Interdomain Interactions in the Chimeric Protein Toxin sCD4(178)-PE40: A Differential Scanning Calorimetry (DSC) Study

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The thermal denaturation of the chimeric protein toxin known as sCD4(178)-PE40 (sCD4-PE40) was studied using differential scanning calorimetry (DSC). sCD4-PE40 consists of HIV-binding domains of the T-cell membrane protein known as CD4 and the cytotoxic domains of *Pseudomonas* exotoxin A (PE40). sCD4-PE40 undergoes two DSC transitions. An endothermic transition associated with unfolding of the CD4 and PE40 components occurs at approximately 46°C in buffered saline at pH 6.5. An exothermic transition associated with precipitation of unfolded protein occurs at higher temperatures. Both transitions are irreversible. DSC studies of solutions of pH 5.0 to 9.5 indicate that sCD4-PE40 shows maximal thermal stability at around pH 6.5. Variable pH experiments are also presented on solutions of sCD4(183) and PE40 revealing how these components denature as independent structural entities. sCD4(183) denaturation occurs at significantly higher temperatures than does the CD4 component of sCD4-PE40. PE40 denaturation occurs at the same temperatures as sCD4-PE40. These results suggest that the native CD4 and PE40 components are independent and noninteracting entities in the chimeric sCD4-PE40 molecule and that unfolding of the less-stable PE40 component induces unfolding of the CD4 component. These destabilizing interdomain interactions of sCD4-PE40 are in contrast to the stabilizing interactions which apparently exist in wild-type *Pseudomonas* exotoxin A between its PE40 domains and the cell binding domain of the native toxin (analogous to the CD4 component in sCD4-PE40). Reasons are discussed why the type of interdomain interactions observed for sCD4-PE40 might be the norm for chimeric proteins.

KEY WORDS: chimeric proteins; chimeric toxins; differential scanning calorimetry; interdomain interactions; sCD4-PE40; alvircept sudotox.

INTRODUCTION

Chimeric protein toxins are being prepared which replace the cell binding domains of *Pseudomonas* exotoxin A, diphtheria toxin, and ricin with protein binding components designed to make these toxins specific for cancer and other diseased cells (reference 1 and references cited therein). One such recombinant toxin known as sCD4(178)-PE40 (hereafter, sCD4-PE40) has been prepared for the purpose of specifically killing HIV-infected T-cells in humans infected with this virus. The targeting moiety for this protein is the amino terminal sequence, residues 1-178, of the T-cell membrane protein known as CD4 which has high affinity for the HIV surface glycoprotein gp120 (2). The CD4 component of sCD4-PE40 is continuous in sequence with domains II, Ib, and III of *Pseudomonas* exotoxin A which collectively have a molecular weight of approximately 39400 daltons (hence the designation "PE40"). The total chimeric protein has 545 amino acids, 4 disulfide bonds, and a theoretical molecular weight of 59,200 (3). The *Pseudomonas* exotoxin domain II is known to facilitate translocation of the catalytically active domain III into the cytosol of the cell (4,5). Domain III catalyzes hydrolysis of $NADH^+$ with transfer of ADP-ribose to elongation factor-2, a ribosomal protein, resulting in cell death (6). sCD4-PE40 selectively kills cells in culture which express gp120 on their surfaces (3) and can eliminate HIV from infected cultures when used in combination with the antiviral agent AZT (7).

The chimeric proteins, of which sCD4-PE40 is one example, could be viewed as representing a unique class of multidomain proteins. Chimeric proteins are unique because their separate protein components are combined on the basis of desired functional capabilities. Thus, specific interdomain interactions which have evolved in naturally occuring multidomain proteins are expected to be absent or at least different in chimeric proteins. Interdomain interactions in naturally occurring multidomain proteins have been studied and modeled (8–11). These studies reveal that the individual domains of multidomain proteins interact to varying degrees with significant effects on the stability of the individual domains. While interdomain interactions in chimeric proteins have not been studied, it is likely that the individual components will also be affected by their neighbors with implications for their structural and biological stability in solution, their thermal stability, and their stability during pharmaceutical processing.

We have undertaken a differential scanning calorimetry (DSC) characterization of sCD4-PE40 to gain an understanding of how the CD4 and PE40 components interact and how each component affects the stability of the other. These studies begin by identifying temperatures of denaturation for the individual components of sCD4-PE40. We then examine the effect of solution pH on the thermal stability of sCD4-PE40 and proteins which closely resemble its CD4 and PE40 components. These studies reveal the nature of interdomain interactions in sCD4-PE40 and how the thermal stability of CD4 and PE40 are altered when these components are incorporated into the chimeric protein. These results may have implications for chimeric proteins in general and highlight the possibility that interdomain interactions in chimeric proteins may be destabilizing.

MATERIALS

sCD4-PE40, CD4(183), and PE40 were obtained from The Upjohn Company. Buffer components were purchased from either Sigma Chemical Company, Mallinkrodt Specialty Chemicals Company, or J. T. Baker, Inc.

