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STORAGE STABILITY OF THE SOLUTION FORMULATION OF sCD4 DETERMINED BY DSC IN COMPARISON WITH TWO FUNCTIONAL ASSAYS*

*C. G. Brouillette***, *S. W. Tendian, B. C. Heard, D. Dunleavy*[a], *A. L. Shorter*[b], *D. G. Myszka*[a] *and I. M. Chaiken*[a]

**Center for Macromolecular Crystallography, University of Alabama at Birmingham, 1918 University Blvd./THT 79, Birmingham, AL 35294
Southern Research Institute, 2000 9th Ave. S., Birmingham, AL 35205
[a]SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, PA 19406
[b]SmithKline Beecham Consumer Health Care, 1500 Littleton Rd., Parsippany, NJ 07054, USA

Abstract

Differential scanning calorimetry (DSC) was used, in conjunction with two functional assays that monitor binding, in a storage stability study on a protein of pharmaceutical interest, the soluble form of the T-lymphocyte multidomain surface receptor, sCD4. DSC monitored structural changes in binding and non-binding domains. ELISA, using the monoclonal antibody OKT4a, and frontal elution affinity chromatography, using the HIV surface glycoprotein, gp120, monitored function of the binding domain. The stability of sCD4 in a solution formulation was followed for up to 30 days at five different *pHs* ranging from 5.0 to 7.9 and five different temperatures ranging from –70°C to 40°C. While the overall trends observed with the three techniques were the same, the ELISA data were somewhat less reproducible than those for the other methods. Furthermore, the results suggest that DSC is more sensitive to structural changes that would reduce the protein's bioactivity. The results of this study indicate DSC's utility, in conjunction with quantitative functional analysis, in the formulation of protein-containing pharmaceuticals or foods, especially those containing multiple-domain proteins.

Keywords: affinity chromatography, biopharmaceutics, conformational epitope, differential scanning calorimetry, ELISA, gp120, multidomain protein, OKT4a, protein stability, sCD4

Introduction

The determination of optimal storage conditions and shelf life for protein pharmaceuticals requires sensitive and reliable assays with which stability to

* This work was supported by NIH Grant AI32687.
** Author to whom all correspondence should be addressed.

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various treatments can be determined. The quantitation of bioactivity, the most critical assay of stability, lacks information regarding the mechanism of inactivation and, in many cases, is less reproducible than simpler, physical measurements. Furthermore, it is simply not feasible to directly measure the bioactivity of many proteins that are not enzymes, and alternative methods that are proven to be correlated with bioactivity are needed.

Maintenance of the native tertiary structure is necessary for bioactivity, and hence, a measure of native structure can be used for the determination of stability. Both physical (conformational changes including denaturation, aggregation and precipitation) and many chemical modifications of proteins which lead to loss of bioactivity (because they also lead to or result from conformational changes) can be detected and quantified by analyzing the equilibrium between the folded and unfolded states of the protein as a function of some perturbant, such as chaotropic agents (e.g., urea) or temperature. Not only is information on the thermodynamic stability of the protein's native conformation acquired in this manner [1, 2], but the same experiment can be used to quantitate the amount of native or correctly folded protein present, as it is used in the study reported here on recombinant soluble CD4 (sCD4).

The AIDS virus infection is initiated by the binding of the HIV-1 envelope protein, gp120, to the T-cell surface receptor, CD4. Inhibition of this interaction was the target of drug intervention using the recombinant form of the extracellular domain, sCD4, as a receptor decoy [3, 4]. While clinical trials on sCD4 were disappointing for a number of reasons, physicochemical studies on sCD4 have been useful to demonstrate and validate the use of novel techniques to determine the stability of biopharmaceuticals. The determination of solution conditions that maximize stability is a challenging problem for a protein such as sCD4, since it is a rod-shaped molecule composed of four structural domains arranged in tandem. These domains are denoted D1 through D4, starting with the domain furthest from the membrane [5–8]. Consequently, unlike many globular proteins, sCD4 folding is not two-state, meaning that stabilization of one domain does not necessarily translate to the rest of the molecule [2]. High-sensitivity DSC has become an established technique of great utility in determining the conformational thermodynamic stability of proteins [9–11]. In the present study, we describe a multiple temperature and *pH* stability study in which data from DSC, used to quantitate the presence of native sCD4 by monitoring the heat absorbed when it thermally unfolds, were compared with results obtained from two in vitro functional assays (i.e., solid-phase binding) for sCD4, monoclonal antibody binding in a standard ELISA and HIV gp120 binding by frontal elution affinity chromatography [12]. The stability of sCD4, monitored by these three techniques, was followed for up to 30 days at five different *pH*s ranging from 5.0 to 7.9 and five different temperatures ranging from

–70°C to 40°C. The results demonstrate that stability data obtained from DSC and binding assays provide synergistic and reliable indicators of sCD4 function.

