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A Multidimensional Spectrophotometer for Monitoring Thermal Unfolding Transitions of Macromolecules

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ABSTRACT We describe a multidimensional spectrometer that is capable of (nearly) simultaneous measurement of circular dichroism, steady-state fluorescence, and absorbance values on the same sample in a standard 1 × 1 cm cuvette. With a computer controlled thermoelectric cell holder, this instrument is capable of measuring the above types of spectral data at various wavelengths as a function of temperature. We have developed software to control the various acquisition functions and to convert the data files to a format appropriate for use with the nonlinear least squares program, NONLIN (Johnson and Frasier, 1985). We have tested various features of this instrument and we have applied this instrument and data analysis procedure to study the thermal unfolding of ribonuclease A, under conditions were the unfolding of this protein involves an intermediate state.

INTRODUCTION

There has been renewed interest in the protein folding problem in recent years, due to advances site-directed mutagenesis, protein design, and structure determination (Oxender and Fox, 1987; Gierasch and King, 1989). Knowledge of the fundamental thermodynamics of protein unfolding processes is needed to correlate with structural and functional information for the multitude of new proteins that have been made and isolated (Privalov, 1989; Schellman, 1987). Related, important questions are whether an unfolding process is two-state or multistate, and what is the nature of the unfolded and intermediate states produced by various unfolding conditions.

Spectroscopic methods have traditionally been valuable for directly monitoring protein unfolding transitions. Among the solution phase spectroscopic methods that can be used are fluorescence, circular dichroism (CD), difference UV/vis spectroscopy, light scattering, and nuclear magnetic resonance (Schmid, 1989; Pace et al., 1989). The intrinsic chromophores (or nuclei) in proteins provide a rich source of such signal changes, with the only requirements being that the signals be different for the folded and unfolded (and intermediate) states, that the signals be adequately resolved, and that the magnitude of the signal be linearly related to the concentration of species. Among the more common and convenient spectroscopic methods for tracking an unfolding transition are measurements of circular dichroism in the deep UV region (where the signal responds to changes in secondary structure) and measurements of fluorescence of aromatic residues (where, for example, changes in the fluorescence intensity of tryptophans reflect changes in the microenvironment of these residues).

The classic paper of Lumry et al. (1966) explained that one can attempt to identify whether a protein unfolding reaction is two-state or multistate (with an intermediate) by monitoring the transition by two or more independent methods and by comparing the resulting thermodynamic parameters (i.e., transition temperature, T_{un}, enthalpy change, ΔH°_{un}, etc). If the transition profiles are not identical, when monitored by different methods, this indicates a multistate process, with certain spectroscopic signals sensing the intermediate(s) more than other signals. A problem with this strategy of using two or more separate instruments, such as CD and fluorescence, to monitor a transition is that errors and complications can be introduced if the conditions (protein concentration, temperature measurement, pH, buffer composition) are not adequately matched between samples. The experimenter must then decide whether the differences are due to errors in the measurements or to the existence of a multistate transition.

In addition to testing the two-state model, the use of more than one method to monitor an unfolding transition is a useful practice to confirm the results, because a given method may be insensitive to the unfolding process, and because baseline trends (i.e., the intrinsic change in a signal with change in temperature or other perturbant) may obscure tracking the transition between states.

For the above reasons, we have developed a multidimensional spectrophotometer that will record CD, fluorescence, light scattering, and absorbance data on the same sample in a nearly simultaneous manner. In this article we describe the instrument and its limitations, and we demonstrate its ability to monitor the unfolding of a protein that exhibits a multistate transition. The combined measurements are possible because both CD and fluorescence measurements share the need for a powerful light source, and

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Abbreviations used: A^{1cm}_{280}, light absorbance, with a 1-cm cuvette path length, at 280 nm; CD, circular dichroism; ΔG°_{un}, ΔH°_{un}, and ΔS°_{un}, standard free energy change, enthalpy change, and entropy change for an unfolding transition; F, steady-state fluorescence intensity; f_{un}, mole fraction of molecules in unfolded state; K_{un}, equilibrium constant for unfolding transition; S and S°, general spectroscopic signal at any temperature and at the reference temperature, respectively; δS, standard deviation for observed signal; s, temperature dependent slope of spectral signal for a pure state; T and T_{un}, temperature and transition temperature.

