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Research Education

CALCULATION OF THERMODYNAMIC PARAMETERS OF PROTEIN UNFOLDING USING FAR-ULTRAVIOLET CIRCULAR DICHROISM

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Abstract: Circular dichroism (CD) is an exceptional tool for rapid determination of thermodynamic parameters associated with the folding/unfolding of proteins. CD has the advantage that the measurements are quick, reliable and can be made under physiological conditions in a very short time. In this article, we summarize methods to obtain and analyze CD spectra to determine the conformational changes associated with the unfolding of proteins as a function of denaturants and temperature. Finally, recent developments with CD studies are discussed.

Keywords: circular dichroism; denaturation; equilibrium; secondary structure; stability; unfolding.

1. Introduction

Circular dichroism (CD) is a valuable and rapid spectroscopic technique for studying the secondary structure, binding properties and conformational changes that occur during the folding and unfolding of proteins (Beychok, 1966; Kelly and Price, 1997). CD results from differential absorption of the left- and right-handed circularly polarized light. This angle, also called as ellipticity, is very small and generally measured in millidegrees (mdeg). Detail information on the technique can be found in a number of reviews (Alder *et al.*, 1973; Beychok, 1966; Kelly *et al.*, 2005; Kelly and Price, 2000; Woody, 1995) as well as in a website that illustrates the production of CD of chiral molecules with animated graphics (*http://www.enzim.hu/~szia/cddemo/*). On the basis of wavelength studied, CD can be of two types- far-UV CD and near-UV CD. Far-UV CD is used to study the secondary structure of proteins, while near-UV CD is used to study the tertiary structure of proteins (Kelly *et al.*, 2005; Kelly and Price, 1997). The application of far-UV CD is varied and is the most commonly used CD technique. This manuscript describes the use of far-UV CD (hereafter called as CD) to study protein folding and stability as a function of denaturants and temperature.

The secondary structural elements of proteins such as alpha helices and beta sheets have highly asymmetrical structure around the space, resulting in a characteristic CD spectrum. When unfolded, the proteins tend to lose these well-organized structures resulting in change in the CD curve. For example, proteins with high α-helical content have distinctive negative bands at 222 and 208 nm and a positive band at 193 nm, while proteins with high β-sheets have a negative band at 216 nm and a positive band at 195 nm. After unfolding, the proteins lose these characteristic bands and the CD ellipticity changes, as the unfolded proteins have very low ellipticity above 210 nm and negative bands at 195 nm. Thus, CD can be used to study the folding/unfolding pathway and the effect of denaturant on the conformation of the protein.

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Thermodynamically, the native folded state of the protein has only relatively limited stability over the unfolded state(s) under physiological conditions. This free energy of stabilization of protein is a consequence of a sum of independent contributions from large stabilizing and destabilizing forces. The stabilizing forces include electrostatic, hydrophobic, van der Waals and hydrogen bonding contributions, while the major destabilizing force is conformational entropy (Lee and Vasmatzis, 1997; Myers and Pace, 1996; Pace, 1975; Pace *et al.*, 1996). Protein stability is also affected by environmental factors such as temperature, pressure, pH, mutation, ionic strength, solvent, and presence of denaturing agents. Of these, chemical and thermal denaturation of proteins are used as standard techniques to characterize the energetics of protein folding (Ahluwalia *et al.*, 2012; Bolen and Santoro, 1988; Kauzmann, 1959; Pace, 1975, 1986; Privalov, 1979). To determine the true thermodynamic parameters, the unfolding of a protein must be reversible. The unfolding and refolding of a protein is a thermodynamically driven process and thus analyses of the unfolding curves provide important parameters regarding the structure, stabilization and folding energetics of the protein.