Materials and methods

Preparation of sCD4 and design of stability study

Recombinant human sCD4 (residues 1–369) was expressed in CHO cells and purified as described previously [13, 14]. Protein concentration was determined from the absorbance at 280 nm, using an extinction coefficient of 1.45 mL mg^{-1} cm^{-1} [2]. To insure complete equilibration to the solution conditions, purified sCD4 was dialyzed into the buffered solutions to be studied: 50 *mM* 3-(-N-morpholino)-propanesulfonic acid (MOPS) *pH* 7.9, *pH* 7.1, 50 *mM* 2-(N-morpholino)-ethanesulfonic acid (MES) *pH* 6.3, 50 *mM* sodium acetate ($NaC_2H_3O_2$) *pH* 5.5, and *pH* 5.0. Aliquots of 2 mL of dialyzed sCD4 samples were sterile-filtered into ampoules, at an sCD4 concentration of 114 μ*M* (5 mg mL^{-1}), and stored at –70°C, –20°C, 5°C, 25°C, or 40°C for 3, 6, 10, or 20 days, in most cases. At the conclusion of the allotted storage time, the sample was transferred to a 5°C condition and assayed for activity, within 48 h for samples at intermediate *pH*s, and within 24 h for samples at the *pH* extremes (5.0 and 7.9). It was previously determined (and can be seen from Fig. 3) that no significant change in activity occurs at 5°C within this time period of the stability study.

Differential scanning calorimetry

Samples were prepared for DSC by first centrifuging solutions to remove any precipitate that might be present (all samples were spun, but only a few, incubated at 40°C for extended time periods, had a visible pellet afterwards), followed by dilution with dialysate buffer to bring the protein concentration in the range from 35 μ*M* to 65 μ*M*. After brief deaeration under water-aspirator vacuum, the samples were loaded into the calorimeter; the cell volume was 1.2 mL.

Calorimetry was performed on a high-sensitivity MC-2 differential scanning calorimeter (Microcal, Inc., Northampton, MA). Data were collected from 10–90°C at ca. 60°C h^{-1}. Data collection and analysis were accomplished with a 386 PC, using software provided by the manufacturer. The instrument is calibrated by the manufacture and calibration constants for correct determination of temperature and heat capacity are provided. The calibration constants are regularly checked. For temperature, if the measured melting points of two standards differ by more than 0.2°C from their reported values (28.2 and 78.9°C), the x-axis calibration constant is changed. An internal calibration pulse is used to

calibrate heat capacity; if the measured value differs by more than 2% of the entered value, then the y-axis calibration constant is changed. After baseline and concentration normalization, the excess heat capacity curve was integrated to determine the area under the endothermic transition, i.e., the unfolding enthalpy. The denaturation temperature (T_m) was determined from the maximum of the heat capacity curve. The percent remaining native structure was obtained by first dividing the unfolding enthalpy measured on the sample stored at a given condition by that obtained on day 0 at 5°C and the same *pH*; this fraction was then multiplied by 100.

Analytical affinity chromatography

Affi-Prep 10 is a macroporous affinity chromatography matrix containing N-hydroxysuccinimide esters at the end of a 10-atom spacer arm, which couple to primary amines spontaneously in aqueous solution. To immobilize HIV gp120, 3mL of Affi-Prep 10 were washed with 200 mL of cold 10 m*M* sodium acetate, *pH* 4.5. Three mg of gp120 in 10 mM HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), *pH* 7.4, were mixed with the gel slurry and agitated gently on a shaker for 1 h at room temperature, followed by incubation overnight at 4°C. The primary amines on gp120 react spontaneously with the active N-hydroxysuccinimide esters on the gel; remaining active esters on the resin were blocked by incubation for 1 h in the presence of 0.1 *M* ethanolamine-HCl, *pH* 8.5. The resin was washed with 10 mL of 0.5 *M* NaCl, and 1 mL was packed into an Omni glass analytical column with an internal diameter of 6.6 mm. The column was attached to a Beckman System Gold HPLC which consisted of a 116-solvent module and a 406-analog interface. All chromatography experiments were performed at room temperature and in buffer containing 150 m*M* NaCl and 10 m*M* sodium phosphate, *pH* 7.4. Column effluent was monitored at 215 nm with an Applied Biosystems 785A absorbance detector.