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because the dynode voltages obtained during the CD measurements are related to the sample's absorbance. Wada et al. (1980) have previously reported the construction of a multidimensional spectrophotometer having similar functions. Our instrument has the added feature of being able to record the standard deviation of each type of data, which then enables proper weighting of a global nonlinear least squares analysis of multiple data sets. Also, our instrument utilizes a computer controlled thermoelectric cell holder, which allows for convenient control of temperature in studies of thermally induced transitions.

DESCRIPTION OF INSTRUMENT AND SOFTWARE

Instrument

Fig. 1 is a schematic of our custom modified Aviv 62DS CD spectrophotopolarimeter. The commercial instrument was modified by the manufacturer to include a Hammamatsu 948 photomultiplier (PM) tube at 90° to the excitation beam. This PM tube has a separate preamplifier, and the output voltage of this side PM tube is fed to the instrument's A/D converter. The controlling software can alternately acquire the voltage signal from the side PM tube and the in-line CD PM tube. A filter holder accepts common 10 × 10 cm cut-off or interference filters, to enable selection of fluorescent light. With this configuration it is possible to collect steady-state fluorescence (or light scattering) data and excitation spectra, by driving the instrument's excitation monochromater. This right angle detection channel is conveniently built into a removable sample chamber for easy interconversion with other sample chambers.

The sample holder accepts standard 1 × 1 cm rectangular cuvettes. (Smaller path cuvettes are possible, but they must be centered if fluorescence measurements are to be made.) Thermoregulation is achieved by a thermoelectric cell holder and feedback circuitry (see below). The temperature of either the thermoelectric block or the contents of the cuvette is digitally measured with a thermistor probe. A stirring bar that is magnetically coupled to a motor in the base of the cell holder provides a means of mixing.

Attention must be given to the preparation of the sample. Far UV CD spectra measurements frequently require a short sample path length due to the sample's (and occasionally the buffer's) high light absorbance in this wavelength range. Likewise fluorescence measurements are better with an $A\frac{1cm}{280}$ of less than 0.1, to avoid inner filter effects. These requirements can be in conflict with the higher concentrations needed for good near UV CD measurements (on the order of $1\,A\frac{1cm}{280}$). However, it is possible to obtain measurements for all of these data types, by using a 1 cm cuvette with a sample concentration just high enough to allow near CD measurements, on the order of $A\frac{1cm}{280} \approx 0.5$. At this concentration changes in the secondary structure can still be observed by monitoring wavelengths on the red-edge of the far UV CD spectra (230–240 nm). This certainly is not enough information to determine secondary structure spectra; however, it is sufficient to monitor changes in the secondary structure. Furthermore, this concentration allows tryptophan fluorescence measurements to be made, by excitation at the red edge (e.g., 295 nm) of the aromatic absorption band. An alternative approach is to optimize conditions for fluorescence and far UV CD measurements, and not bother collecting near UV CD signals. The justification for this is that the tryptophan fluorescence and near UV CD signals are both sensitive the tryptophan's environment and so are probably redundant. Therefore the same information could probably be gathered by using a less concentrated sample and measuring fluorescence and far UV CD signals at multiple wavelengths.

The thermoelectric cell holder, designed by AVIV Associates, is a modification of their standard holder and has an opening for the 90° light signal. To accomplish this, the Peltier elements have been repositioned and enlarged and their power supply has been enhanced.

We have developed a program to control the thermopiles and to reach thermal equilibrium. This program uses the thermistor in the cuvette as the feedback reading to control the thermopiles and allows the temperature of the sample to be controlled to within 0.05°C. When equilibrium has been reached at a new temperature, the collection of spectroscopic data begins. With this arrangement, less than 5 min are required to achieve equilibration for a 2°C increase in temperature. Large temperature steps do not take much longer, since the Peltier devices work very rapidly and the damping of the temperature oscillations determines the approach to equilibrium.

The rest of the spectropolarimeter is the standard 62DS, which has a 450 W xenon lamp, dual prism monochromater, and uses a 486 CPU, 33 MHz computer for control and data acquisition.

Acquisition software

The standard Aviv software is capable of interpreting the Aviv macro level computer language, which allows the creation of custom-made programs to drive the instrument. Aviv provides several programs for controlling the instrument. With assistance from J. Laureno of Aviv Associates, we have modified certain of these programs and have written some of our own code to achieve the functions described below.