METHODS

Solution Preparation. Protein solutions were prepared

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by overnight dialysis of bulk drug solutions at 4°C using SpectraPor 7® dialysis tubing with molecular weight cut-offs of 10,000 (sCD4-PE40) or 3,500 (sCD4(183) and PE40). Dialysis solutions were prepared with ionic strengths controlled by NaCl. All buffers were 10 mM in concentration and consisted of sodium citrate for pH 5.0 and 5.5, 2-[N-Morpholino]ethane-sulfonic acid (MES) for pH 6.5, sodium phosphate for pH 7.5, Tris(hydroxymethyl)aminomethane hydrochloride (Tris) for pH 8.5, and glycine for pH 9.5. Dialyzed solutions were adjusted to the desired concentration by dilution with dialysis buffer. These solutions were sometimes clarified by filtration through Millex-GV filter cartridges. Solutions were degassed prior to calorimetry by gently stirring solutions under reduced pressure for 10–20 min at room temperature.

Protein concentrations of sCD4-PE40 and sCD4(183) were determined by UV absorbance of unheated solutions at 280 nm using extinction coefficients of 1.10 ml/mg-cm for sCD4-PE40 and 0.90 ml/mg-cm for sCD4(183). These extinction coefficients were based upon UV absorbance measurements of protein standards of known concentration. The concentration of protein standards was determined by amino acid analysis in which the number of moles of each amino acid recovered are plotted against the number of moles of that particular amino acid per mole protein. This latter term is known from the sequence of the protein. Concentrations of PE40 were determined by a reverse-phase HPLC method utilizing a C-18 column and elution with a linear gradient of increasing trifluoracetic acid (TFA)/acetonitrile solution and decreasing TFA/water. PE40 peak areas detected at 214 nm were compared against standard curves generated using sCD4-PE40 standards. We make the assumption in this procedure that solutions of sCD4-PE40 and PE40 will have similar response factors in this RP-HPLC assay at 214 nm. The use of sCD4-PE40 standards was necessary to conserve PE40.

DSC and Other Controlled Heating Experiments. DSC experiments were conducted using a Microcal MC-2 differential scanning calorimeter (Amherst, MA). The MC-2 utilizes two identical 1.2 ml total-fill cells. The sample cell was filled with protein solution prepared as described above while the reference cell was filled with dialyzate. Scanning rates ranged from 28.5 to 60 deg/hr. Raw data in cal/min reflect the rate of heat consumption or heat release due to protein thermal transitions. This raw data was divided by the scan rate using the DSC computer software to convert to cal/deg which in turn was divided by the protein concentration to obtain a DSC profile in units of cal/gm-deg or kcal/mol-deg. These concentration-normalized DSC profiles reflect the change in apparent heat capacity of the protein solution as a function of temperature.

Enthalpies of denaturation (ΔH) were estimated from the peak areas of the protein endotherms. These peak areas were obtained by connecting baselines immediately before and after the transition to isolate the area of the transition. In some cases sloping baselines after the transition made it impossible to extrapolate a baseline under the transition. ΔH measurements are not reported in these cases.

The pH of solutions were adjusted at room temperature to values of 5.0, 5.5, 6.5, 7.5, 8.5, or 9.5 using either 1.0 M NaOH or HCl. The pH of each solution is known to decrease during heating, to varying degrees depending on the buffer component. The pH of each solution at the temperature of the endotherm (T_d) was estimated using equations 1 and 2:

$$pH_{Td} = pH_{25} - (pK_a^{25} - pK_a^{Td}) \quad (1)$$

$$pK_a^{25} - pK_a^{Td} = (1 / 2.303)(\Delta H_{ion} / R)(1 / 298.15 - 1 / T_d) \quad (2)$$

In these equations pH_{25} and pH_{Td}, and pK_a^{25} and pK_a^{Td} refer to the solution pH and buffer pK_a values at 25°C (room temperature) and at the temperature of the thermal transition. ΔH_{ion} refers to the enthalpy of ionization for the buffer component in the solution. The following ΔH_{ion} values were used: citric acid (pH 5.0 and 5.5 solutions), essentially zero (12); MES (pH 6.5 solution), 4.16 kcal/mol (13); sodium phosphate (pH 7.5 solution), 0.98 kcal/mol (12); Tris (pH 8.5 solution), 11.36 kcal/mol (12); glycine (pH 9.5 solution), 10.75 kcal/mol (12). Equation 1 is based on the observation that the temperature-dependent change in pH of a buffer solution is due almost entirely to changes in pK_a of the main buffer component (14). Equation 2 is a modification of the van't Hoff equation and assumes a constant ΔH_{ion} over the temperature range of our studies.

Some heating experiments were conducted by immersing sample tubes containing sCD4-PE40, sCD4(183), or sCD4(183) + PE40 solutions in a Neslab® programmable water bath which was run at a heating rate of 0.5 – 1.0 deg/min. Separate aliquots of protein solution were withdrawn from the water bath at selected temperatures and were chilled immediately. Samples were stored at −80°C until assay.