Samples of sCD4 were prepared by first centrifuging solutions to remove any precipitate (none was visibly detected in the samples subjected to affnity chromatography), followed by dilution with chromatographic buffer to bring the protein concentration to 120 m*M*. 30 mL of the sCD4 solutions were continuously applied to the gp120 resin at a flow rate of 200 μL min^{-1}. Between each chromatography run, the column was washed with 0.5 mL of 100 m*M* phosphoric acid, at a flow rate of 0.5 mL min^{-1}, to remove any remaining sCD4 and to regenerate an active gp120 surface. The data from each storage condition were analyzed by first normalizing the elution profiles to a percent of the maximum absorbance achieved by the end of the elution profile (see righthand axis of Fig. 5). The percent active protein in each sample subjected to a specific storage condition was then obtained from the following equation,

$$\% \text{ Active protein} = 100 \frac{c - a}{c - b}$$

using normalized absorbance values for the sample (dashed line) and control (solid line), as indicated by points "a" "b" and "c" in Fig. 5 as an example. The expression "c-a" equals the fractional change in absorbance in the retained chromatographic plateau of the sample, and "c-b" is the fractional absorbance change in the retained chromatographic plateau of the control on day 0 at 5°C and the same *pH*.

Enzyme-linked immunosorbant assay (ELISA)

An automated ELISA [15] was also used to determine the activity of the sCD4 samples subjected to different storage conditions. In this assay, 96-well microtiter plates were coated with the primary antibody, a goat anti-mouse IgG, and incubated overnight at 4°C. The plates were then washed with phosphate buffered saline (PBS) containing 0.05% Tween 20. OKT4a, a murine monoclonal antibody directed against sCD4, was then pipetted onto the plates, and the coated plates were incubated overnight at 4°C. The plate was then washed with PBS and blocked with 0.5% bovine serum albumin in PBS at 37°C for 30 min. After removal of the blocking solution from the plate, sCD4 samples and standards were added to the plate, and the plate was incubated again for one hour at 37°C. Horseradish peroxidase-conjugated rabbit anti-sCD4 IgG was added to the plate, followed by incubation for one hour at 37°C. The plate was then washed with PBS, O-phenylenediamine substrate solution was added, and the reaction was quenched with sulfuric acid after 7 min. The absorbance of each well was measured at 492 nm, based on a reference of 620 nm. The active sCD4 concentration in each sample was calculated relative to an internal sCD4 standard, and the percent active sCD4 reported in Fig. 7 was obtained by dividing the concentration by that obtained on day 0 at 5°C and the same *pH* and multiplying by 100.

Results and discussion

DSC

The temperature midpoint (T_m) and enthalpy (ΔH) of sCD4 unfolding were measured by DSC. A typical excess heat capacity profile obtained at *pH* 7.1 is shown in Fig. 1. The measured ΔH for this sample, which contained 64.8 nmoles of sCD4 (concentration =51 μM), was 9.6 mcal (148.2 kcal mol^{-1}), and the T_m was 59.0°C. The good correspondence between the amount of native protein assayed by DSC and the measured ΔH for the sample is seen in the plot shown in

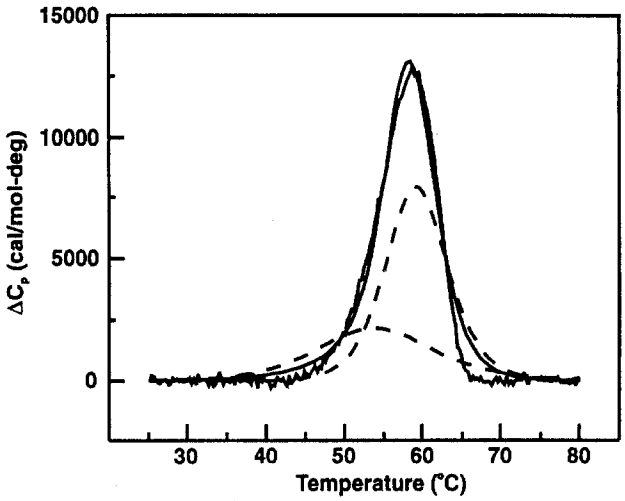

Fig. 1 Excess heat capacity profile for sCD4, 51 μ*M*, *pH* 7.0. A calculated three-state sequential unfolding model (smooth solid line) was found to fit the raw data (solid line) better than did a simple two-state model. The dashed peaks are the two constituent transitions of the calculated curve