Although CD is not a very high resolution technique, such as X-ray crystallography or NMR, the method has the lead that the data can be obtained and analysed in a very short time (within few hours) and very minute quantity of protein (≤ 10µg) is sufficient for a single experiment. Moreover, the experiment can be performed under physiological conditions, making it valuable for biological studies. This review covers methods and equations for obtaining the protein folding data using far-UV CD as a function of denaturants and temperature to determine the conformational stability and various thermodynamical parameters associated with protein folding.

2. Materials and Methods

2.1 Denaturant-induced unfolding: The equilibrium unfolding experiments with GdnHCl/urea were performed in 10 mM potassium phosphate buffer (pH 8.0) containing 1 mM EDTA. The GdnHCl/urea stock solutions were prepared using their molecular weight and then the concentration were checked by refractive index measurements using the standard equations given in Table 1. The defined volumes of GdnHCl/urea solution were added to the protein samples to obtain an increasing denaturant concentration (Tripathi *et al.*, 2010). For all unfolding experiments the mixtures were incubated at the desired temperature for 4 h to ensure that the equilibrium was achieved before the measurements were made. The unfolding reactions were studied by measuring the intrinsic fluorescence and the Far-UV CD signals. The data were analyzed according to the linear free energy model for two-state transition of proteins.

Table 1
Information for preparing Urea and GdnHCl stock solutions

Property	*Urea*	*GdnHCl*
Molecular Weight	60.056 g/mol	95.533 g/mol
Solubility, 25°C	10.49 M	8.54 M
d/d_o[a]	$1 + 0.2658W$ $+ 0.0330\,W^2$	$1 + 0.2710W$ $+ 0.0330\,W^2$
Molarity[b]	$117.66(\Delta)$ $+ 29.753(\Delta)^2$ $+ 185.56(\Delta)^{3???}$	$57.147(\Delta)$ $+ 38.68(\Delta)^2$ $- 91.60(\Delta?)^3$
Grams of Denaturant/Gram of H_2O to prepare		
6 M	0.495 g	1.009 g
8 M	0.755 g	1.816 g
10 M	1.103 g	—

[a]*W* is the weight fraction of denaturant in the solution, *d* is the density of the solution and d_o is the density of H_2O.
[b]ΔN is the difference in refractive index between the denaturant solution and water (or buffer) at the sodium D line.

2.2. Thermal denaturation: Temperature denaturation experiments were performed in a 10 mm path length quartz cuvette at a protein concentration of 2 mM in 10mM CGH buffer of various pH values. Readings at 222 nm were recorded between the temperatures from 45°C to 95°C with a linear increment of 1°C min^{-1} (Tripathi *et al.*, 2010). The data were corrected for the baseline contribution of the buffer at all conditions.

2.3. CD measurements: Far-UV CD measurements were carried out on a JASCO J-810 spectropolarimeter, equipped with a peltier-type temperature controller and a thermostated cell holder, interfaced with a thermostatic bath. The instrument was calibrated with ammonium (+)-10-camphor sulfonate and was under constant nitrogen flush. Spectra were recorded in a 1 mm path length quartz cuvette at a protein concentration of 2 mM in the above-mentioned buffer with or without GdnHCl/urea (Tripathi *et al.*, 2010). Five consecutive scans were accumulated and the average spectra stored. All Data were fitted using Origin 7.0 Server Software (Northampton, MA, USA).

3. Results and Discussion

3.1. Using denaturant-induced unfolding profiles

Chemicals, such as guanidine HCl (GdnHCl) and urea are most commonly used protein unfolding denaturants. The spectral changes associated with the GdnHCl- or urea- induced unfolding can be studied by far-UV CD spectroscopy. Addition of these chemicals causes loss of the characteristic CD bands, resulting into ellipticity data which can be used to calculate the free energy of stabilization of protein folding. To calculate the accurate thermodynamic parameters, the unfolding of a protein must be reversible and thus, the reversibility of the chemical unfolding should be confirmed by matching the CD ellipticity curve before denaturation and after renaturation.

