Published in final edited form as:

Nat Protoc. 2006 ; 1(6): 2527–2535. doi:10.1038/nprot.2006.204.

Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions

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Abstract

Circular dichroism (CD) is an excellent spectroscopic technique for following the unfolding and folding of proteins as a function of temperature. One of its principal applications is to determine the effects of mutations and ligands on protein and polypeptide stability. If the change in CD as a function of temperature is reversible, analysis of the data may be used to determine the van't Hoff enthalpy (ΔH) and entropy (ΔS) of unfolding, the midpoint of the unfolding transition (T_M) and the free energy (ΔG) of unfolding. Binding constants of protein-protein and protein-ligand interactions may also be estimated from the unfolding curves. Analysis of CD spectra obtained as a function of temperature is also useful to determine whether a protein has unfolding intermediates. Measurement of the spectra of five folded proteins and their unfolding curves at a single wavelength takes approximately eight hours.

INTRODUCTION

There are numerous reviews on the theory and use of circular dichroism (CD)¹⁻⁷ as well as a website with animated graphics <http://www.enzim.hu/~szia/cddemo/>, which illustrates the production of circularly polarized light, the circular dichroism of optically active molecules, and how passing plane polarized light through an asymmetric molecule results in the production of elliptically polarized light. An introduction to CD and a description of how to estimate the secondary structure of a protein from its CD spectrum are in the first protocol in this series⁸. Briefly, circular dichroism is defined as the difference between the absorption of left-handed, ϵ_R , and right-handed, ϵ_L , circularly polarized light. CD is reported either in units of $\Delta\epsilon$, the difference in absorbance of ϵ_R and ϵ_L by an asymmetric molecule, or in degrees ellipticity, which is defined as the angle whose tangent is the ratio of the minor to the major axis of the ellipse. $[\theta]$, the molar ellipticity in $\text{deg} \cdot \text{cm}^2/\text{dmol} = 3298\Delta\epsilon$. For illustrations of the phenomena of CD see Beychok².

When proteins are folded they often have highly asymmetric secondary structural elements, such as α -helices and β -pleated sheets (β -helices), which have characteristic CD spectra (Figure 1). The spectra of some highly helical model proteins are shown in Figure 2. When proteins

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http://faculty.umdj.edu/neuroscience/faculty_greenfield.asp

SUPPLEMENTAL MATERIALS

Two files are in supplemental materials. Equations.doc has routines in SigmaPlot™ formats for fitting a variety of unfolding/folding curves for proteins with various numbers of subunits, e.g. monomers, dimers, trimers, etc. to estimate the enthalpy, T_M of and heat capacity changes of folding. CCA.doc has instructions for using DOS versions of the convex constraint algorithm for deconvoluting sets of CD spectra. A copy of the CCA algorithm that works in a command window in Windows 95, 98 and XP is also supplied.

unfold they lose these highly ordered structures and the CD bands change. Some proteins tend to unfold completely and their unfolded spectrum resembles that of a mixture short polypeptides (e.g. see the spectrum of unfolded collagen, Figure 1). Other proteins and polypeptides only partially unfold and may have considerable residual alpha helical content in a “molten globule state”⁹⁻¹³. The changes in CD as a function of temperature, at characteristic wavelengths, can be used to determine the thermodynamics of unfolding (or more precisely change of state), i.e., the van't Hoff enthalpy (ΔH) and entropy (ΔS) of unfolding, the midpoint of the unfolding transition (T_M) and the free energy (ΔG) of folding. If the apparent T_M of unfolding is changed as a function of protein concentration or perturbed by changes in salt or pH, the heat capacity change of unfolding (ΔC_p) can also be estimated from CD data. In addition, analysis of CD spectra obtained as a function of temperature may also be useful in determining whether a protein has unfolding or folding intermediates.

The thermodynamics of protein unfolding can be investigated by either monitoring the ellipticity at a single wavelength (A) or collecting complete spectra (B) as a function of temperature. Two additional protocols on the use of CD to study protein folding cover the determination of the free energy of folding and binding constants from CD data collected as a function of denaturants, osmolytes or ligands¹⁴ and determination of the kinetics of folding from CD data collected as a function of time¹⁵.

(A) Characterization of unfolding by following the change in ellipticity at a single wavelength as a function of temperature

When the CD of a protein and polypeptide or other asymmetric polymer such as a polynucleotide or polysaccharide changes as a function of temperature, the change can be utilized to analyze the thermodynamics of unfolding or folding. In the simplest case a molecule undergoes an unfolding transition between two states: folded, F, and unfolded, U. At any temperature, T, the constant of folding, K, is:

$$K = [F] / [U] \quad (1)$$

[F] and [U] are the concentrations of the folded and unfolded forms respectively. The free energy of folding is

$$\Delta G = -RT \ln K \quad (2)$$

R is the Gas constant = 1.98 cal/mol, and T is the absolute temperature (Kelvin).

The fraction folded at any temperature is α .

$$\alpha = [F] / ([F] + [U]) \quad (3)$$

$$\alpha = (\theta_t - \theta_U) / (\theta_F - \theta_U) \quad (4)$$

θ_t is the observed ellipticity at any temperature, θ_F is the ellipticity of the fully folded form and θ_U is the ellipticity of the unfolded form.