Set-up and execution programs

A compiled Quick Basic program, named SCANMAKE.EXE, has been written in-house. This program takes information entered from the keyboard to create an executable program (SCANTW.AVI) for the desired experiment. SCANMAKE.EXE requests the experimental set-up for a thermal unfolding experiment. This information includes: starting temperature, final temperature, number of intermediate temperatures (note that temperature scans can be either ascending or descending), type of and number of observables to record (i.e., whether CD or fluorescence or dynode voltage), excitation wavelengths, slit widths, time over which the signal is to be averaged (the latter three conditions can be different for different types of observables). SCANMAKE.EXE then creates the necessary accessory files for SCANTW.AVI to run.

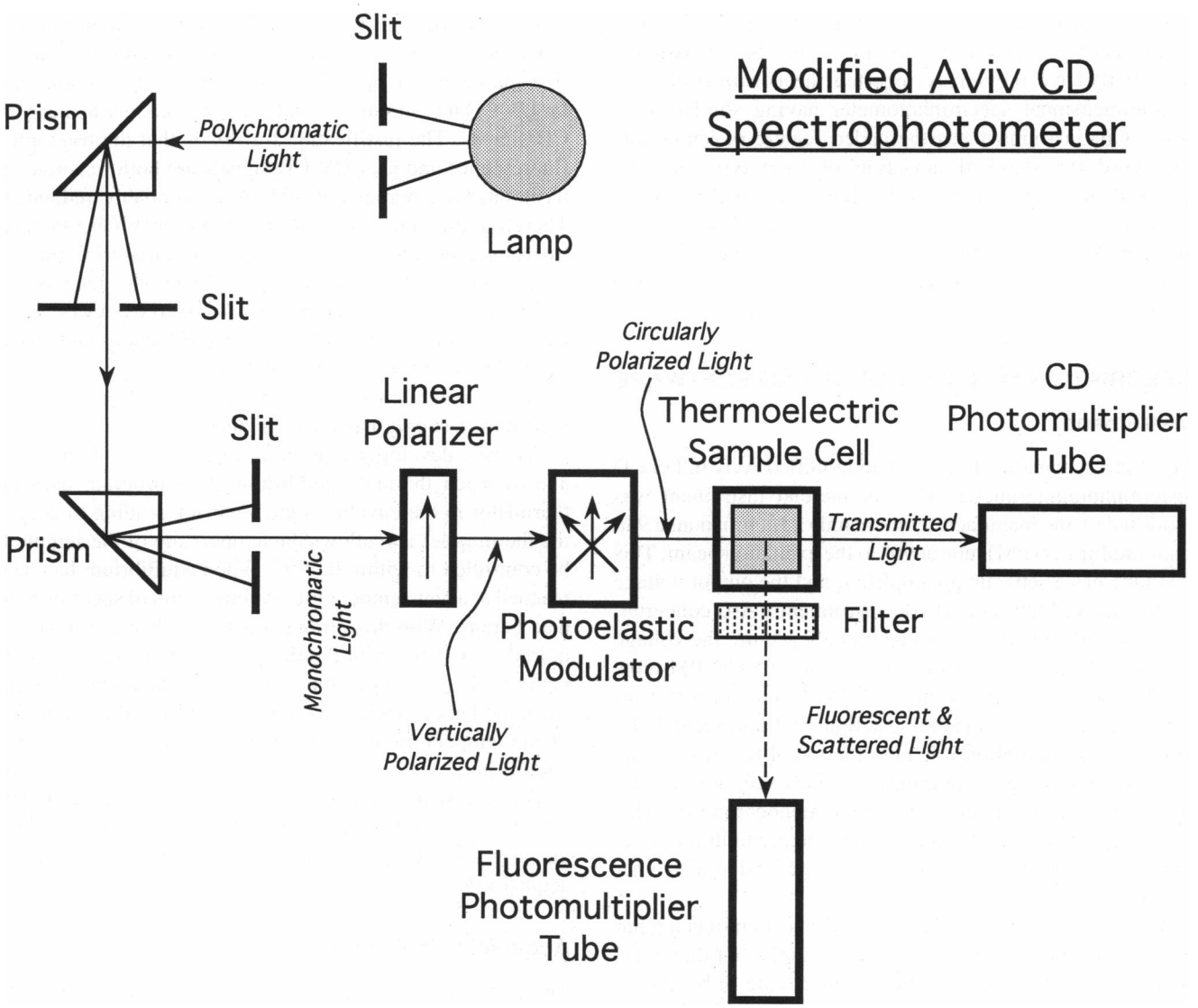

FIGURE 1 Block diagram of the modified AVIV 62-DS circular dichroism spectrophotometer. Modification of the thermoelectric cell holder and the addition of a side "fluorescence" PM tube allows measurement of steady-state fluorescence and fixed-angle light scattering. This figure is reproduced from Ramsay and Eftink (in press) with permission of Academic Press Inc.