If the change in CD ellipticity is a monophasic (two states) transition between folded and unfolded states, the data can be used to determine the free energy of folding of a protein as a function of denaturants. Figure 1A shows the change in ellipticity at 222 nm for protein samples as a function of increasing denaturant concentration (Tripathi *et al.*, 2010). The protein shows a two-state unfolding transition. A sigmoidal loss of the CD signal can be observed between 1.0 and 3.0 M GdnHCl, and at denaturant concentrations above 3.0 M almost complete unfolding of the protein was observed.

The equilibrium constant, K, of a monomeric protein or polypeptide can be calculated by the equation:

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

Where [F] is the concentration of folded protein and [U] is the concentration of unfolded protein.

The fraction folded is:

$$\alpha = ([\theta]-[\theta]_U) / ([\theta]_N-[\theta]_U) \quad (2)$$

In equation 2, $[\theta]$ is the observed ellipticity at 222 nm at different concentrations of denaturant, and $[\theta]_N$ and $[\theta]_U$ are the ellipticities at the same wavelength for the folded (N) and unfolded (U) conformations of the protein samples, respectively.

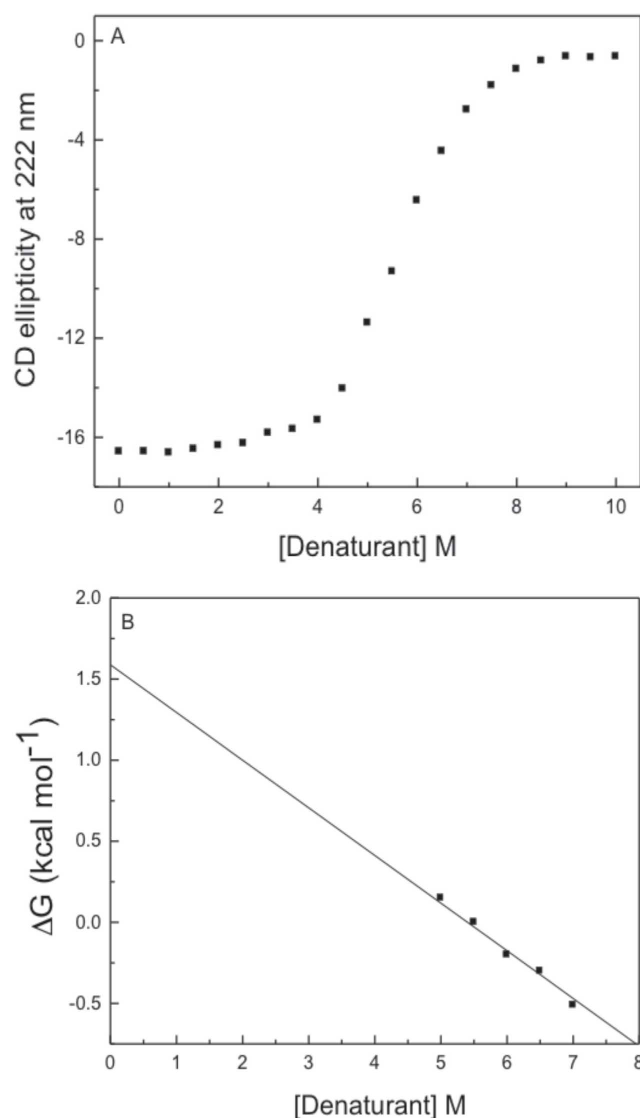

Figure 1: **Solvent-induced equilibrium unfolding curves (A) Denaturant-induced unfolding followed by monitoring CD ellipticity at 222 nm. (B) The linear free energy extrapolation curve with respect to increasing denaturant concentrations. The ΔG_D^{H20} is the intercept on the Y-axis, obtained using the linear extrapolation method**