To fit the change of CD at a single wavelength as a function of temperature, T (Kelvin), one uses the Gibbs-Helmholtz equation that describes the folding as a function of temperature. If one is following the unfolding of a monomer, the following equations (7-10) are fit:

$$\Delta G = \Delta H (1 - T/T_M) - \Delta C_p ((T_M - T) + T \ln (T/T_M)) \quad (5)$$

$$K = \exp (-\Delta G / (RT)) \quad (6)$$

$$\alpha = K / (1 + K) \quad (7)$$

$$[\theta]_t = \alpha ([\theta]_F - [\theta]_U) + [\theta]_U \quad (8)$$

T_M is the temperature where $\alpha = 0.5$ and ΔC_p is the change in heat capacity going from the folded to the unfolded state. ΔC_p is usually set at 0 for the initial calculations of the thermodynamics of folding of a monomeric protein.

Initial values of ΔH , T_M , $[\theta]_F$ and $[\theta]_U$ are estimated and used as initial parameters for nonlinear least squares fitting routines. The values giving the best fit to the raw data are found using the fitting procedures found in the commercial curve fitting programs described in **Materials**. One can fit the unfolding of homo and heterodimers and trimers using similar equations^{16,17}. If a protein exhibits multiple unfolding transitions, CD data can be fit by the sum of multiple transitions.

Occasionally there are regions in the curves of ellipticity as a function of temperature, preceding or following the unfolding transitions, where the ellipticity shows a change that is almost linear as a function of temperature. One can add linear¹⁸ or parabolic¹⁹ corrections to the equations to subtract these contributions. The entropy of unfolding, ΔS , at the temperature where $K=1$, can be calculated using the relationship: $\Delta G = \Delta H - T\Delta S$. Equations for fitting unfolding/folding curves in SigmaPlot format are available at <http://www2.umdj.edu/cdrwjweb> and in SUPPLEMENTAL MATERIALS in the file equations.doc.

ΔC_p is difficult to estimate from CD measurements for a monomer because there isn't enough information in a single unfolding curve, obtained under one set of conditions, to estimate this parameter¹⁹. For multimers, ΔC_p can be determined from global analysis of unfolding as a function of both concentration and temperature²⁰⁻²⁴. If the apparent T_M of unfolding ($\alpha = 0.5$) of a monomer changes as a function of pH or other perturbant, the ΔC_p of unfolding can be evaluated from the slope of the plot of the enthalpy at the apparent T_m , obtained under each condition, as a function of the T_m (Kelvin). This value can then be used as fixed parameter for determining the enthalpy and T_M of unfolding using equations 5-8.

Direct fitting of unfolding curves is easy for monomers, dimers and trimers (Figure 3a), but it is difficult for higher order multimers. An alternative method is to determine the midpoints of the unfolding curves by taking derivatives of the spectra (Figure 3b) and to determine the enthalpy of unfolding using the van't Hoff equation (Figure 3c). At two different temperatures, T_1 and T_2 :

$$\ln (K (T_2) / K (T_1)) = \Delta H / R (1 / T_1 - 1 / T_2) \quad (9)$$

To determine the enthalpy of unfolding from van't Hoff analysis, the natural logarithm ($\ln$) of the folding constant, K is plotted as a function of $1/T$ where T is the absolute temperature (Kelvin). The data is truncated to the region around the region giving a linear curve with the highest slope. The enthalpy of unfolding equals the slope of the line times the gas constant, R , which is equal to 1.98 cal/mol.

(B) Characterizing the unfolding of a protein by examining spectra as a function of temperature

The information about the unfolding pathway is greatly increased by collecting full spectra as a function of temperature. The set of spectra can then be deconvoluted using either the convex constraint algorithm²⁵⁻²⁷ or singular value decomposition²⁸⁻³¹ to get information about the number of folded states contributing to the set of spectra. The change in the fraction of each basis spectra determined using the CCA algorithm, contributing to the total CD as a function of temperature, may also be used to estimate the thermodynamics of unfolding.

Comparison with related methods—When the unfolding of a protein is reversible and two-state and there is only a single unfolding transition, the thermodynamic parameters evaluated by CD are almost identical to those that can be estimated using scanning calorimetry^{32,33}. When there are multiple unfolding transitions, CD and scanning calorimetry will usually give the same total enthalpy of unfolding, but the value of the enthalpy of the individual transitions may vary due to the difficulty of separating overlapping transitions. For an example of the unfolding of tropomyosin and tropomyosin mutants, which have closely spaced multiple reversible unfolding transitions, see Singh and Hitchcock-DeGregori³⁴.

CD and other spectroscopic measurements, such as measuring changes in absorption and fluorescence will give similar results if the unfolding of the protein or polypeptide represents a two-state transition between totally folded and unfolded forms. Different methods may give varying results if there are multiple transitions. For example, if a region with aromatic chromophores loses its tertiary structure packing before the molecule loses all of its secondary structure, examining the unfolding by looking at the CD of the aromatic region, or the fluorescence or aromatic groups, will give different thermodynamic parameters than examining the CD of backbone α -helices by following the unfolding at 222 nm^{20,35}. For the most accurate determination of protein unfolding thermodynamics and pathways, it is always preferable to use multiple methods.

Applications—Measuring CD as a function of temperature can be used to determine: the effects of mutations on protein stability, as well as the binding constants of interacting proteins and protein-ligand complexes.

CD can be used to determine whether the T_M or slope of a transition changes when mutations are present. If the unfolding is reversible, true thermodynamic parameters can be obtained. Even if the unfolding is irreversible, because the protein aggregates at high temperature, the method can still give information about relative stability, especially if the same apparent unfolding parameters are obtained at different rates of heating, showing that kinetics of aggregation are not perturbing the results.

If two proteins bind to each other only when they are folded, and the protein complex unfolds cooperatively and reversibly, one can use the change in stability upon binding to measure the binding constant. In the simplest case both proteins (e.g. X and Y) are monomers, $[X_F]$ and $[Y_F]$ are the concentrations of folded X and Y and $[X_U]$ and $[Y_U]$ are the concentrations of unfolded X and Y , respectively.