The controlling/data acquisition program, SCANTW.AVI, controls the thermal scan, functioning of the spectropolarimeter, acquisition of data, and calculation of the standard deviations of each data point. SCANTW.AVI begins by instructing the spectrophotometer to go to the starting wavelength, slit width, and sample temperature. The temperature is judged to be stable when it is within 0.09°C of the set temperature, and when the temperature derivative with respect to time approaches zero. The time for actual acquisition of spectral data depends on the number of data types (i.e., number of different wavelengths, CD or fluorescence) and the specified acquisition time for each measurement. For a typical acquisition (including determination of standard deviations, see below) of five types of data, the acquisition may be about 3 min at a given temperature. Because it takes about 5 min for equilibration to the next temperature, the effective thermal scan rate is about 8 min per temperature. The scans can be time consuming (i.e., for a scan of 30 temperatures, 4 h can be required, for an effective scan rate of approximately 0.25°C/min). However, the user can choose to collect fewer types of data and to average for a shorter period of time. Because it is essential to allow chemical equilibrium to be reached for the conformational transition, we consider the slow scans to be necessary in many cases to extract valid thermodynamic information. Lepcock et al. (1992) have discussed how both calorimetric and van't Hoff enthalpy changes for the unfolding of a protein can be skewed if the relaxation time for the unfolding reaction is slow (long) compared to the thermal scanning rate. For example, the unfolding of the protein ribonuclease A has a relaxation time of approximately 0.33 min near its denaturation temperature, and thus the thermal scanning rate should be <0.5°C/min in order to determine valid thermodynamic data. As an alternate data acquisition strategy, we are developing a continuous

scanning version of SCANTW.AVI, which will be able to collect data at a higher rate.

To acquire an individual data point, SCANTW.AVI actually records data for a user-specified period of time (i.e., for 1–30 s) in what is essentially a "kinetics mode" acquisition routine. It then calculates the average signal over this period, **S**, and the standard deviation for the data point, **δS**, and records this information in a data-log file. The direct determination of the standard deviation of each data point is an important feature because it enables us to assign a statistical weight to each data point when performing a global analysis of multiple data sets. To illustrate the significance of this approach, the CD measurements at 280 nm are much lower in magnitude than the CD measurements at 222 nm. Without weighting, a global analysis of these two data sets would, in most cases, essentially ignore the former data set. By including the standard deviation for each data measurement, we can give appropriate weight to two or more data sets of different magnitudes (and also we can include the fact that some data are intrinsically more noisy than are other data).

After the first data type at the first temperature is recorded, SCANTW.AVI directs the instrument to acquire the second, third, etc., data type (i.e., to move to a new wavelength, or to record the voltage from the side PM tube). Next, SCANTW.AVI directs the thermoelectric cell holder to adjust to the second temperature and the acquisition is repeated, until the final temperature is reached.

The dynode voltage of the in-line CD PM tube is acquired simultaneously with the CD measurements. As we show below, the dynode voltage is directly proportional to the absorbance of a solution and changes of this dynode voltage can be used to monitor a transition, just as can the CD and fluorescence signals. The acquisition of the fluorescence (or light scattering) signals is nearly simultaneous, because the output from the in-line and side PM tubes is sequentially directed to the A/D board.

Conversion of files

When the experiment is complete, the average data points and their standard deviations at each temperature are extracted from the data-log file with a program (EXTRACT.EXE) that searches for signature words in the file. The extracted data and standard deviations are saved as ASCII files, whose format is made to be compatible with the nonlinear least squares program, NONLIN (Johnson and Frasier, 1985; Straume, et al., 1991). At this stage the data files are essentially in the format of $\mathbf{S}_{ij}$, $\mathbf{\delta S}_{ij}$, T_j, i, where the first subscript, i, is a flag that indicates the type of data and excitation wavelength (i.e., CD at wavelength λ_i, CD at wavelength λ_{i+1}, dynode voltage at λ_i, fluorescence at λ_i, light scattering, etc), and the second subscript, j, is for temperature.