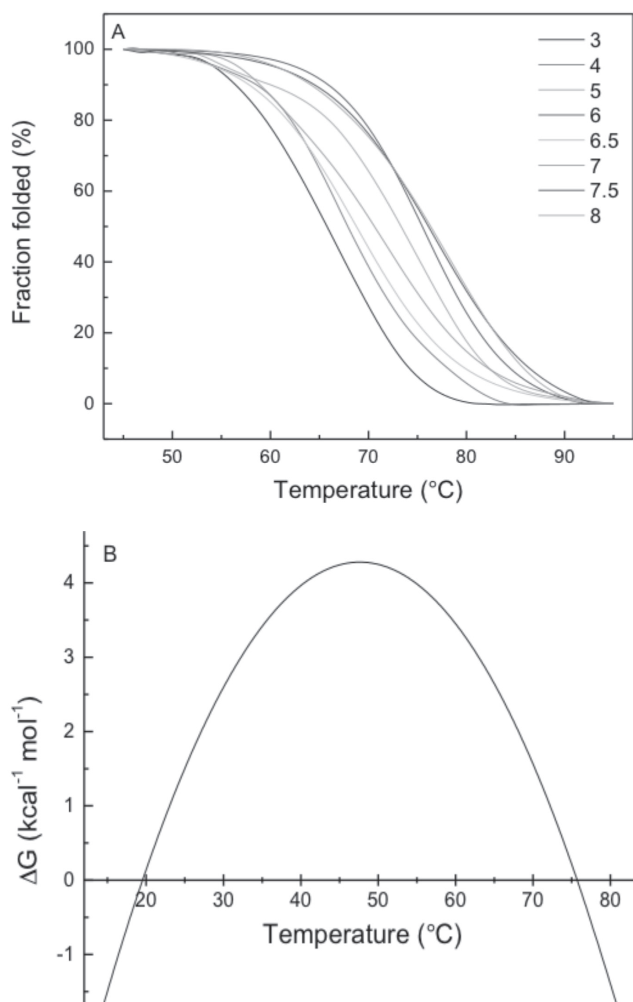

Figure 2: **Energetic characterization of protein (A) Temperature-induced unfolding curves at different pHs. Unfolding was monitored by the changes in far-UV CD ellipticity at 222 nm and plotted as percent fraction folded. A linear extrapolation of the baselines in the pre- and post-transition region was used to determine the fraction of folded protein within the transition region by assuming a two-state mechanism of unfolding. The experiments were performed at pH values of 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, and 8.0. (B) Conformational stability curve. The free energy of unfolding is plotted as a function of temperature at pH 8.0, using Gibbs-Helmholtz equation and energetic parameters were derived from the global fits of the thermal unfolding data**

The equilibrium constant at any concentration of denaturant [D]n for a monomeric protein is:

$$K = P_t\alpha \ / \ P_t(1\text{-}\alpha) = \alpha \ / \ (1\text{-}\alpha) \quad (3)$$

Where P_t is the total protein concentration.

The equilibrium constant for an oligomeric protein with similar subunits the equation is:

$$K = P_t\alpha \ / \ [nP_t(1\text{-}\alpha)]^n \quad (4)$$

Where P_t is the molecular concentration of the oligomeric protein when all the protein is folded, i.e., the total concentration of chains/n.

The equilibrium constant for an oligomeric protein where all the chains are different the equation is:

$$K = P_t\alpha \ / \ [P_t(1\text{-}\alpha)]^n \quad (5)$$

Assuming a two-state model of denaturation of the protein, the denaturant-induced denaturation curves can be used to determine the free energy of stabilization in the absence of denaturants (ΔG_D^{H20}) by linear extrapolation of the ΔG_D values to zero denaturant concentration, according to the following equation (Bolen and Santoro, 1988; Pace, 1986):

$$\Delta G_D^{H20} = \text{-R}T \ln K \quad (6)$$

Where R is the universal gas constant and T is temperature in Kelvin. Figure 1B shows the graph for the calculation of ΔG_D^{H20} value.