Fig. 2 for protein amounts ranging from 15.8 nmol (0.7 mg) to 620 nmol (27.3 mg). Similar correspondence has been observed with other proteins [16]. Error associated with the ΔH measurement can arise from several sources that include

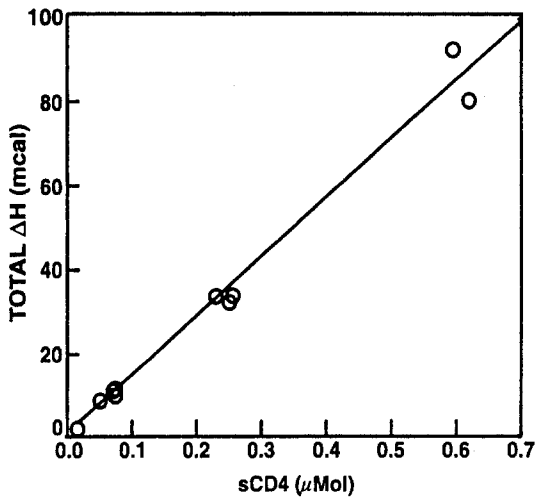

Fig. 2 Measured total unfolding enthalpy as a function of the sCD4 amount. DSC was performed on samples ranging from 15.8 nmol to 620 nmol at *pH* 7.0. The integrated area of the endothermic transition, as illustrated in Fig. 1, is plotted *vs.* the amount of sCD4 assayed. The fitted regression line is shown (r^2=0.992; y-intercept=0.051 mcal)

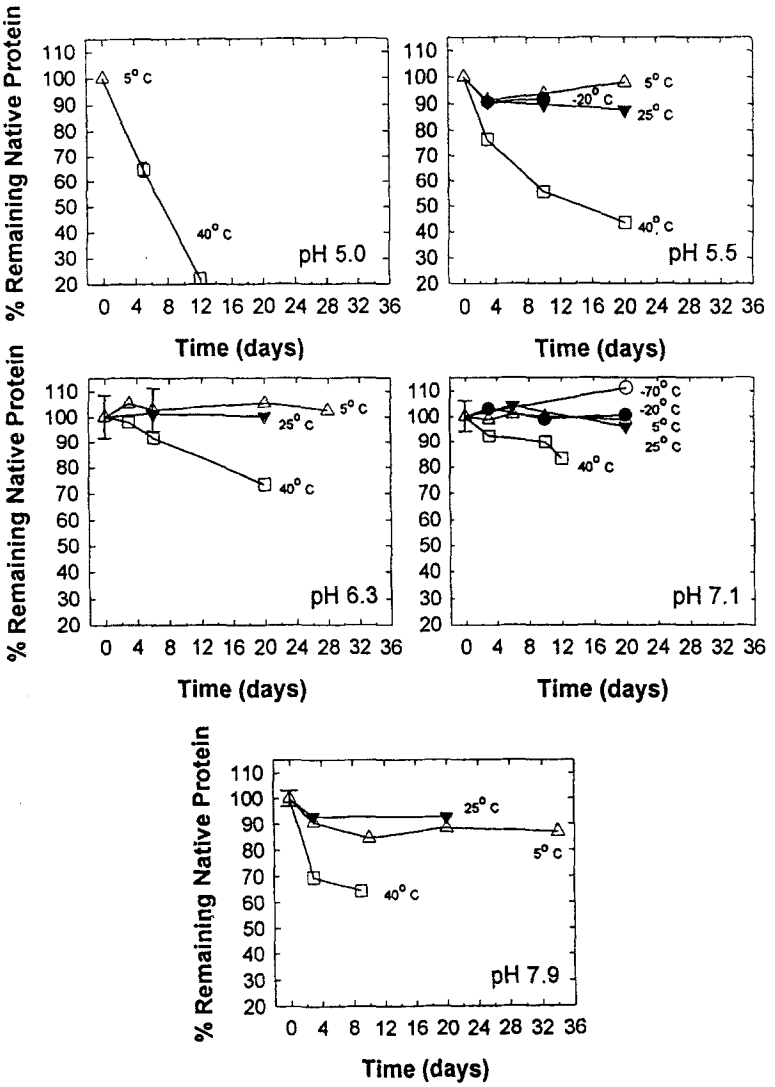

Fig. 3 Stability of sCD4 as a function of time, obtained by DSC. Each panel represents measurements on samples at a single *pH*. The different incubation temperatures are represented by the following symbols: o (–70°C), • (–20°C), Δ (5°C), ▼ (25°C), □ (40°C). Where shown, error bars represent the range of values obtained in two to three replicates