Nonlinear least squares analyses

We have written subroutines for NONLIN that describe various thermal unfolding models (see below) and can be used to simultaneously fit the several data sets obtained above. A more detailed explanation of the curve fitting process is given in Ramsay and Eftink (in press). For the analysis of a single data set (i.e., one type of data versus temperature), a minimum of six fitting parameters are required. These are the ΔH°_{un}, ΔS°_{un} (or T_{un}), signal for the folded (low temperature) state, signal for the unfolded (high temperature) state, and the slopes of the signals for the folded and unfolded states (see below for the definitions of these parameters). NONLIN has options to allow all fitting parameters to be fitted or to allow one or more of the fitting parameters to be fixed.

A global analysis of multiple, linked data sets can be easily achieved with NONLIN (Johnson and Fraiser, 1985). For example, several types of data can be globally fitted to a two-state unfolding model with linked thermodynamic fitting parameters. As mentioned above, it is necessary to include the directly measured standard deviations of each type of data in order to perform such a global analysis with proper weighting. NONLIN also provides estimates of the 67% confidence limits of each fitting parameter and the cross-correlation coefficient for pairs of fitting parameters.

For graphical output of the fits, we use the commercial program SIGMAPLOT (version 5.0, Jandel Scientific). With this program we can enter the function (combination of Eqs. 1–7 below) to be simulated, and, with the fitting parameters determined with NONLIN, we can graphically present the simulated curve and the data points. We have created a template for the display of four panels, such as is illustrated in Fig. 4.

MODELS FOR CONFORMATIONAL TRANSITIONS

Because our principal application of the above instrument and analysis procedure is to study the thermal unfolding of globular proteins, we briefly present here the two-state model for the unfolding of a protein and its possible relationship to the type of data acquired by the above instrument. (We will also fit a multistate model[1] to our data; the latter model will not be presented here, but is given elsewhere (Ramsay and Eftink, 1994)).

For a two-state unfolding transition, N $\rightleftarrows$ U, where N is the native state and U is the unfolded state, the equilibrium constant, K_{un}, free energy change for unfolding, ΔG°_{un}, and fraction of unfolded species, f_{un}, are

$$K_{un} = [U]/[N] \quad (1)$$

[1] The multistate model is based on the notion that a globular protein has two domains, which unfold independently in both a thermodynamic and spectroscopic sense. In addition to the native and completely unfolded states, two folding intermediates are considered. One intermediate has the first domain unfolded; the other intermediate has the second domain unfolded. While this is a four-state model, when one of the domains is much less stable than the other, the model collapses to essentially a three-state model. The multistate analysis referred to in the text, Fig. 4 and Table 1 has thermodynamic parameters ΔH°_{un1} and T_{un1} for the unfolding of the less thermally stable domain and ΔH°_{un2} and T_{un2} for the unfolding of the more stable domain.

$$\Delta G^\circ_{un} = -RT \cdot \ln K_{un} \quad (2)$$

$$f_{un} = k_{un}/(1 + K_{un}) \quad (3)$$

The free energy change is related to the enthalpy change, ΔH°_{un}, entropy change, ΔS°_{un}, and transition temperature, T_{un} (defined as the temperature at which $\Delta G^\circ_{un} = 0$ and hence $T_{un} = \Delta H^\circ_{un}/\Delta S^\circ_{un}$) by the relationship

$$\Delta G^\circ_{un} = \Delta H^\circ_{un} - T \cdot \Delta S^\circ_{un} \quad (4)$$

$$\Delta G^\circ_{un} = \Delta H^\circ_{un} \cdot (1 - T/T_{un}) \quad (5)$$

If a spectroscopic signal, S, is different for the N and U states, and if the signal is linearly related to the fraction of N and U states via Eq. 6, then S can be used to monitor the unfolding transition and thermodynamic parameters can be obtained by analysis of such data.

$$S = f_N \cdot S_N + f_U \cdot S_U = (1 - f_U) \cdot S_N + f_U \cdot S_U \quad (6)$$

Here, S_N and S_U are the signals for the pure states N and U at a given temperature. In the work presented here, the signals may be CD signals at various wavelengths, absorbance values (actually dynode voltages) at various wavelengths, or fluorescence intensities.

The signals for the pure states will usually have an intrinsic dependence on temperature. We call this dependence the slope, $s_N = dS_N/dT$ and $s_U = dS_U/dT$, of the "baseline" regions for the N and U states. Assuming that this baseline slope is linear within the temperature range of study, the values of S_N and S_U at any temperature can be given as

$$S_N = S^\circ_N + s_N \cdot T \quad (7a)$$

$$S_U = S^\circ_U + s_U \cdot T \quad (7b)$$

where S°_N and S°_U are the values of S_N and S_U at the reference temperature (conveniently chosen at 0°C).