The ΔG_D values vary linearly with denaturant concentration and a least squares analysis can be used to fit the data to the equation:

$$\Delta G_D = \Delta G_D^{H20} - m[\text{D}]_n \quad (7)$$

Where, m, the slope, is the measure of the dependence of ΔG_D on GdnHCl concentration. It directly reflects the amount of protein surface exposed to solvent upon denaturation. $[\text{D}]_n$ is the concentration of the denaturant. The denaturant concentration at which $\Delta G_D = 0$, i.e. midpoint of GdnHCl denaturation at any temperature, is given by the $C_{1/2}$ so that the:

$$\Delta G_D^{H20} = C_{1/2} \times m \quad (8)$$

Similarly, the denaturant-induced unfolding experiments can be performed at various temperatures in order to determine the temperature dependence of ΔG_D^{H20}, m value, and $C_{1/2}$ values.

To elucidate whether the stability of any protein is due to hydrophobic or electrostatic interactions, separate GdnHCl- and urea-induced denaturation experiments can be performed (Tripathi *et al.*, 2008; Tripathi *et al.*, 2010). Urea, being neutral, provides information about the total stabilization energy (i.e. hydrophobic and electrostatic interactions), whereas the ionic

character of GdnHCl suppresses the effects due to electrostatic interactions and only the contributions of hydrophobic interactions are measured (Monera *et al.*, 1994; Tripathi *et al.*, 2007).

3.2. Using temperature-induced unfolding profiles

Before beginning any temperature-induced denaturation experiments, the reversibility of the thermal denaturation process should be checked. After denaturation, the sample should be immediately quenched cool to measure the reversibility of the reaction. The data from the first denaturation experiment should be matched with the second denaturation experiment. If both the data fall in line, the thermal-induced unfolding experiments can be performed on that particular protein and thermodynamic data can be generated.

The thermodynamics of protein folding can be studied by monitoring the ellipticity changes at a particular wavelength with increasing temperature as a function of pH. For calculations of various thermodynamic parameters, we take the thermal unfolding of a monomer which undergoes two state unfolding with temperature (Tripathi *et al.*, 2010). Most of the proteins which follow a reversible two-step folding transition without any intermediate state the temperature dependence characterization can be done using standard equations (Greenfield, 2006). Figure 2A shows eight thermally induced unfolding curves, normalized to fraction folded at various pH values ranging from 3 to 8.

To extract the relevant energetic parameters we made two assumptions: (i) that the unfolding transition takes place in two-states and, (ii) that there is no temperature dependence of ΔCp under experimental conditions. All pH-dependent thermal denaturation curves can be fitted to a two-state equilibrium unfolding model (Pace, 1986). In order to calculate the value of T_m (the midpoint of unfolding transition), ΔG_D values can be calculated from the transition region of the curve and plotted as a function of the absolute temperature and the value of T_m can be determined graphically at $\Delta G_D = 0$. The change in entropy (ΔS_m) associated with the temperature-induced denaturation is the slope of the plot of ΔG versus T. The Van't Hoff enthalpy, ΔH_m (or, the change in enthalpy when $\Delta G_D = 0$) can be calculated by using the relationship,

$$\Delta G_D = \Delta H_m - T_m \Delta S_m \tag{9}$$

These data can be used to estimate the heat capacity change for unfolding, ΔC_p, using the Kirchoff equation:

$$d(\Delta H_m) / d\, \Delta T_m = \Delta C_p \tag{10}$$

The ΔC_p of a protein describes the amount of curvature in the plot of ΔG as a function of temperature. The higher the ΔC_p, the more sharply ΔG depends on temperature. Practically, it is better to measure ΔH_m as a function of Tm by carrying out CD thermal scans at various pH values. The value of ΔC_p obtained from the slope of the plot of ΔH_m versus Tm is considered a better estimate than that obtained directly as the difference between the native and denatured baselines in a single CD or DSC experiment through Van't Hoff analysis of the unfolding curve, because the latter methods are fraught with errors due to arbitrariness in baseline determination (Ladbury *et al.*, 1994; Privalov and Khechinashvili, 1974).