Assays on Heated Solutions. Bioactivity measurements were conducted at room temperature on heated solutions of sCD4-PE40, sCD4(183), and PE40 prepared as described above. Gp120 binding activity and nicotinamide adenine dinucleotide (NAD) glycohydrolase activity were measured on these solutions to assess the functional integrity of the CD4 and PE40 components, respectively. Bioactivity results are expressed in units of percent (%), obtained as the ratio of apparent concentration of active protein (by bioassay measurement and comparison with standards) to total protein concentration (by UV absorbance or RP-HPLC). Gp120 binding activities were determined using a procedure described by McQuade et al. (15). NAD glycohydrolase activities were determined by measuring the rate of sCD4-PE40-dependent hydrolysis of NAD, using reverse-phase HPLC (RP-HPLC) to quantitate nicotinamide which is one of the two hydrolysis products. The RP-HPLC method uses a Zorbax Rx C8 column (4.6 mm ID × 25 cm) eluted with 2% acetonitrile and 20 mM potassium phosphate buffer, pH 3.5. Detection was at 254 nm. After a 60-min reaction of sCD4-PE40 with NAD at 30°C, quantities of nicotinamide were compared to quantities generated from sCD4-PE40 standard solutions to determine activity.

Size-exclusion HPLC (SEC) was also conducted on solutions heated in the programmable water bath. Prior to analysis, samples were diluted to 200 μg/ml with their respective buffers and chromatographed on a Zorbax GF-250 column with an isocratic mobile phase of 0.1 M potassium phosphate, 0.9 M KCl, pH 8.0 and with UV detection at 221 nm.

RESULTS

Structural Changes Associated with the sCD4-PE40 Thermal Transitions. DSC profiles for sCD4-PE40 in buffers of pH 6.5 and 8.5 are shown in Figure 1. These profiles were obtained using a scanning rate of 28.5 deg/hr. sCD4-PE40 in pH 6.5 buffer shows a cooperative endotherm with a transition temperature (T_d) at 45.5°C followed by an exotherm at 58°C. The apparent enthalpy (ΔH) of the pH 6.5 endotherm is 2.7 cal/gm. sCD4-PE40 in pH 8.5 buffer shows a broader endotherm with T_d at 41.0°C followed much later by an exotherm at 83°C. The apparent enthalpy (ΔH) of the pH 8.5 endotherm is 1.9 cal/gm.

Both the endotherm and exotherm were irreversible: Reheating of solutions previously heated beyond the transition resulted in no transition (data not shown). While sCD4-PE40 is expected to behave as a multidomain protein, there is no clear indication of separable, independent transitions. The apparent enthalpies observed for the endotherms in Figure 1 are low compared to other proteins. Ramsay and Freire (9) determined ΔH for the A and B fragments of diphtheria toxin to be approximately 5.2 and 4.7 cal/gm, respectively, which are ΔH values more typical of proteins denaturing in the 40 to 60°C range (16).

Bioassays were conducted on sCD4-PE40 solutions heated to temperatures extending through the DSC transitions. The gp120 binding assay was used to assess the structural integrity of the CD4 domains. The NAD glycohydrolase assay was used to assess the structural integrity of the cytotoxic domain (domain III) of PE40. Figures 2A and 2B summarize the results of these bioassays for sCD4-PE40 in buffers of pH 6.5 and 8.5, respectively. In buffer of pH 6.5, both bioassays indicate a loss of integrity of their respective structural components between 40 and 50°C (Figure 2A). In buffer of pH 8.5, bioassays indicate a total loss of structural integrity by 50°C (Figure 2B). The NAD glycohydrolase assay may show slight decreases at 40°C which are not apparent in the gp120 binding activity. These studies clearly show that the sCD4-PE40 endotherm involves thermal denaturation of both the CD4 and the PE40 components with resulting total losses of bioactivity for both components.

Size exclusion chromatography (SEC) was used to detect changes in molecular size occurring during the sCD4-PE40 thermal transitions. Figure 3A shows SEC chromatograms of sCD4-PE40 at pH 6.5 heated to temperatures extending through the DSC endotherm. The chromatogram of sCD4-PE40 heated to 40°C shows a single component which is associated with native monomer sCD4-PE40. The chromatogram at 50°C has almost no monomer and has converted

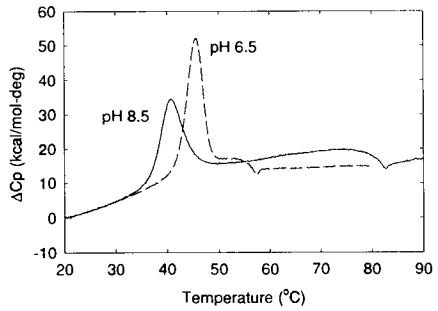

Figure 1. DSC profiles of sCD4-PE40 solutions at pH 6.5 or 8.5 (pH at room temperature). Heating rates were 28.5 deg/hr in these scans. Ionic strength (μ) for both solutions = 0.16. Protein concentrations were both approximately 4.0 mg/ml.

