of 1.0 residue out of the 12 amino groups per rp24 molecule becomes substituted, which means that there is a practically complete consumption of the reagent. To achieve a modification of 11 amino groups, an excess of 8 mol of MA per mole of amino groups is necessary, complete modification is found at a 16-fold excess. Since the reactivity of the different lysines correlates with the surface accessibility (Suckau et al., 1992), this indicates that there is no increase in the reactivity of low-reactive residues at higher modification levels caused by an unfolding of modified protein. Therefore, the charge conversion from a positive to a negative charge which is connected with each substitution does not induce an overall unfolding of the protein at higher degrees of modification.

CD and fluorescence measurements of rp24 and of the three maleic anhydride modified proteins, rp24F, rp24G, and rp24I, also provide evidence that the general fold of the protein is basically preserved up to a modification extent of nine amino acid residues out of the total number of 11 ϵ-amino groups of lysine residues and of the N-terminus. Spectral changes indicating conformational changes were observed for the rp24G and rp24I samples containing as many as 11 and 12 modified amino groups, respectively. The far-UV CD spectra of unmodified and modified HIV-1 rp24 indicate a high content of α-helix structure confirming recently published data for the unmodified protein (Ehrlich et al., 1994; Hausdorf et al., 1994; Misselwitz et al., 1995). Different to rp24 and rp24F, for rp24G with 11 modified residues a small reduction and for the completely modified rp24I a pronounced reduction of the α-helical content are indicated.

GdnHCl-induced unfolding curves were monitored by CD, are sigmoidal and single phasic, and were taken to determine the stability of the protein assuming a "two-state" model of unfolding. The value of the free energy of unfolding $\Delta G_u^{H_2O}$ of 18.1 kJ/mol determined earlier for unmodified rp24 (Misselwitz et al., 1995) is on the lower conformational stability limit of other globular proteins (Pace, 1990). High modification as in rp24G leads to a decrease of the conformational stability, and the determined $\Delta G_u^{H_2O}$ value is reduced to 15.4 kJ/mol. The stability of completely modified rp24I is further reduced to a $\Delta G_u^{H_2O}$ value of 12.1 kJ/mol and is comparably low as the $\Delta G_u^{H_2O}$ value of unmodified rp24 at pH 2 where a destabilized acid-induced form of the protein was found (Misselwitz et al., 1995).

Maleylation of rp24 has surprisingly small effects on the spectroscopic properties of rp24. Up to a modification level of about nine modified amino functions per protein molecule, the CD spectra in the far- and near-UV regions are practically not changed, and thus, within the detection limits of these spectroscopic measurements, conformational changes can be neglected. Antibody binding, however, is sensitively influenced already at low substitution levels. This is surprising in view of the fact that the peptide epitope does not contain Lys residues. Thus a direct effect on the epitope by chemical modification of one of its residues is not possible, and the modification must influence the binding properties of the epitope in an indirect way. Most probably modification facilitates the exposure and thus increases the accessibility of the epitope whereas the epitope is hidden in the unmodified protein in a more rigid and less accessible conformation. There is no direct structural evidence that the epitope is exposed in the modified proteins before antibody binding. A loop location of the epitope was suggested earlier (Höhne et al., 1993). Possibly, at low modification levels increased flexibility or decreasing stability of this loop cause an enhancement of the antibody binding prior to any changes of the overall fold of the protein which could be observable in its spectra.

In addition, the kinetic measurements (Glaser & Hausdorf, 1996) provide evidence for the existence of at least two forms of rp24 with different binding properties for CB4-1. They support the view that modification of lysine residues does not change the affinity of the epitope directly, but rather shifts the equilibrium between the conformations in favor of a conformation with high affinity. This high-affinity form is present in the unmodified rp24 only in a small ratio of 1%−2%, whereas the significantly higher ratio of about 10% in modified immobilized rp24F could be induced by the modification.

The association behavior of rp24 might influence the antibody binding provided that the peptide epitope is located

at the interface region of the associating protein molecules. In that case an enhanced antibody binding of maleylated rp24 samples could be caused by a shift of the dimer−monomer equilibrium to monomers which might occur with increasing extent of maleylation. This alternative explanation, however, is not supported by the FPLC analyses, which demonstrated reduced elution volumes of the maleylated samples but not the increased elution volumes expected for a dissociation into monomers.

A conclusive explanation of the increased antibody binding caused by maleylation demands very detailed structural data. Results of future studies on the crystal and solution structures of rp24 and of rp24−antibody complexes could provide this information.

Acid-induced stable conformational states have been described for a variety of proteins (Goto & Fink, 1989; Buchner et al., 1991; Dryden & Weir, 1991; Hagihara et al., 1993, 1994; Hlodan & Pain, 1994). At low pH the unmodified rp24 undergoes a structural change that is characterized by the following spectral features: (1) a significant secondary structure content; (2) loss of CD in the aromatic region; (3) red shift of the maximum of the tryptophan fluorescence but reduced exposure of the tryptophan residues to the solvent in comparison to the completely unfolded state, and (4) a strong binding of ANS, indicating the exposure of hydrophobic clusters in this protein conformation accessible to the dye (Misselwitz et al., 1995). Similar acid-induced spectral features were observed for the modified rp24 forms. The reduced ANS fluorescence, observed with increasing degree of modification, may indicate a disturbed binding of the dye to hydrophobic clusters, or differences in the conformations of the acid-induced forms of unmodified and modified protein samples, or effects of the increasing number of negative charges on the fluorescence properties of ANS. The spectral features of HIV-1 rp24 forms at low pH are similar to properties of other proteins for which a "molten globule" state was described (Goto & Fink, 1989; Dryden & Weir, 1991; Hagihara et al., 1994; Hodlan & Pain, 1994). This structural state is characterized as a compact denatured state with a significant native-like secondary structure and a fluctuating tertiary structure and is considered as a major intermediate of protein folding (Dobson, 1992; Kuwajima, 1992; Ptitsyn, 1992). The relevance of the acid-induced conformation of rp24 for the folding of the protein at neutral pH, however, is not established.

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