the protein concentration determination, the selected baseline for the transition, as well as the intrinsic instrument sensitivity, which is quoted by the manufacturer to be 10 μcal/degree. For the data shown in Fig. 2, the mean and standard error of the measured unfolding ΔH are 142±5 kcal mol^{-1}. Based on the instrument's sensitivity and our own experience, the estimated detection limit is about

4 nmol sCD4 (ca. 600 μcal), which indicates that for the storage stability experiments described here, a loss of native protein of as little as 5% should be detectable.

Samples of sCD4 were stored at various temperatures and *pH*s for specified time periods, up to 34 days in some instances. The amount of native protein remaining in each sample was determined by measuring the unfolding enthalpy of the sample and normalizing it to the enthalpy change determined at day 0 and 5°C for the particular *pH* studied. The results are summarized in Fig. 3, which shows five panels, one for each *pH* studied. The *pH* values for this study were selected based on previous measurements which indicated that sCD4 is maximally stable *vs.* thermal denaturation at $pH \approx 6.5$–7.5 (Fig. 4); hence, it was of interest to determine if differences in storage stability within this *pH* range were detectable. At the intermediate *pH*s (5.5–7.1), sCD4 showed no significant change in stability up to 28 days of incubation at temperatures ranging from −70°C to 25°C. However, incubation at 40°C resulted in a significant loss of native protein as early as 3 days in the *pH* 5.5 and 7.1 samples. At the *pH* extremes (5.0 and 7.9), more dramatic losses of native structure were observed over the study's time course, and changes were observed at earlier incubation times as well. These results are consistent with the overall lower thermal stability of sCD4 at these *pH*s (Fig. 4).

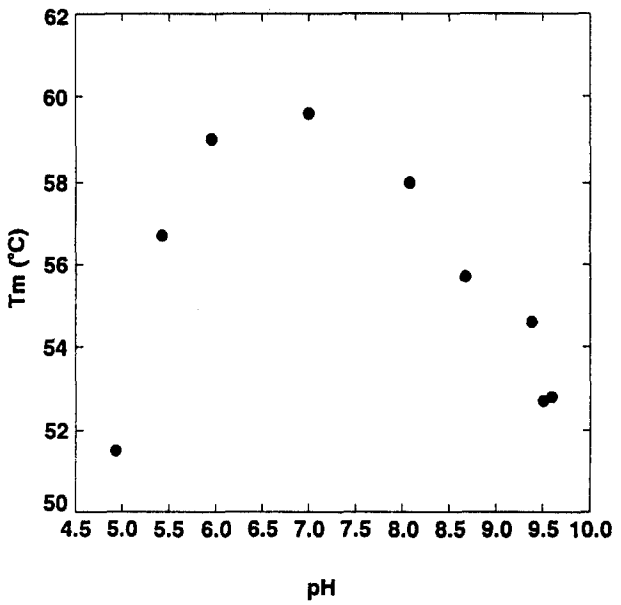

Fig. 4 Unfolding T_m of sCD4 as a function of solution *pH*. The T_m was determined by DSC at a scan rate of 60°C h^{-1}

Analytical affinity chromatography

While calorimetry provides information on the overall structure of sCD4, assays that focus on an activity, such as the presentation of a binding site, could be more sensitive to subtle changes in conformation, although for sCD4, they are limited to information on a single domain. The binding site for the HIV glycoprotein gp120 is in D1. This epitope is conformationally sensitive which means that D1 must be correctly folded to bind to gp120. Hence, the affinity of sCD4 for gp120 reports on the folded state of sCD4's domain 1. For a given sample, to determine the amount of sCD4 active for gp120 binding, analytical affinity chromatography frontal elution profiles were obtained with an Affi-Prep 10 resin containing immobilized gp120. A typical chromatogram of a sample stored for 20 days at *pH* 5.5 and 5°C (dashed line) and the corresponding day 0 control (solid line) are shown in Fig. 5. Two plateaus are apparent in each run. For the control, the earlier plateau is most likely nonproteinacious material present in the samples, which absorbs at 215 nm but does not interact with gp120. The increase in the level of this plateau for the sample indicates the added presence of incorrectly folded sCD4. The later plateau present in each chromatogram represents correctly folded sCD4 that, due to its interaction with immobilized gp120 on the column, shows a delayed elution time.