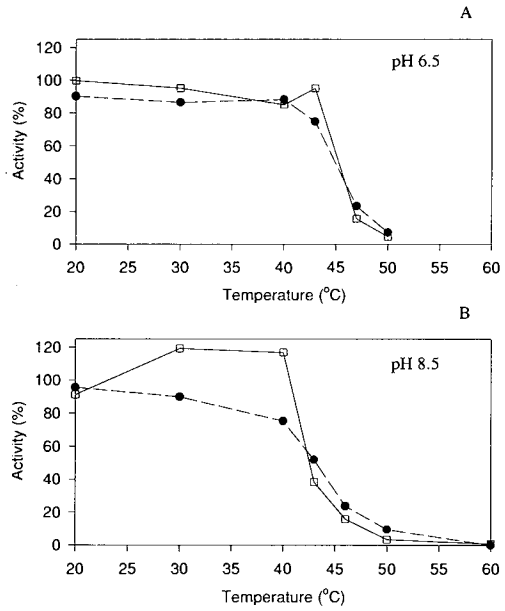

Figure 2. Bioactivity measurements for sCD4-PE40 in pH 6.5 buffer (A) and pH 8.5 buffer (B). These data correlate with the DSC profiles in Figure 1. The gp120 binding activity (□) is a measure of CD4 integrity while the NAD glycohydrolase activity (●) is a measure of PE40 integrity. The data represent apparent protein concentrations, measured by bioassay, expressed as a percent of the total protein measured by UV absorbance. Experimental conditions (heating rate, solution pH, ionic strength, and protein concentration) were essentially identical to those of Figure 1.

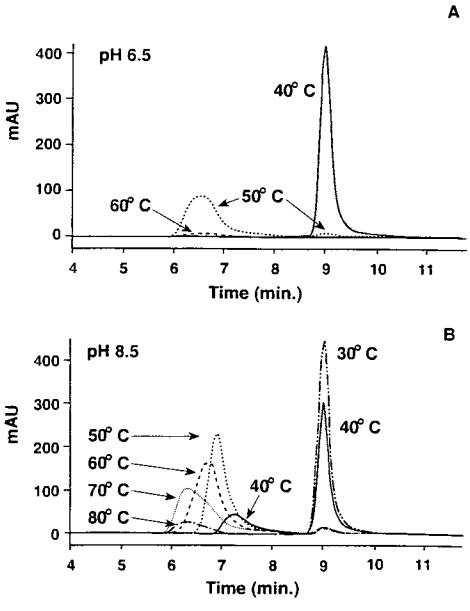

Figure 3. Size-exclusion HPLC chromatograms of sCD4-PE40 solutions of pH 6.5 (A) and pH 8.5 (B) taken to the temperatures indicated, cooled, and analyzed. These data correlate with the DSC profiles in Figure 1. Experimental conditions were essentially identical to those of Figure 1.

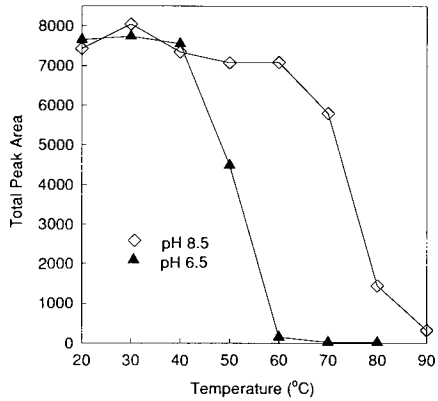

Figure 4. Total peak areas for size-exclusion HPLC chromatograms of sCD4-PE40 solutions during heating. These data correlate with the DSC profiles in Figure 1. Experimental conditions (heating rate, solution pH, ionic strength, and protein concentration) were essentially identical to those of Figure 1.

almost completely to an earlier-eluting oligomer form. Thus, oligomerization occurs at temperatures coinciding with those of the sCD4-PE40 endotherm. Chromatograms of sCD4-PE40 solutions heated to still higher temperatures show no peak area and appear as baselines in this figure. Figure 3B shows a similar set of SEC chromatograms of sCD4-PE40 at pH 8.5. sCD4-PE40 at 30°C (pH 8.5) is completely in the monomer form. However, heating to 40°C results in about 25% conversion to oligomer form. Heating to higher temperatures causes almost complete conversion to oligomer. Furthermore, the oligomer peak elutes progressively earlier in samples heated to higher temperatures, indicating that the size of oligomeric units is increasing during heating.

The total peak areas for sCD4-PE40 solutions of pH 6.5 and 8.5 decrease at different temperatures during heating (Figure 4). The total SEC peak area for the pH 6.5 solution decreased sharply between 50 and 60°C while the SEC peak area of the pH 8.5 solution decreased sharply above 70°C. The decreases in SEC total peak area correspond approximately to the exotherms of sCD4-PE40 in solutions of pH 6.5 and 8.5 (see Figure 1). Visual observations of solutions used in these SEC studies indicated a large increase in turbidity coinciding with the exotherms. These observations indicate that the exotherm is associated with precipitation of the

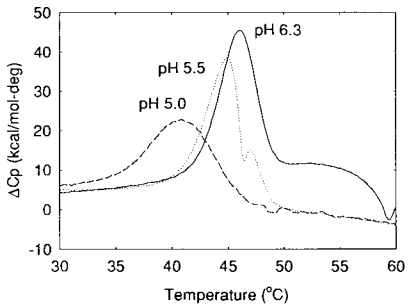

Figure 5. DSC profiles of sCD4-PE40 in solutions of variable pH (μ constant at 0.16): pH < 6.5. Solution pH at T_d was estimated as described in Methods. Heating rate was approximately 52 deg/hr.