The constant of folding of protein X is $k_{FX} = [X_F] / [X_U]$. (10).

The constant of folding of protein Y is $k_{FY} = [Y_F] / [Y_U]$ (11).

The constant of folding of the dimer is $k_{FXY} = [XY_F] / [X_U][Y_U]$ (12).

The association constant $K_A = [XY_F] / [X_F][Y_F]$ (13).

Making the substitutions: $K_A = k_{FXY} / k_{FX} k_{FY}$ (14)

To determine the association constant:

1. Determine the ΔH and ΔS of folding of the individual monomers X and Y and the stability of the dimer XY by following their ellipticity as a function of temperature or denaturant.
2. Calculate the free energy of folding at any temperature using:

$$\Delta G = \Delta H - T\Delta S \quad (15).$$

3. Determine the unfolding constant from the relationship:

$$K_F = \exp(-\Delta G/RT) \quad (16).$$

4. Divide the K_F of folding of the dimer by the K_F values of folding of the two monomers to get the binding constant for the dimer formation.

If a ligand binds to a protein only when it is folded, the increase in stability of the complex compared to the free protein can be used to estimate the binding constant. If the changes in enthalpy of folding upon binding are small, the binding constant can be evaluated using the equation:

$$\Delta T_M = (T T_0 R / \Delta H) \ln(1 + K [L]) \quad (17)$$

where ΔT_M is the change in the midpoint of the thermal denaturation curve, T and T_0 are the T_M values in the present and absence of ligand, ΔH is the enthalpy of unfolding of the protein in the presence of the ligand, K is the equilibrium for the binding of the ligand to the folded protein and [L] is the free concentration of ligand and R is the gas constant^{18,36,37}.

EXPERIMENTAL DESIGN

It is difficult to give a single protocol for collecting data as a function of temperature because the different CD instruments use different methods of controlling temperature. In some instruments temperature change is steady as a function of time (this is called a temperature

ramp). In other instruments, one sets the desired temperature increment and equilibration time at each point. Some manufacturers have sample compartments where measurements can be made on multiple samples at each temperature. The machines also all have different methods of programming the collection of data. Some allow one to make macros to collect spectra as a function of temperatures. Consult the manufacturer for details of individual instruments. The steps described in the procedure for collecting data at a single wavelength, and for collecting spectra as a function of wavelength have been used for data collection on Aviv Model 62D, Aviv Model 202 and Aviv Model 215 instruments equipped with a temperature regulated five compartment cell holder.

Preliminary experiments

Many proteins equilibrate slowly. For accurate measurements of thermodynamic parameters, it is important to set the equilibration time long enough to allow full equilibration. Preliminary experiments should be performed to determine how long it takes a protein to fold and unfold at selected temperatures and to insure that the protein will be fully unfolded at the highest temperature studied. It is suggested to take preliminary spectra at 5, 10, 25, 37 and 50, 70 deg and then take spectra at 50, 37, 25 and 10 deg to see if the protein refolds.⁸ CD, and all other spectroscopic techniques, can only be used to get true thermodynamic properties if the unfolding and folding of a protein is reversible and curves obtained when the sample is heated (for example from 10 to 70 °C) and cooled (from 70 to 10 °C) are identical. Collagen and related proteins fold and unfold very slowly and for accurate determination of the enthalpy and T_M values of folding, unfolding experiments are set to run overnight or even longer periods of time.

MATERIALS

Reagent Setup

Five to six protein samples depending on multi-cell holder rotor capacity, e.g. a single protein at different or replicate concentrations; individual proteins containing different mutations; individual proteins which interact and mixtures of interacting proteins; free proteins and proteins with different ligands added; the same protein concentration dissolved in buffers with different pH values. The preparations of protein samples, determination of protein concentration and buffers suitable for CD have been discussed previously⁸. The samples should be preincubated at the desired starting temperature to make sure it the proteins are fully folded. Most globular proteins which are active at physiological temperature will fold fully at 25 °C within minutes, but collagen-like triple helices can take several days to fold. If one is studying short protein fragments or peptides about which little is known, they should be folded on ice overnight. Folding conditions have been described in greater detail previously⁸.

To retard evaporation, melts can be done in cuvettes with Teflon stoppers. Alternatively, a drop of mineral oil may be carefully placed of the protein solutions to prevent evaporation.

Equipment

Sources of CD instruments and cuvettes and have been previously described⁸.

Software

A commercial graphic program which allows user-defined non-linear least squares curve fitting. Most of the commonly available commercial programs use the Levenberg-Marquardt algorithm³⁸ for fitting curves to non-linear equations. Graphic packages include: GraphPad, <http://www.graphpad.com/>; Psi-Plot, <http://www.polysoftware.com/aboutpsi.htm>; Origin, <http://www.originlab.com>; and SigmaPlot, <http://www.systat.com>. Some of these programs,

e.g. SigmaPlot, allow global fits to multiple parameters. Non commercial software is available to analyze the spectra of proteins obtained as a function of temperature using the convex constraint algorithm (CCA) <http://www2.chem.elte.hu/protein/programs/cca/> or <http://www2.umdj.edu/cdrwjweb/>. Commercial software that can deconvolute CD spectra using singular value decomposition is also available from some CD manufacturers, e.g. Olis and Applied photophysics, and is available as a stand alone applications from Olis <http://www.olisweb.com/software/svd.php>.

Equipment Setup

Procedures for starting CD instruments have been detailed previously⁸. Make sure that the instrument is flushed with nitrogen before firing the lamp and that circulating water baths if they are needed for cooling the lamp or acting as a heat trap for the temperature controllers are turned on before the CD software is started.