The plot of ΔH_m versus T_m gives the value of ΔC_p, the temperature-independent heat capacity change at constant pressure. These values can be used to calculate additional energetic parameters; the enthalpy of unfolding at any temperature T, ΔH(T) and the entropy of unfolding at any temperature T, ΔS(T) using the following equations:

$$\Delta H(\mathrm{T}) = \Delta H_m + \Delta C_p (T - T_m) \tag{11}$$

$$\Delta S(\mathrm{T}) = \Delta H_m + \Delta C_p \ln (T / T_m) \tag{12}$$

Using values of T_m, ΔH_m and ΔC_p, the values of ΔG_D^{H2O} at any temperature T, $\Delta G_D(T)$, can be estimated with the help of the Gibbs-Helmholtz equation,

$$\Delta G_D(T) = \Delta H_m (T_m - T / T_m) - \Delta C_p [(T_m - T) + T \ln (T / T_m)] \tag{13}$$

The description of the free energy change of unfolding as a function of temperature represents the stability curve of the protein (Becktel and Schellman, 1987). The conformational stability curve of the protein over a wide range of temperatures can be drawn using these data

(Figure 2B). The temperature of maximum stability (T_S), interpreted as the temperature where $\Delta S_m = 0$, is derived from the analysis of the curve and is given by:

$$T_S = T_m \exp^{[-\Delta Hm/(Tm\ \Delta Cp)]} \quad (14)$$

4. Other applications of CD

Of late CD has been used in conjunction with many other techniques to yield valuable information. Thorough characterization of folding pathways for many proteins have been performed using rapid hydrogen ion exchange techniques in combination with structural analysis using NMR and/or mass spectroscopy (Dobson and Hore, 1998; Dyson and Wright, 1996; Konermann *et al.*, 2006; Konermann and Simmons, 2003; Krishna *et al.*, 2004; Roder *et al.*, 2004). Synchrotron radiation CD spectroscopy (SRCD) of proteins is finding lot of applications in structural and functional genomics (Miles and Wallace, 2006). The combination of new analysis methods, automated sample handling, and array detection may give SRCD an important role in proteomics programmes. Magnetic circular dichroism (MCD) has been developed which is an optical technique for the detection of electronic structure of both the ground states and excited states. MCD has found useful application in the study of biologically important systems including metalloenzymes and proteins containing metal centers (Mason, 2007). Frequency modulated circular dichroism spectroscopy has plenty of application in studying photolysis of molecules such as ICN. Vibrational circular dichroism provides three-dimensional structural information and thus is a powerful technique as the VCD spectra of enantiomers can be simulated using ab initio calculations, thereby allowing the identification of absolute configurations of small molecules in solution from VCD spectra (Keiderling, 1993; Keiderling *et al.*, 1988). Also, use of CD microscopy is booming to study different amyloid conformations and specific disease signatures (Kaminksy *et al.*, 2006).

5. Conclusions

Using these well established equations a number of information can be obtained including (i) protein secondary structure, (ii) various thermodynamic parameters associated with the folding of proteins (Pace, 1986; Tripathi *et al.*, 2010), (iii) protein folding intermediates (Tripathi *et al.*, 2009), (iv) stability of various proteins (Tripathi *et al.*, 2007), (v) kinetics of protein folding (Kuwajima and Schmid, 1984; Roder and Shastry, 1999), (vi) effect of mutation on the sequence (Newbold *et al.*, 2002), (vii) binding interactions (Zsila *et al.*, 2004), (viii) conformational transition, protein aggregation and misfolding (Dobson, 2003) and (ix) protein-protein, protein-DNA and protein-ligand interactions (Greenfield, 2004, 2006; Rodger *et al.*, 2005). The equations can also be used to analyze CD data collected by other spectroscopic methods like changes in fluorescence, absorption or IR spectra (Eftink, 1995).