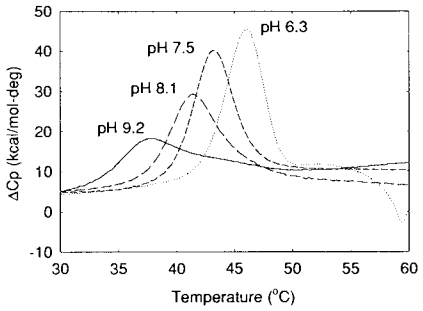

Figure 6. DSC profiles of sCD4-PE40 in solutions of variable pH (μ constant at 0.16): pH > 6.20. Solution pH at T_d was estimated as described in Methods. Heating rate was approximately 52 deg/hr.

sCD4-PE40 sample which results in particles too large to pass through the SEC column.

Effect of pH on the Thermal Stability of sCD4-PE40, sCD4(183), and PE40. Figures 5 and 6 show DSC profiles of sCD4-PE40 in buffered saline solutions ranging from pH 5.0 to pH 9.5 (numerical data are summarized in Table I). These figures show that the thermal stability of sCD4-PE40 is highest in the buffered saline solution at pH 6.3. The sCD4-PE40 endotherm is shifted to lower temperatures (destabilized) and is broadened by pH increasing and decreasing from 6.3. These data suggest that protonation of a carboxylate group (ASP or GLU) at pH below 6.3 destabilizes sCD4-PE40 while deprotonation of a different side chain above pH 6.3 (probably HIS) also destabilizes the protein. Enthalpies of thermal denaturation, listed in Table I, range from 104 kcal/mol (1.76 cal/gm) at pH 9.2 to 165 kcal/mol (2.79 cal/gm) at pH 5.0. These enthalpies and those to follow in Tables II and III have not been corrected for differences in protein and buffer ionization enthalpies. These effects could alter the apparent enthalpy of sCD4-PE40 unfolding by several kcal/mol for each protein group undergoing protonation or deprotonation upon unfolding.

In none of these profiles is there a clear separation of multiple transitions. DSC profiles generated from pH 8.5 and 9.5 solutions (Figure 6) appear skewed to the high temperature side which might be the first indication of multiple overlapping transitions. The profile generated from pH 5.5 solu-

Table I. Summary of DSC Parameters for sCD4-PE40 as a Function of pH

Protein concentration; pH at 25°C.	T_d- endotherm (°C)	Estimated pH at T_d	ΔH- endotherm (kcal/mol)
3.71 mg/ml; pH 5.0	40.8	5.0	165
5.10 mg/ml; pH 5.5	45.0	5.5	160
4.87 mg/ml; pH 6.5	46.0	6.3	149
4.66 mg/ml; pH 7.5	43.2	7.5	149
4.03 mg/ml; pH 8.5	41.4	8.1	134
4.74 mg/ml; pH 9.5	37.6	9.2	104

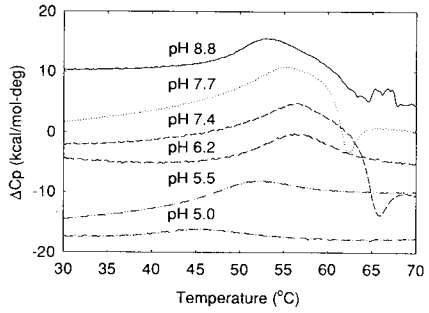

Figure 7. DSC profiles of sCD4(183) in solutions of variable pH (μ constant at 0.16). Solution pH at T_d was estimated as described in Methods. Heating rate was approximately 52 deg/hr for these scans.

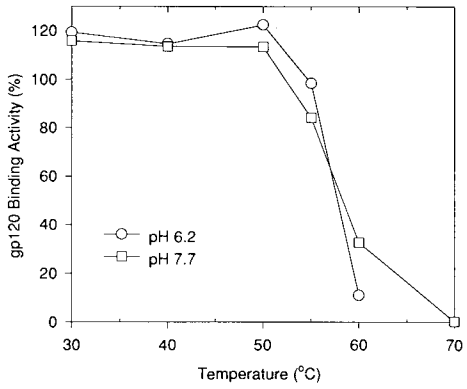

Figure 8. The effect of increasing temperature on gp120 binding activity of sCD4(183). These data correlate with the DSC profiles from Figure 7 labelled pH 6.2 and 7.7. Experimental conditions were equivalent between the correlating data sets in this figure and Figure 7. The data represent an apparent protein concentration, as measured by gp120 binding assay, expressed as a percent of the total protein concentration measured by UV absorbance.

tion (Figure 5) which appears to show two adjacent transitions is more likely to be the endotherm with a superimposed exotherm associated with protein precipitation. The isoelectric point of sCD4-PE40 is approximately 5.5 (unpublished observation). Solutions of pH 5.5 are extremely prone to sCD4-PE40 precipitation.

To learn more about how the CD4 and PE40 components may be denaturing in the sCD4-PE40 endotherm, DSC runs were conducted on sCD4(183) in buffered saline solutions across the pH of 5.0 to 9.5 (Figure 7; Table II). sCD4(183) has the amino-terminal sequence 1 through 183 of full-length CD4. Thus, the sCD4(183) sequence corresponds exactly with the CD4 component of sCD4-PE40 but differs in residues 180 through 183. The concentration of sCD4(183) for the DSC experiments was approximately equimolar to the concentration used in the sCD4-PE40 pH study.