PROTOCOL

Data Collection at a single wavelength in one direction

<TIMING> 7-8 hours for collecting initial baselines and spectra and performing an unfolding study in one directions, 12 to 14 hours for unfolding and refolding.

In the following steps, suggested values are in parentheses.

- 1 Set the desired temperature to start the measurements (0).
- 2 Take initial spectra of the protein samples to determine the wavelengths that have maximal ellipticity. For example proteins with high helical content have peaks at 222, 208 and 193 nm. Unfolding studies are usually performed at the 222 nm because data at higher wavelengths usually have lower absorption and higher signal to noise.

TROUBLESHOOTING

- 3 Set the desired wavelength, e.g. 222 nm for proteins with a high content of α -helices, 218 nm for proteins with a high content of β -structure or 225 or 200 for collagen-like peptides.
- 4 Set the sample changer or rotor for the desired number of samples (5).
- 5 Set the desired temperature range (0 to 70 °C). To take spectra of the unfolded proteins after the melt, set the temperature to remain at the final temperature after the experiment is completed. If one is studying an unknown protein, and the protein is not fully unfolded by 70, one can collect a second set of data between 70 and 90°C and combine the data sets. To check whether unfolding is reversible, set the machine to collect the reverse scan from 70 deg to 0 deg using a negative increment at the completion of the run.

<Critical Step> Do not set the upper temperature greater than 90 °C, as prolonged use at high temperature may damage the temperature controller.
- 6 Set the desired temperature interval (0.2 °C).
- 7 Set the equilibration time at each point (0.2 min).
- 8 Set the temperature tolerance (0.15 °C).
- 9 Set the number of seconds to collect data at each point (5 sec) and the time constant for machine response (100 ms).

10 Set the rate of heating (5 deg/minute).

11 Start the data collection.

12 Periodically examine the progress of unfolding by looking at the unfolding curves.

13 Save the data in columns of ellipticity (y) versus temperature (x).

<CRITICAL STEP> Make sure the data is saved on the hard drive or other permanent medium, as data will be lost if the machine is turned off or the power fails before the data is saved.

<PAUSE POINT> The data collection can be ended here. If the machine has not been automatically set to return to the initial temperature, make sure to cool the machine before removing the samples to prevent burns and to be able to remove cell stoppers, which tend to stick at elevated temperatures because of difference in the expansion of the Teflon stoppers and quartz cell. If the stoppers stick in the cuvettes, plunging them upside down in an ice bath will usually loosen them.

Data Analysis

<TIMING> 8 hours

14 Import the data into a Graphics program with columns of ellipticity versus temperature and plot the curves.

15 If one has collected data both as a function of heating and cooling, plot the data sets on the same graph and see if they overlay one another.

TROUBLESHOOTING

16 If the data show only one folding transition, initially fit the data using curves describing the unfolding of a monomer using equations 7-10 described above. Set the initial values for the ellipticity of the folded and unfolded proteins by examining the graphs. Estimate the T_M from the midpoint between the starting and ending ellipticity values. Initially set the estimated enthalpy to between $-20,000$ and $-50,000$ cal/mol and the value of ΔC_p to 0. If there are multiple transitions the data can be initially fit by the sum of two or more transitions. The number of transitions and their midpoints can be estimated by taking the first derivative of the folding curve. The maxima will be at the midpoints of the folding transitions.

TROUBLESHOOTING

17 Run the curve fitting routine and plot the fitted curve to the raw data. If the agreement is good you are done. If the fits are good around the midpoint of the transition but deviate at the ends, include linear corrections for pre and post transitions (see the sample fitting curves equations.doc in SUPPLEMENTAL MATERIALS.)

18 If the same protein gives different calculated values for the T_M , which increase as a function of concentration, try fitting the data by equations which assume that the protein is forming dimers or trimers and evaluate the T_M at $K=1$ (see equations.doc in SUPPLEMENTAL MATERIALS). If the correct oligomerization state is assumed, the calculated T_M at $K=1$ should be independent of protein concentration.

TROUBLESHOOTING

19 If the protein or protein complex is a dimer or trimer, one may fit the data globally to determine the T_M at $K=1$, and ΔH and ΔC_p of folding.

Option A—Convert the data to mean residue ellipticity⁸, and fit the data to folding equations using three variables where the independent variables are temperature and protein concentration and the dependent variable is mean residue ellipticity (e.g. column 1, temperature, column 2, protein concentration, column 3, mean residue ellipticity). Equations for performing global fits are in SUPPLEMENTAL MATERIALS in the file equations.doc.

Option B—Fit the curves collected at each concentration individually using the equations for unfolding of a monomer with corrections for any pre or post transition linear changes in ellipticity. Use the parameters determined for the ellipticities of the fully folded and unfolded proteins and any linear changes as a function of temperature to convert the data to a scale of 1 to 0 where 1 is the fully folded protein and 0 is the fully unfolded protein. Then fit the data globally where the independent variables are temperature and protein concentration and the dependent variable is the fraction folded.

Collecting spectra as a function of temperature

<TIMING> 6 to 24 hours

20 Program the CD instrument to collect spectra as a function of temperature if the machine has a macro writing program using the following parameters. If the machine does not run macros, set the same parameters to collect the spectra, but the temperature will have to be changed manually.