Figure 6 shows the results of the stability study monitored by affinity chromatography. The results obtained by this method are very similar to those obtained by DSC, with the possible exception of the 40°C data at *pH*s 5.5 and 7.9, which show slightly more apparent native protein than do the DSC results

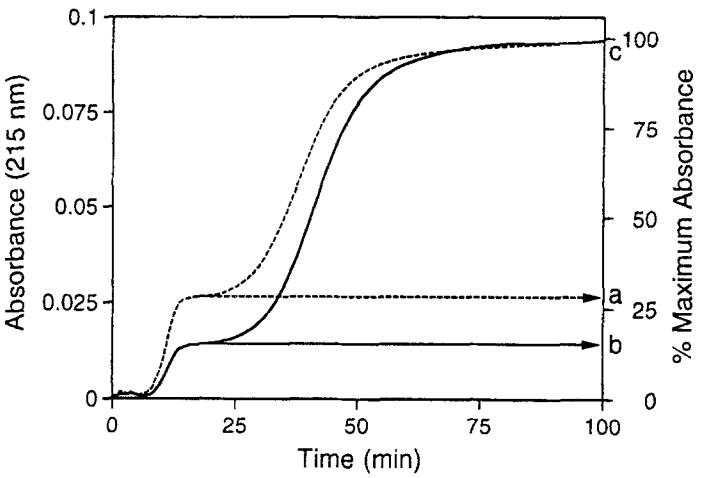

Fig. 5 Analytical gp120 Affinity Chromatography of sCD4. Frontal elution profiles obtained by monitoring effluent at 215 nm; the control (solid line) was obtained from a day 0 sample stored at *pH* 5.5 and 5°C; an sCD4 sample stored for 20 days at *pH* 5.5 and 5°C is also shown (dashed line)

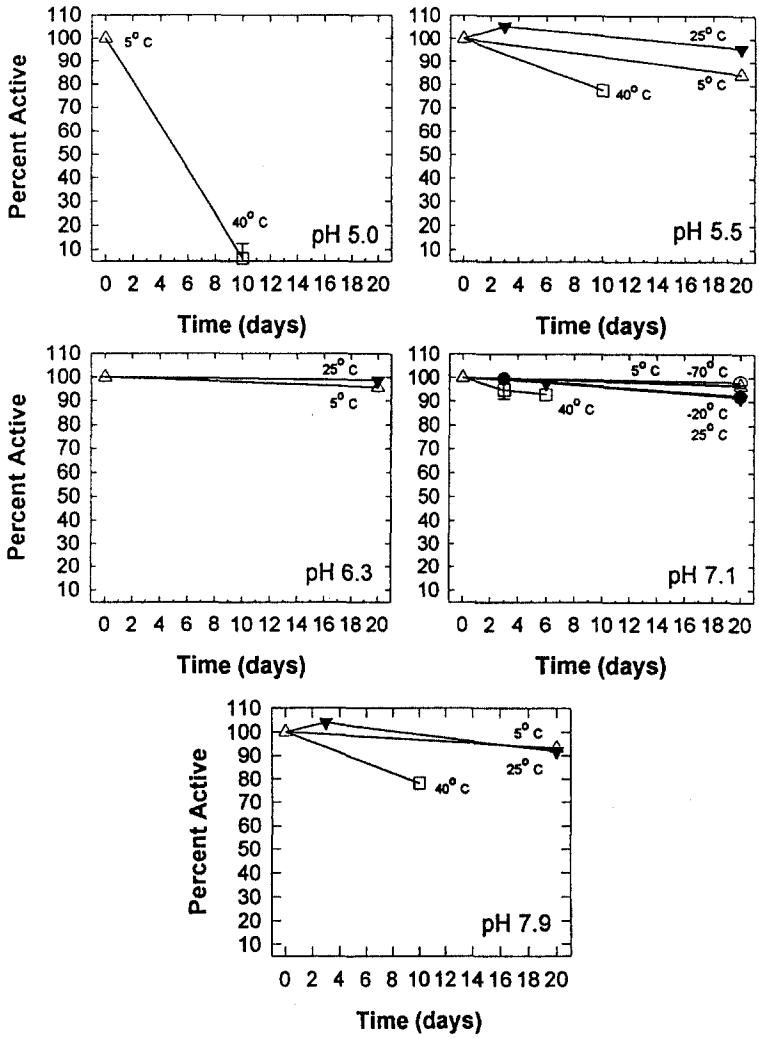

Fig. 6 Stability of sCD4 as a function of time, obtained by Analytical gp120 Affinity Chromatography. This figure is set up similarly to Fig. 3, with the same symbols used for the various incubation temperatures