The sCD4(183) thermal transitions for all but the pH 5.0 sample occurred above 50°C. The sCD4(183) transitions were higher in temperature and considerably broader than the sCD4-PE40 transitions under comparable pH conditions. In solutions of pH 7.5 through 9.5, an exotherm, likely due to protein precipitation, followed the endotherm. Figure 8 shows that loss of gp120 binding activity for sCD4(183) in buffers of pH 6.5 and 8.5 coincides with the temperature of the DSC endotherm. Comparison of the gp120 binding data of sCD4(183) (Figure 8) and sCD4-PE40 (Figures 2A and 2B) reveals that the loss of CD4 integrity occurs at a higher temperature in sCD4(183) than in sCD4-PE40.

An abbreviated pH stability study was conducted using "PE40" whose sequence replicates exactly that of the PE40 domains in sCD4-PE40 extending from residues 182 through 545 (Figure 9; Table III). Limited quantities of this protein allowed only three DSC runs of different pH to be conducted. PE40 thermal transitions in buffers of pH 6.5, 7.5, and 8.5 coincided closely in temperature with those seen for sCD4-PE40 under similar pH conditions. Peak shapes and widths were also very similar between sCD4-PE40 and PE40. The PE40 transition was destabilized and broadened as pH increased from pH 6.5 to 8.5, similar to our observation with sCD4-PE40.

The above thermal data on sCD4-PE40, sCD4(183), and PE40 indicate that unfolding of the CD4 component with accompanying loss of gp120 binding activity occurs concomitant with unfolding of the PE40 component when these separate proteins are incorporated into the sCD4-PE40 chimeric protein. Otherwise, unfolding of the CD4 component occurs at a temperature at least 10 degrees higher than the unfolding of PE40. To determine whether the destabilization of CD4 by PE40 was a local effect due to covalent attachment or to a non-specific effect of being in the same solution, bioactivity measurements were conducted on a pH 6.5-buffered saline

Table II. Summary of DSC Parameters for sCD4 (183) as a Function of pH

Protein concentration; pH at 25°C.	T_d- endotherm (°C)	Estimated pH at T_d	ΔH- endotherm (kcal/mol)
1.32 mg/ml; pH 5.0	45.4	5.0	22
1.88 mg/ml; pH 5.5	51.8	5.5	46
1.34 mg/ml, pH 6.5	56.6	6.2	49
1.44 mg/ml, pH 7.5	56.4	7.4	—
1.75 mg/ml, pH 8.5	55.0	7.7	—
1.86 mg/ml, pH 9.5	53.1	8.8	—

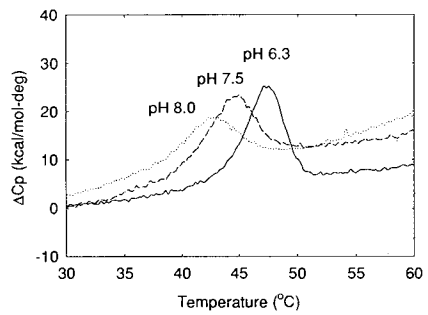

Figure 9. DSC profiles of PE40 in solutions of variable pH (μ constant at 0.16). Solution pH at T_d was estimated as described in Methods. Heating rate was approximately 52 deg/hr for these scans.

Table III. Summary of DSC Parameters for PE40 as a Function of pH

Protein concentration; pH at 25°C.	T_d- endotherm (°C)	Estimated pH at T_d	ΔH- endotherm (kcal/mol)
0.82 mg/ml; pH 6.5	47.1	6.3	81
0.85 mg/ml; pH 7.5	45.0	7.5	90
0.82 mg/ml; pH 8.5	42.4	8.0	78

solution containing both sCD4(183) and PE40 which had been heated in a water bath to temperatures ranging from 30 to 60°C (Figure 10). The mole ratio of sCD4(183) and PE40 in this experiment was 1: 0.86. DSC was not a useful technique in this experiment because the sCD4(183) transition is broad and of low enthalpy, making it difficult to distinguish this transition from the larger and more well-defined PE40 transition. The NAD glycohydrolase activity of the PE40 at 30°C was found to be 62% when compared against equimolar concentrations of sCD4-PE40. While the reason for this lower enzymatic activity is unclear, the temperatures at which the decrease in activity occurs still provide a useful indicator of the thermal unfolding transition for native PE40, as indicated by the correlation of bioactivity data in Figures 2A and 2B with the DSC data in Figure 1. Figure 10 shows that PE40 in the presence of near-equimolar sCD4(183) undergoes thermal unfolding between 40 and 55°C, as indicated by the decrease in NAD glycohydrolase activity. sCD4(183) in the presence of PE40 thermally unfolds between 50 and 60°C, as indicated by the decrease in gp120 binding activity. Thus, the data indicate that sCD4(183) and PE40 unfold independently when present in solution as separate protein molecules.