21 Set the number of sample compartments utilized (5)

22 Set the equilibration time at each temperature (2 to 5 minutes)

23 Set starting temperature (0 °C)

24 Set the temperature interval (5 deg) and number of repeat increments, or set each temperature increment manually to take points more closely spaced where the ellipticity changes rapidly as a function of temperature (determined from steps 12- 14)

25 Set the wavelength range for data collection and the number of repeats⁸.

26 Save the macro, e.g. with the name “tempscan”

27 Run the macro “tempscan” or manually collect data at each desired temperature.

TROUBLESHOOTING

28 Save the data

29 Import the data into a graphics program or spread sheet. For use with the convex constraint algorithm, the first column should be the wavelengths, and the following columns should have the ellipticity values collected at each temperature. Save the file in a comma delineated format with a name that is no longer than 8 characters with an extension of no more than 3, e.g. “data.dat” for analyzing the data using the CCA algorithm. Make a different set for each protein sample.

30 To combine data sets collected using different protein concentrations, convert the data to mean residue ellipticity.⁸

31 Analyze the data using the convex constraint algorithm or singular value decomposition to determine the number of basis sets need to reconstruct the data. Detailed instructions for use of the CCA algorithm program are in SUPPLEMENTAL MATERIALS in the file CCA.doc. If only two basis sets are needed to reconstruct the entire set the protein does not have a folding intermediate that is detectable by CD.

Anticipated Results

A. Melts at a single wavelength—Analysis of CD spectra of proteins as a function of temperature should allow one to determine the thermodynamics of folding and estimate the binding constants of interacting proteins. Determination of the enthalpy of folding/unfolding using direct fitting or van't Hoff plots should give results within experimental error of one another and the T_M values of folding determined from the direct fits, and from the 1st derivative of the melts should be similar to each other.

Figures 2 and 3 illustrates the use of circular dichroism to examine the interaction of model proteins containing the C- and N-terminal domains (NTD and CTD, respectively) of tropomyosin, an actin binding protein, with a fragment of the tropomyosin binding domain of troponin (TnT₇₀₋₁₇₀), a protein that regulates contraction of striated muscles in response to calcium³⁹⁻⁴³. The NTD and CTD model proteins form a binary complex, which binds the troponin T fragment to form a ternary complex. The troponin fragment contains a mutation, I27N, found to cause cardiac myopathy. Figures 2a and 2b show the spectra of the model proteins and protein complexes in millidegrees and in mean residue ellipticity, respectively. All are highly helical, and have large ellipticities at 222 nm, which can be used to follow unfolding.

Figure 3 shows how changes in the ellipticity of the model peptides and peptide complexes as a function of temperature can be used to derive the thermodynamic parameters of unfolding and the dissociation constants of protein-protein complexes. The CTD model peptide is a two-chain coiled-coil where the peptide chains are linked by a disulfide cross-bridge. It unfolds as a monomer. NTD is a two-chain coiled coil that unfolds as a dimer. The protein fragment TnT (I79N)₇₀₋₁₇₀ is a monomer. Also shown are a 1:1 complex of the NTD and CTD model proteins, which unfolds as a heterotrimer, and a 1:1:1 complex of the NTD, CTD and TnT model proteins, which unfolds as a tetramer. Figure 3a shows the change in ellipticity of the five samples as a function of temperature. When the NTD and CTD model proteins are mixed there is an increase in ellipticity and stability compared to the addition of the individual unfolding curves, showing that they form a complex. When TnT(I79N)₇₀₋₁₇₀ is added there is an even greater increase in total ellipticity and large increase in T_M compared to the unmixed components. The data in Figure 3a for the CTD, NTD, TnT(I79N)₇₀₋₁₇₀ fragments and the NTD/CTD and NTD/CTD/TnT(I79N)₇₀₋₁₇₀ complexes are fit by equations for the unfolding of a monomer, dimer, monomer, heterotrimer and generic curve respectively. Figure 3b shows the van't Hoff plots of the data in Figure 3a. Both methods give almost identical estimates of the enthalpy of unfolding (Table 1). Figure 3c shows the first derivative of the data in Figure 1a. The T_M values determined from the maxima are also close to those determined by direct fitting of the data. The data in Figure 2 was used to determine the dissociation constants of the CTD/NTD and CTD/NTD/TnT(I79N)₇₀₋₁₇₀ complexes, which are 2.7×10^{-6} and 1.7×10^{-11} M at 25 °C, respectively.

B. Spectra collected as a function of wavelength—Analysis of spectra collected as a function of temperature, showing the use of the convex constraint algorithm to deconvolute the spectra to obtain the minimum number of basis spectra needed to deconvolute the data. Figure 4a and 4d illustrate the spectra of the CTD model used above and the same model protein with the mutation Q263L⁴⁰ (the numbering is that of intact tropomyosin) determined as a

function of temperature, respectively. The CD spectra suggests that the wild-type CTD model protein at 10 °C is 60 to 70 % helical, in agreement with NMR studies, which show that it is 73% helical (63% of the helices are in a canonical coiled coil and the rest are in linear α -helices) and the rest is disordered⁴¹. Replacing Q263 at the coiled-coil interface of the CTD with a leucine increases the helical content to approximately 90% (almost all coiled-coil) and also increases the stability of the molecule⁴⁰.

Analysis of the change in the spectrum of the wild-type CTD model protein shows that it unfolds in a two-state transition. When the set of spectra in 4a are deconvoluted into basis spectra using the CCA algorithm into three spectra (Figure 4b), two of the basis spectra are almost identical, showing that only two conformations are needed to fit the all of the data in Figure 4a. The percentage of each of the basis curves contributing to the spectra are shown in Figure 4c. In 4c the fraction of each of the two curves corresponding to the unfolded structure are added. The change in the spectrum of the curve corresponding to the folded form unfolds with a T_M of 25 °C (within experimental error of the T_M of unfolding when the ellipticity change at 222 nm is followed as a function of temperature).