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Abbreviations

CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; CGH buffer, citrate-glycine-hepes buffer; GdnHCl, guanidine hydrochloride

References

Ahluwalia, U., Katyal, N., and Deep, S. (2012). Models of Protein Folding. J Protein Proteomics *3*, 85-93.

Alder, A.J., Greenfield, N.J., and Fasman, G.D. (1973). Circular dichroism and optical rotatory dispersion of proteins and polypeptides. Methods Enzymol *27*, 675-735.

Becktel, W. J., and Schellman, J.A. (1987). Protein stability curves. Biopolymers *26*, 1859-1877.

Beychok, S. (1966). Circular dichroism of biological macromolecules. Science *154*, 1288-1299.

Bolen, D.W., and Santoro, M.M. (1988). Unfolding free energy changes determined by the linear extrapolation method. 2. Incorporation of delta G degrees N-U values in a thermodynamic cycle. Biochemistry *27*, 8069-8074.

Dobson, C.M. (2003). Protein folding and misfolding. Nature *426*, 884-890.

Dobson, C. M., and Hore, P. J. (1998). Kinetic studies of protein folding using NMR spectroscopy. Nat Struct Biol *5 (Suppl)*, 504-507.

Dyson, H.J., and Wright, P.E. (1996). Insights into protein folding from NMR. Annu Rev Phys Chem *47*, 369-395.

Eftink, M.R. (1995). Use of multiple spectroscopic methods to monitor equilibrium unfolding of proteins. Methods Enzymol *259*, 487-512.

Greenfield, N.J. (2004). Circular dichroism analysis for protein-protein interactions. Methods Mol Biol *261*, 55-78.

Greenfield, N.J. (2006). Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. Nat Protoc *1*, 2527-2535.

Kaminksy, W., Jin, L.W., Powell, S., Maezawa, I., Claborn, K., Branham, C., and Kahr, B. (2006). Polarimetric imaging of amyloid. Micron *37*, 324-338.

Kauzmann, W. (1959). Some factors in the interpretation of protein denaturation. Adv Protein Chem *14*, 1-63.

Keiderling, T.A. (1993). Vibrational Circular Dichroism of Proteins Polysaccharides and Nucleic Acids. In Physical Chemistry of Food Processes, I.C. Baianu, H. Pessen, T. Kumosinski, and V. Norstrand—Reinhold, eds. (New York), pp. 307-337.

Keiderling, T.A., Yasui, S.C., Narayanan, U., Annamalai, A., Malon, P., Kobrinskaya, R., and Yang, L. (1988). Vibrational Circular Dichroism of Biopolymers. In Spectroscopy of Biological Molecules New Advances, E.D. Schmid, F.W. Schneider, and S. F., eds., pp. 73-76.

Kelly, S.M., Jess, T.J., and Price, N.C. (2005). How to study proteins by circular dichroism. Biochim Biophys Acta *1751*, 119-139.

Kelly, S.M., and Price, N.C. (1997). The application of circular dichroism to studies of protein folding and unfolding. Biochim Biophys Acta *1338*, 161-185.

Kelly, S.M., and Price, N.C. (2000). The use of circular dichroism in the investigation of protein structure and function. Curr Protein Pept Sci *1*, 349-384.

Konermann, L., Pan, J., and Wilson, D.J. (2006). Protein folding mechanisms studied by time-resolved electrospray mass spectrometry. Biotechniques *40*, 135-141.

Konermann, L., and Simmons, D.A. (2003). Protein-folding kinetics and mechanisms studied by pulse-labeling and mass spectrometry. Mass Spectrom Rev *22*, 1-26.

Krishna, M.M., Hoang, L., Lin, Y., and Englander, S.W. (2004). Hydrogen exchange methods to study protein folding. Methods *34*, 51-64.