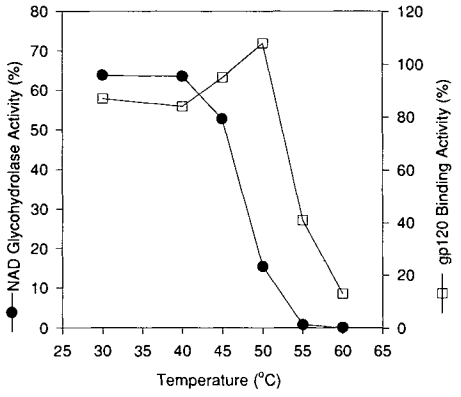

Figure 10. The effect of increasing temperature on bioactivity measurements for sCD4(183) and for PE40 in a single solution containing both proteins. sCD4(183) (0.53 mg/ml; 26 μM) and PE40 (0.89 mg/ml; 22 μM) were both in pH 6.5 buffer with ionic strength (μ) of 0.16. Heating was at approximately 30 deg/hr in a Neslab programmable water bath. Gp120 binding acitivity (□) and NAD glycohydrolase activity (●) were measured as described in Methods to assess the integrity of sCD4(183) and PE40, respectively. The data represent apparent protein concentrations, measured by each bioassay, expressed as a percent of the total protein concentration measured by UV absorbance or RP-HPLC.

DISCUSSION

The DSC data provide evidence that the CD4 and PE40 components of sCD4-PE40 are independent and non-interacting when both components are in their native state. This conclusion is reached by comparing the thermal stability of PE40 with the thermal stability of this component in the sCD4-PE40 molecule. The PE40 component of sCD4-PE40 denatures in the main endotherm as indicated by the loss of NAD glycohydrolase activity coinciding with the endotherm. PE40 which is not a part of the chimeric protein denatures at about the same temperature. Thus, the PE40 component of SCD4-PE40 behaves under the stress of heat as though it is entirely independent of the rest of the chimeric protein. Our expectation is that the PE40 component of sCD4-PE40 should denature at a higher temperature if the PE40 component was interacting with the intrinsically more stable CD4 component. The CD4 component is said to be intrinsically more stable since sCD4(183) was found to denature at about 55°C. This type of stabilizing interdomain interaction is illustrated by wild-type *Pseudomonas* exotoxin A of 67 kDa (PE67) (see Figure 11). PE67 consists of a cell binding domain (domain I, analogous to CD4 in the sCD4-PE40 molecule) and the cytotoxic domains which are identical to PE40. PE67 monitored by intrinsic fluorescence spectroscopy denatures in a single transition at approximately 56°C at neutral pH (17). This indicates that interdomain interactions between the cell binding domain of PE67 and domains II and III of this molecule (identical to PE40) have stabilized the latter from their intrinsic denaturation temperature around 45°C (this study) to 56°C. This is the expected result of stabilizing interdomain interactions which appear to be absent in sCD4-PE40.

A comparison of the NAD glycohydrolase activity between sCD4-PE40 and PE67 provide another line of evidence supporting the conclusion that the components of sCD4-PE40 are non-interacting in the native state and those of PE67 are interacting. The NAD glycohydrolase activity of sCD4-PE40 is measured without the need for denaturing solution conditions. However, the NAD glycohydrolase activity of PE67 is measured only after partial denaturation of domain I using dithiothreitol and urea, and after ligation of

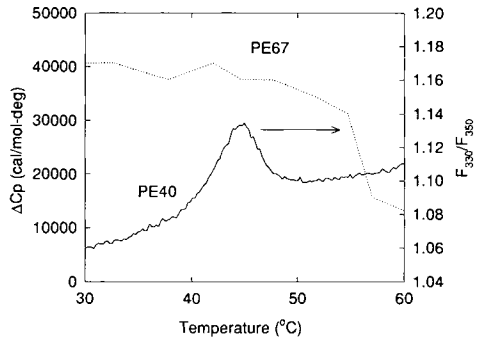

Figure 11. Thermal denaturation of *Pseudomonas* exotoxin A (PE67; fluorescence) and PE40 (by DSC), both at pH 7.5. PE40 denaturation occurs at around 45°C in the free state as seen in Figure 9. PE67 denaturation occurs at 56°C (data redrawn from ref. 17). These data suggest that unfolding of the PE40 component which is present in PE67 is delayed by stabilizing interactions with a higher-melting binding component in the naturally-occurring toxin.

domain III (18). In other words, interdomain interactions in native PE67 obscure its active sites for NAD glycohydrolase activity while the absence of those interactions in sCD4-PE40 allow free access to the active sites of the chimeric molecule.

The DSC data indicate another important aspect of interdomain interactions in sCD4-PE40: The unfolded PE40 component *induces* unfolding of the CD4 component of sCD4-PE40. Our DSC studies indicate that the free form of CD4 (represented by sCD4(183)) denatures at approximately 56°C with an accompanying loss of gp120 binding activity while the CD4 component of sCD4-PE40 denatures between 40 and 50°C, also indicated by a loss of gp120 binding activity. These data indicate that there is an effect of unfolded PE40 which induces the nearby CD4 component on the same molecule to unfold. Moreover, we have shown that this is an intramolecular interaction of the PE40 and CD4 components, since PE40 and sCD4(183) in the same solution as separate protein molecules denature independently. While the molecular basis for PE40 destabilization of CD4 is not known, we speculate that hydrophobic or electrostatic interactions of the unfolded PE40 and CD4 components are the basis for this effect.