When the set of spectra from the CTD molecule containing the Q263L mutation are deconvoluted, in contrast, at least three curves are needed to fit the data. The helical peptide unfolds to give a spectrum similar to that of the wild-type CTD, but then it begins to aggregate to give a spectrum with some β -sheet characteristics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The work was supported by a NIH grant, GM-36326 to NJG and Dr. Sarah E. Hitchcock-DeGregori and by the Circular Dichroism Facility at Robert Wood Johnson Medical School (UMDNJ). I would like to thank the late Dr. Gerald D. Fasman for supplying copies of programs to deconvolute data set using the convex constraint algorithm and Dr. Takashi Konno for supplying a singular value decomposition program.

REFERENCES

1. Velluz, L.; Legrand, M.; Grosjean, M. Optical Circular Dichroism: Principles, Measurements, and Applications. Verlag Chemie Academic Press Inc; New York and London: 1965.
2. Beychok S. Circular dichroism of biological macromolecules. *Science* 1966;154:1288–99. [PubMed: 5332570]
3. Adler AJ, Greenfield NJ, Fasman GD. Circular dichroism and optical rotatory dispersion of proteins and polypeptides. *Methods Enzymol* 1973;27:675–735. [PubMed: 4797940]
4. Johnson WC Jr. Protein secondary structure and circular dichroism: a practical guide. *Proteins* 1990;7:205–14. [PubMed: 2194218]
5. Woody RW. Circular dichroism. *Methods Enzymol* 1995;246:34–71. [PubMed: 7538625]
6. Kelly SM, Jess TJ, Price NC. How to study proteins by circular dichroism. *Biochim. Biophys. Acta* 2005;1751:119–39. [PubMed: 16027053]
7. Miles AJ, Wallace BA. Synchrotron radiation circular dichroism spectroscopy of proteins and applications in structural and functional genomics. *Chem. Soc. Rev* 2006;35:39–51. [PubMed: 16365641]
8. Greenfield NJ. Using circular dichroism spectra to estimate protein secondary structure. *Nature Protocols* 2006;1:2876–90.
9. Ohgushi M, Wada A. 'Molten-globule state': a compact form of globular proteins with mobile side-chains. *FEBS Lett* 1983;164:21–4. [PubMed: 6317443]

10. Dolgikh DA, Abaturov LV, Brazhnikov, Lebedev Iu O, Chirgadze Iu N. Acid form of carbonic anhydrase: "molten globule" with a secondary structure. Dokl. Akad. Nauk. SSSR 1983;272:1481–4. [PubMed: 6418497]
11. Bolotina IA. Secondary structure of proteins from circular dichroism spectra. V. Secondary structure of proteins in a "molten globule" state. Mol. Biol. (Mosk.) 1987;21:1625–35. [PubMed: 3128730]
12. Kuwajima K. The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. Proteins 1989;6:87–103. [PubMed: 2695928]
13. Goto Y, Calciano LJ, Fink AL. Acid-induced folding of proteins. Proc. Natl. Acad. Sci. USA 1990;87:573–7. [PubMed: 2153957]
14. Greenfield NJ. Determination of the folding of proteins as a function of denaturants, osmolytes or ligands using circular dichroism. Nature Protocols 2006;1:2733–41.
15. Greenfield NJ. The use of circular dichroism to study the kinetics of protein folding and unfolding. Nature Protocols 2006;1:2891–9.
16. Greenfield NJ. Circular dichroism analysis for protein-protein interactions. Methods Mol. Biol 2004;261:55–78. [PubMed: 15064449]
17. Greenfield NJ. Analysis of circular dichroism data. Methods Enzymol 2004;383:282–317. [PubMed: 15063655]
18. Pace CN, McGrath T. Substrate stabilization of lysozyme to thermal and guanidine hydrochloride denaturation. J. Biol. Chem 1980;255:3862–5. [PubMed: 7372654]
19. Yadav S, Ahmad F. A new method for the determination of stability parameters of proteins from their heat-induced denaturation curves. Anal. Biochem 2000;283:207–13. [PubMed: 10906241]
20. Eftink MR. Use of multiple spectroscopic methods to monitor equilibrium unfolding of proteins. Methods Enzymol 1995;259:487–512. [PubMed: 8538469]
21. Johnson CR, Morin PE, Arrowsmith CH, Freire E. Thermodynamic analysis of the structural stability of the tetrameric oligomerization domain of p53 tumor suppressor. Biochemistry 1995;34:5309–16. [PubMed: 7727392]
22. Eftink MR, et al. Thermodynamics of the unfolding and spectroscopic properties of the V66W mutant of Staphylococcal nuclease and its 1-136 fragment. Biochemistry 1996;35:8084–94. [PubMed: 8672513]
23. Boice JA, Dieckmann GR, DeGrado WF, Fairman R. Thermodynamic analysis of a designed three-stranded coiled coil. Biochemistry 1996;35:14480–5. [PubMed: 8931544]
24. Horng JC, Moroz V, Raleigh DP. Rapid cooperative two-state folding of a miniature alpha-beta protein and design of a thermostable variant. J. Mol. Biol 2003;326:1261–70. [PubMed: 12589767]
25. Perczel A, Hollósi M, Tusnády G, Fasman GD. Convex constraint analysis: a natural deconvolution of circular dichroism curves of proteins. Protein Eng 1991;4:669–79. [PubMed: 1946324]
26. Greenfield NJ, Hitchcock-DeGregori SE. Conformational intermediates in the folding of a coiled-coil model peptide of the N-terminus of tropomyosin and alpha alpha-tropomyosin. Protein Sci 1993;2:1263–73. [PubMed: 8401212]
27. Safar J, Roller PP, Gajdusek DC, Gibbs CJ Jr. Thermal stability and conformational transitions of scrapie amyloid (prion) protein correlate with infectivity. Protein Sci 1993;2:2206–16. [PubMed: 7905316]
28. Staskus PW, Johnson WC Jr. Conformational transition of hyaluronic acid in aqueous-organic solvent monitored by vacuum ultraviolet circular dichroism. Biochemistry 1988;27:1522–7. [PubMed: 3365406]
29. McPhie P, Shrager RI. An investigation of the thermal unfolding of swine pepsinogen using circular dichroism. Arch. Biochem. Biophys 1992;293:46–53. [PubMed: 1731638]
30. Konno T. Conformational diversity of acid-denatured cytochrome c studied by a matrix analysis of far-UV CD spectra. Protein Sci 1998;7:975–82. [PubMed: 9568904]
31. Ionescu RM, Smith VF, O'Neill JC Jr. Matthews CR. Multistate equilibrium unfolding of Escherichia coli dihydrofolate reductase: thermodynamic and spectroscopic description of the native, intermediate, and unfolded ensembles. Biochemistry 2000;39:9540–50. [PubMed: 10924151]