Kuwajima, K., and Schmid, F.X. (1984). Experimental studies of folding kinetics and structural dynamics of small proteins. Adv Biophys *18*, 43-74.

Ladbury, J.E., Sturtevant, J.M., and Leontis, N.B. (1994). The thermodynamics of formation of a three-strand, DNA three-way junction complex. Biochemistry *33*, 6828-6833.

Lee, B., and Vasmatzis, G. (1997). Stabilization of protein structures. Curr Opin Biotechnol *8*, 423-428.

Mason, W.R. (2007). Practical Guide to Magnetic Circular Dichroism Spectroscopy, A.

Miles, A.J., and Wallace, B.A. (2006). Synchrotron radiation circular dichroism spectroscopy of proteins and applications in structural and functional genomics. Chem Soc Rev *35*, 39-51.

Monera, O.D., Kay, C.M., and Hodges, R.S. (1994). Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions. Protein Sci *3*, 1984-1991.

Myers, J.K., and Pace, C.N. (1996). Hydrogen bonding stabilizes globular proteins. Biophys J *71*, 2033-2039.

Newbold, R.J., Deery, E.C., Payne, A.M., Wilkie, S.E., Hunt, D.M., and Warren, M.J. (2002). Guanylate cyclase activating proteins, guanylate cyclase and disease. Adv Exp Med Biol *514*, 411-438.

Pace, C.N. (1975). The stability of globular proteins. CRC Crit Rev Biochem *3*, 1-43.

Pace, C.N. (1986). Determination and analysis of urea and guanidine hydrochloride denaturation curves. Methods Enzymol *131*, 266-280.

Pace, C.N., Shirley, B.A., McNutt, M., and Gajiwala, K. (1996). Forces contributing to the conformational stability of proteins. Faseb J *10*, 75-83.

Privalov, P.L. (1979). Stability of proteins: small globular proteins. Adv Protein Chem *33*, 167-241.

Privalov, P.L., and Khechinashvili, N.N. (1974). A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. J Mol Biol *86*, 665-684.

Roder, H., Maki, K., Cheng, H., and Shastry, M.C. (2004). Rapid mixing methods for exploring the kinetics of protein folding. Methods *34*, 15-27.

Roder, H., and Shastry, M.R. (1999). Methods for exploring early events in protein folding. Curr Opin Struct Biol *9*, 620-626.

Rodger, A., Marrington, R., Roper, D., and Windsor, S. (2005). Circular dichroism spectroscopy for the study of protein-ligand interactions. Methods Mol Biol *305*, 343-364.

Tripathi, T., Na, B.K., Sohn, W.M., Becker, K., and Bhakuni, V. (2009). Structural, functional and unfolding characteristics of glutathione S-transferase of Plasmodium vivax. Arch Biochem Biophys *487*, 115–122.

Tripathi, T., Rahlfs, S., Becker, K., and Bhakuni, V. (2007). Glutathione mediated regulation of oligomeric structure and functional activity of Plasmodium falciparum glutathione S-transferase. BMC Structural Biology *7*, 67.

Tripathi, T., Rahlfs, S., Becker, K., and Bhakuni, V. (2008). Structural and stability characteristics of a monothiol glutaredoxin: glutaredoxin-like protein 1 from Plasmodium falciparum. Biochim Biophys Acta *1784*, 946-952.

Tripathi, T., Röseler, A., Rahlfs, S., Becker, K., and Bhakuni, V. (2010). Conformational stability and energetics of Plasmodium falciparum glutaredoxin. Biochimie *92*, 284-291.

Woody, R.W. (1995). Circular Dichroism. Methods Enzymol *246*, 34-71.

Zsila, F., Bikádi, Z., Fitos, I., and Simonyi, M. (2004). Probing protein binding sites by circular dichroism spectroscopy. Curr Drug Discov Technol *1*, 133-153.