The lack of stabilizing interactions between native components and the destabilizing interactions of unfolded components may be properties inherent in chimeric proteins. Chimeric proteins are the products of human invention and are born of the need for multiple functional properties on the same molecule. Stabilizing interdomain interactions common in multidomain proteins are not as likely to occur in chimeric proteins since these are based on specific hydrogen bonding interactions and complementary interactions of hydrophobic surfaces on separate domains (11,19). These stabilizing interactions, presumed to be "selected" by evolution, are evident in yeast phosphoglycerate kinase and aspartate transcarbamoylase (8,11), diphtheria toxin (9), and in *Pseudomonas* exotoxin A, as discussed above. Chimeric proteins, without the benefit of evolutionary refinement, are not likely to possess these specific, stabilizing interdomain interactions. Instead, chimeric proteins are more likely to show non-specific interactions of their unfolded components. Extending the example of sCD4-PE40, we expect that the thermal stability of chimeric proteins will be controlled by the least stable of the chimeric molecule's structural components. PE40 is that least-stable component in the sCD4-PE40 molecule. Further research on the thermal stability of chimeric proteins will determine whether this expectation is generally observed.

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REFERENCES

1. I. Pastan, V. Chaudhary, and D. J. FitzGerald. Recombinant toxins as novel therapeutic agents. *Annu. Rev. Biochem.* **61**:331–354 (1992).
2. E. A. Berger, T. R. Fuerst, and B. Moss. A soluble recombinant polypeptide comprising the amino-terminal half of the extracellular region of the CD4 molecule contains an active binding site for human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA.* 85:2357–2361 (1988).
3. V. K. Chaudhary, T. Mizukami, T. R. Fuerst, D. J. FitzGerald, B. Moss, I. Pastan, and E. A. Berger. Selective killing of HIV-infected cells by recombinant human CD4-*Pseudomonas* exotoxin hybrid protein. *Nature.* 335:369–372 (1988).
4. J. Hwang, D. J. FitzGerald, S. Adhya, and I. Pastan. Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in E. coli. *Cell.* 48:129–136 (1987).
5. V. S. Allured, R. J. Collier, S. F. Carroll, and D. B. McKay. Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-Ångstrom resolution. *Proc. Natl. Acad. Sci. USA.* 83:1320–1324 (1986).
6. B. H. Iglewski and D. Kabat. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. *Proc. Natl. Acad. Sci. USA.* 72:2284–2288 (1975).
7. P. Ashorn, B. Moss, J. N. Weinstein, V. K. Chaudhary, D. J. FitzGerald, I. Pastan, and E. A. Berger. Elimination of infectious human immunodeficiency virus from human T-cell cultures by synergistic action of CD4-*Pseudomonas* exotoxin and reverse transcriptase inhibitors. *Proc. Natl. Acad. Sci. USA.* 87:8889–8893 (1990).
8. J. F. Brandts, C. Q. Hu, and L.-N. Lin. A simple model for proteins with interacting domains. Applications to scanning calorimetry data. *Biochemistry.* 28:8588–8596 (1989).
9. G. Ramsay and E. Freire. Linked thermal and solute perturbation analysis of cooperative domain interactions in proteins. Structural stability of diphtheria toxin. *Biochemistry.* 29:8677–8683 (1990).
10. E. Freire and R. L. Biltonen. Statistical mechanical deconvolution of thermal transitions in macromolecules. I. Theory and application to homogeneous systems. *Biopolymers.* 17:463–479 (1978).
11. E. Freire, K. P. Murphy, J. M. Sánchez-Ruiz, M. L. Galisteo, and P. L. Privalov. The molecular basis of cooperativity in protein folding. Thermodynamic dissection of interdomain interactions in phosphoglycerate kinase. *Biochemistry.* 31:250–256 (1992).
12. R. M. Izatt and J. J. Christensen. Heats of proton ionization, pK_a, and related thermodynamic quantities. In G. D. Fasman (ed.) *CRC Handbook of Biochemistry and Molecular Biology, 3rd Edition, Physical and Chemical Data Volume I,* CRC Press, Boca Raton, FL, 1985, pp. 151–269.
13. N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Singh. Hydrogen ion buffers for biological research. *Biochemistry.* 5:467–477 (1966).
14. D. D. Perrin and B. Dempsey. *Buffers for pH and Metal Ion Control,* Chapman and Hall, London. 1974.
15. T. J. McQuade, T. W. Pitts, and W. G. Tarpley. A rapid solution immunoassay to quantify binding of the human immunodeficiency virus envelope glycoprotein to soluble CD4. *Biochem. Biophys. Res. Commun.* 163:172–176 (1989).
16. P. L. Privalov and N. N. Khechinashvili. A thermodynamic approach to the problem of stabilization of globular protein structure: A calorimetric study. *J. Mol. Biol.* 86:665–684 (1974).
17. J. X. Jiang and E. London. Involvement of denaturation-like changes in *Pseudomonas* exotoxin A hydrophobicity and membrane penetration determined by characterization of pH and thermal transitions. *J. Biol. Chem.* 265:8636–8641 (1990).
18. S. Lory and R. J. Collier. Expression of enzymic activity by exotoxin A from *Pseudomonas aeruginosa. Infect. Immun.* 28:494–501 (1980).
19. E. Freire and K. P. Murphy. Molecular basis of co-operativity in protein folding. *J. Mol. Biol.* 222:687–698 (1991).