under these conditions. Two possible explanations for this observation exist. The binding of gp120 to *folded* sCD4 could increase the amount of sCD4 in the folded state at any given temperature by shifting the equilibrium between the folded and unfolded states, a result of Le Chatelier's principle [17]. The second possibility is that D1 is thermally more stable than the rest of the protein. Deconvolution of the DSC endotherm supports the notion that the protein unfolds in two steps, with one portion of the protein exhibiting greater thermal stability

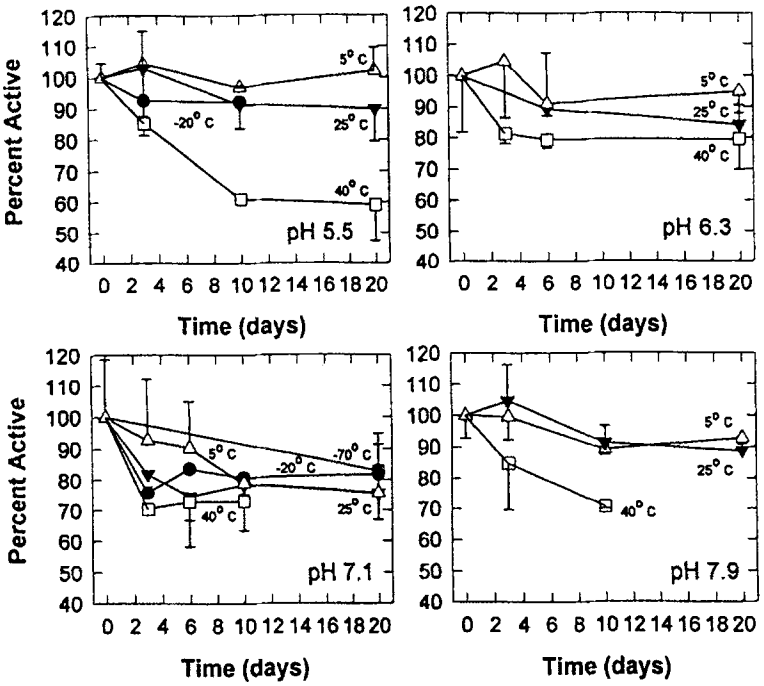

Fig. 7 Stability of sCD4 as a function of time, obtained by ELISA. This figure is set up similarly to Fig. 3, with the same symbols used for the various incubation temperatures

than the remaining portion (Fig. 1). Equilibrium isothermal unfolding confirms the interpretation of the DSC data that sCD4 unfolding is not 2-state and likely unfolds in two steps (2). DSC of a D1D2 recombinant protein suggests that it is within the second, higher temperature unfolding transition observed in sCD4 that D1 unfolds (unpublished observation). Therefore, at temperatures that induce unfolding of the remainder of the protein, D1 may still be folded and able to bind to gp120.

ELISA

Like the epitope for gp120, the epitope for OKT4a, the monoclonal antibody used in this assay, is conformationally sensitive. The primary sequence of this epitope partially overlaps with that for gp120 [18]. Interestingly, the ELISA data, shown in Fig. 7, seemed more sensitive to changes in structure at 40°C and, in general, were consistent with the DSC data, although the standard deviation in the measurements was greater. It is known that the binding site for gp120 is small and highly defined, with phenylalanine 43 playing a particularly important role [6]. If the OKT4a epitope were larger, it would be expected to be more sensitive to overall structure, and this greater sensitivity could translate

into a greater sensitivity to temperature-induced conformational changes. A discernible difference in the results obtained at *pH* 7.1 was also seen. Since the results of the DSC and gp120-binding data were comparable at this pH, there is no ready explanation for the discrepancy of the ELISA data. It is possible, however, that the concentration reading of the day 0 time point was spuriously high compared to subsequent readings, which would cause the subsequent readings to appear as if native protein had been lost. In fact, the average concentration obtained for this particular control assay, before normalization, was higher than that for the controls obtained at the other pHs (day 0 at *pH* 7.1 =6.3 mg mL^{-1}, compared to a range of 4.8–5.6 mg mL^{-1} for *pH* 5.5, 6.3 and 7.9).