32. Thompson KS, Vinson CR, Freire E. Thermodynamic characterization of the structural stability of the coiled-coil region of the bZIP transcription factor GCN4. *Biochemistry* 1993;32:5491–6. [PubMed: 8504069]
33. Taylor JW, Greenfield NJ, Wu B, Privalov PL. A calorimetric study of the folding-unfolding of an alpha-helix with covalently closed N and C-terminal loops. *J. Mol. Biol* 1999;291:965–76. [PubMed: 10452900]
34. Singh A, Hitchcock-DeGregori SE. Local destabilization of the tropomyosin coiled coil gives the molecular flexibility required for actin binding. *Biochemistry* 2003;42:14114–21. [PubMed: 14640678]
35. Ramsay GD, Eftink MR. Analysis of multidimensional spectroscopic data to monitor unfolding of proteins. *Methods Enzymol* 1994;240:615–45. [PubMed: 7823851]
36. Schellman JA. Macromolecular Binding. *Biopolymers* 1975;14:999–1018.
37. Schellman JA. The effect of binding on the melting temperature of biopolymers. *Biopolymers* 1976;15:999–1000.
38. Marquardt DW. An algorithm for the estimation of non-linear parameters. *J. Soc. Indust. and Appl. Math* 1963;11:431–441.
39. Greenfield NJ, Montelione GT, Farid RS, Hitchcock-DeGregori SE. The structure of the N-terminus of striated muscle alpha-tropomyosin in a chimeric peptide: nuclear magnetic resonance structure and circular dichroism studies. *Biochemistry* 1998;37:7834–43. [PubMed: 9601044]
40. Greenfield NJ, Palm T, Hitchcock-DeGregori SE. Structure and interactions of the carboxyl terminus of striated muscle alpha-tropomyosin: it is important to be flexible. *Biophys. J* 2002;83:2754–66. [PubMed: 12414708]
41. Greenfield NJ, et al. The structure of the carboxyl terminus of striated alpha-tropomyosin in solution reveals an unusual parallel arrangement of interacting alpha-helices. *Biochemistry* 2003;42:614–9. [PubMed: 12534273]
42. Palm T, Greenfield NJ, Hitchcock-DeGregori SE. Tropomyosin ends determine the stability and functionality of overlap and troponin T complexes. *Biophys. J* 2003;84:3181–9. [PubMed: 12719247]
43. Palm T, Graboski S, Hitchcock-DeGregori SE, Greenfield NJ. Disease-causing mutations in cardiac troponin t: identification of a critical tropomyosin-binding region. *Biophys. J* 2001;81:2827–37. [PubMed: 11606294]
44. Greenfield N, Fasman GD. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* 1969;8:4108–16. [PubMed: 5346390]
45. Bentz H, Bächinger HP, Glanville R, Kühn K. Physical evidence for the assembly of A and B chains of human placental collagen in a single triple helix. *Eur. J. Biochem* 1978;92:563–7. [PubMed: 738278]
46. Tiffany ML, Krimm S. Effect of temperature on the circular dichroism spectra of polypeptides in the extended state. *Biopolymers* 1972;11:2309–16. [PubMed: 4634868]
47. Woody RW. Circular dichroism and conformation of unordered poly-peptides. *Adv Biophys Chem* 2:31–79.

Figure 1. Circular dichroism (CD) spectra of polypeptides and proteins with some representative secondary structures

a, CD spectra of poly-L-lysine in the 1, α -helical (black) and 2 antiparallel β -sheet (red) conformations at pH 11.1, and 3 extended conformation at pH 5.7, 3 (green)⁴⁴ and placental collagen in its 4, native triple-helical (blue) and 5, denatured (cyan) forms⁴⁵. Note that the extended conformation of poly-L-lysine was originally described as a “random coil” but its spectrum is similar to the conformation of poly-L-proline II^{46,47}, which forms an extended left-handed helix.

Figure 2. Spectra of unbound tropomyosin and troponin model proteins and protein-protein complexes

a, raw data. b, mean residue ellipticity. (green circles) CTD; red squares (NTD); (blue triangles up); TNT₇₀₋₁₇₀I79N; pink triangles down (1:1 mixture of CTD and NTD); (cyan diamonds) 1:1:1 mixtures of the NTD, CTD and TnT₇₀₋₁₇₀I79N model peptides. (open triangles up) add the curves for the unbound CTD and NTD peptides; (open triangles down) add the curves for the CTD, NTD and TnT₇₀₋₁₇₀I79N model peptides. The peptide concentrations were all 17.5 μM in 10 mM potassium phosphate, pH 6.5. Data were collected in cells with 1 mm path lengths. Abbreviations: NTD, model protein of the N-terminal domain of tropomyosin containing residues 1-14 of rat striated alpha tropomyosin and the last 18 residues of the yeast transcription factor, GCN4. The peptide is N-acetylated^{39,43}. CTD, peptide containing residues 251-284 of rat striated tropomyosin with GCG at N-terminus. The peptides are cross-linked by a disulfide bond⁴⁰. TNT₇₀₋₁₇₀I79N, residues 70-170 of human cardiac troponin with the mutation I79N and glycine at the N-terminus⁴³.