Conclusions

DSC has been shown to be a useful and convenient assay which can aid in the development of solution formulations for protein-containing pharmaceuticals and foods [16, 19]. Storage stability is a critical criterion in the formulation of a protein pharmaceutical, and this report is the first, to our knowledge, to describe its use to follow the long-term stability of a protein under various storage conditions. The DSC experiment requires minimal amounts of protein and is not labor-intensive (hands-on time for a single experiment is less than four hours). While DSC cannot be automated (vis-à-vis ELISA), the results are highly reproducible and generally applicable to any protein. Furthermore, multidomain proteins offer a particular challenge, because most functional assays are specific for a single domain. Partially folded byproducts resulting from long-term storage may be less likely to be detected, as suggested by the comparison of gp120-binding and DSC data shown here. In fact, the use of binding assays, in general, suffers from the possibility that the ligand will shift the equilibrium between the folded and unfolded species. Finally, the potential to obtain thermodynamic parameters on a protein's stability enables the quantitative extrapolation of stability results to untested storage conditions, which is information unattainable from functional assays.

References

1 "Section I. Unfolding and Refolding of Proteins," in Meth. Enzymol., 131 (1986) 3.
2 S. W. Tendian, D. G. Myszka, R. W. Sweet, I. M. Chaiken and C. G. Brouillette, Biochemistry, 34 (1995) 6464.
3 R. W. Sweet, A. Truneh and W. A. Hendrickson, Curr. Opin. Biotech., 2 (1991) 622.
4 J. P. Moore and R. W. Sweet, Perspectives in Drug Discovery and Design, 1 (1993) 235.
5 S. E. Ryu, P. D. Kwong, A. Truneh, T. G. Porter, J. Arthos, M. Rosenberg, X. Dai, N.-H. Xuong,R. Axel, R.W. Sweet and W. A. Hendrickson, Nature, 348 (1990) 419.
6 S.-E. Ryu, A. Truneh, R. W. Sweet and W. A. Hendrickson, Structure, 2 (1994) 59.
7 J. Wang, Y. Yan, T. P. J. Garrett, J. Liu, D. W. Rodgers, R. L. Garlick, G. E. Tarr, Y. Husain, E. L. Reinherz and S. C. Harrison, Nature, 348 (1990) 411.

8 T. P. J. Garrett, J. Wang, Y. Yan, J. Liu and S. C. Harrison, J. Mol. Biol., 234 (1993) 763.
9 P. L. Privalov and N. N. Khechinashvili, J. Mol. Biol., 86 (1974) 665.
10 P. L. Privalov and S. A Potekhin, Meth. Enzymol., 131 (1986) 4.
11 J. M. Sturtevant, Ann. Rev. Phys. Chem., 38 (1987) 463.
12 I. Chaiken, S. Rose and R. Karlsson, Anal. Biochem., 201 (1992) 197.
13 K. C. Deen, S. McDougal, R. Inacker, G. Folena-Wasserman, J. Arthos, M. Rosenberg, P. J. Maddon, R. Axel and R. W. Sweet, Nature, 331 (1988) 482.
14 J. Arthos, K. C. Deen, M. A. Chaikin, J. A. Fornwald, G. Sathe, Q. J. Sattentau, P. R. Clapham, R. A. Weiss, J. S. McDougal, C. Pietropaolo, R. Axel, A., Truneh, P. J. Maddon and R. W. Sweet, Cell, 57 (1989) 469.
15 D. Schrader, M. B. Seaman, A. L. Shorter and R. Roman, Laboratory Robotics and Automation J., 133 (1991)
16 K.-I. Izutsu, S. Yoshioka and Y. Takeda, Chem. Pharm. Bull., 38 (1990) 800.
17 H. Fukada, K. Takahashi and J. M. Sturtevant, Biochemistry, 26 (1987) 4063.
18 Q. J. Sattentau, J. Arthos, K. C. Deen, M. Hanna, D. Healey, P. Beverley, R. W. Sweet and A. Truneh, J. Exp. Med., 170 (1989) 1319.
19 S. De Cordt, I. Avila, M. Hendrickx and P. Tobback, Biotech. and Bioeng., 44 (1994) 859.