Figure 3. Typical curves of ellipticity as a function of temperature used to determine the thermodynamics of folding

In all the panels the proteins, symbols and conditions are the same as Figure 2. a, Changes in ellipticity of tropomyosin and troponin model proteins and protein complexes as a function of temperature. The raw data are fit with equations for the unfolding of monomers (CTD and TnT₇₀₋₁₇₀I79N), a dimer (NTD); a heterotrimer (NTD/CTD complex) and a generic hyperbolic curve (CTD/NTD/ TnT₇₀₋₁₇₀I79N complex), respectively. b, Van't Hoff plots of the data in panel a. c, the first derivatives of the curves in panel a. The maxima correspond to the midpoints (T_M s) of the unfolding/folding curves.

Figure 4. Example of the analysis of set of spectra using the convex constraint algorithm.

a and d, spectra of a model protein containing the C-terminal domain of rat striated muscle tropomyosin (CTD) and a fragment containing the mutation of a glutamine to leucine (CTDQ263L)⁴⁰ collected as a function of temperature, respectively. b, and e, the data in panels a and d deconvoluted into 3 basis curves using the convex constraint algorithm²⁵. c and f, the fraction of each basis curve contributing to each spectrum at each temperature. The fractional weights of the two curves corresponding to the unfolded protein are summed in panel c because they were almost identical. The protein samples were 1.67 μ M in 100 mM NaCl, 10 mM sodium phosphate buffer pH 6.5. Data were collected at 5 deg increments with two minutes equilibration at each point in cells with path lengths of 1 cm. Abbreviations: CTD, model protein containing residues 251-284 of rat striated tropomyosin with GCG at N-terminus. CTDQ263L, the same peptide with the mutation Q263L. The peptides are cross-linked by disulfide bonds.

TROUBLESHOOTING

Step	Problem	Solution
2	The CD spectra have very low ellipticity.	Check the protein concentration ⁸
3	The signal to noise is very low.	Try opening the slit width to 2 nm, as the exact wavelength is not critical. Try increasing the time of data collection. Signal to noise increases as the square root of the time of the signal averaging time.
12	The samples precipitate when heated.	Try using a different buffer or include stabilizing agents such as low concentrations of glycerol.
15	The samples refold to give the same spectra as the starting material but the unfolding and refolding curves are displaced from each other.	The samples are not at thermal equilibrium during the measurements. Try increasing the equilibration time.
17	The curve of ellipticity as a function of temperature appears to have a typical sigmoidal shape, but none of the folding equations fit the data and the error of the fit is large.	Make sure that the initial parameters are close to the actual values of the data and that the correct units are used for the initial estimates of the ellipticity of the folded and unfolded protein (e.g. millidegrees or mean residue ellipticity). Make sure the initial estimate of the T_M of folding is close to the midpoint of the transition. If this doesn't work, try increasing or decreasing the initial estimates of the enthalpy of folding.
18	The calculated T_M values for different concentrations of protein are not close to each other, even when the data are modeled to fit the dissociation of dimers or trimers.	Determine the oligomerization state of the protein using independent methods such as gel filtration or ultracentrifugation, and use the van't Hoff equation to determine the thermodynamic parameters.
27	The macro doesn't work properly	Check the users manual to make sure you have programmed the machine properly. Usually machines will have sample macros. Try to program a single temperature step with using only one or two samples.
31	A. When the spectral are deconvoluted using the CCA algorithm some basis curves only contribute to a spectrum obtained at a single temperature. B. When the spectra are analyzed using the CCA algorithm, some of the curves have very similar shapes but are displaced from each other. C. When the data is deconvoluted using singular value decomposition (SVD), while it is clear there are more than two states, only one principal component is resolved.	A. If a spectrum is noisy and has outlying points, the CCA algorithm will identify the spectrum as a unique basis set. Try removing the spectrum from the data set. B. The CD spectra of the cells may change as a function of temperature, leading to shifts in the baseline. Obtain spectra of the cuvettes filled with water as a function of temperature and correct the individual data set for the contribution of the cells. C. If the spectra of the folded and unfolded proteins have maxima (nodes) that are identical to each other, SVD the solution may be "singular" and only one spectrum will deconvoluted.

Table 1

The Enthalpies of Unfolding of Model Proteins and Protein-Protein Complexes Calculated From the Change in Ellipticity as a Function of Temperature*

Sample	Direct Fit*	van't Hoff Equation
	ΔH , Kcal/mol	ΔH , Kcal/mol
CTD (monomer)	17.7 $\pm$ 0.3	17.2 $\pm$ 0.3
NTD (dimer)	30.3 $\pm$ 0.2	30.1 $\pm$ 0.2
TnT ₇₀₋₁₇₀ (monomer)	21.5 $\pm$ 0.2	21.6 $\pm$ 0.1
Mix CTD and NTD (trimer)	36.8 $\pm$ 0.3	36.5 $\pm$ 0.2
Mix CTD, NTD and TnT ₇₀₋₁₇₀ (tetramer)	ND	57.8 $\pm$ 0.3

* The raw data and fitted curves are shown in Figure 3.