Combining Eqs. 1–7 we obtain an equation that relates the observed signal, S, to the fitting parameters ΔH°_{un}, ΔS°_{un} (or T_{un}), S°_N, s_N, S°_U, and s_U.

The signals may be measured at more than one wavelength, or multiple types of spectroscopic data (i.e., CD and fluorescence) may be combined in a global analysis. In such a case, the signal changes, S_i, may be indexed for the type of data, *i*. Likewise, the values of $S^\circ_{N,i}$, $s_{N,i}$, $S^\circ_{U,i}$, and $s_{U,i}$ must be indexed for the type of data and can be called "local" fitting parameters (i.e., local for each data set). However, the thermodynamic parameters, ΔH°_{un} and ΔS°_{un}, should be the same for all data sets and can be called "global" fitting parameters (i.e., if, in fact, the two-state model is correct). A global analysis combines multiple data sets and performs a nonlinear least squares fit with local and global parameters. The analysis proceeds by minimizing the variance (summing over *i* data sets and including the standard deviation of each type of data) as follows

$$SSR = \sum (S_{i,calc} - S_{i,exp})^2/\delta S_i^2 \quad (8)$$

where $S_{i,calc}$ are the calculated values of the observed signal for data set *i* using Eqs. 1–7, $S_{i,exp}$ are the experimentally observed signals for data set *i*, and δS_i is the standard deviation (experimentally determined, see above) for the data points.

TESTING OF INSTRUMENT, ACQUISITION, AND DATA ANALYSIS ROUTINES

In this section we will 1) show that the signal changes observed with our modified CD instrument are linear in concentration of species; 2) demonstrate the means of achieving thermal equilibration; 3) and present data for the thermal unfolding of a protein, ribonuclease A, as a test case for our multidimensional/global analysis approach.

Check of instrument linearity

Because the fluorescence attachment for the AVIV 62-DS is new, we have studied the linearity of response of the side PM to concentration of a fluorophore. Fig. 2 shows the apparent signal (open circles) of the side PM tube to varying concentrations of L-tryptophan in aqueous solution, using an excitation wavelength of 280 nm and observing emission through a 320 nm interference filter. At optical densities greater than 0.2 AU, there is a significant inner filter effect, which attenuates the apparent fluorescence signal. This inner filter effect can be corrected for with the

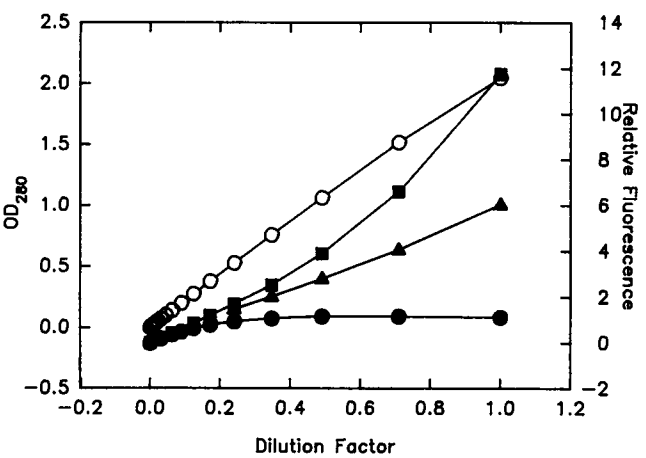

FIGURE 2 Test of the linearity of the side PM tube as a function of the concentration of a fluorescent substance, L-tryptophan, using a standard 1 × 1 cm cuvette. Excitation wavelength, 280 nm; emission observed through a 10-nm wide interference filter centered at 320 nm. Open circles (○) are the absorbance at 280 nm, determined by a separate UV-vis spectrophotometer, for the samples. Closed circles (●, right axis) are the observed fluorescence signals; note the departure from linearity when A_{280} is greater than about 0.2, due to the inner filter effect. Filled squares (■, right axis) are the corrected fluorescence signal using Eq. 9 with a value of $L_{ex} = 0.5$, which is the customarily assumed value. Filled triangles (▲, right axis) are the corrected fluorescence signal with $L_{ex} = 0.35$. Note that the latter correction gives a linear fluorescence signal for solutions of tryptophan having an absorbance over the range of 0 to 2.0 (i.e., highest concentration of about 360 micromolar).

equation (Parker, 1968)

$$I_{corr} = I_{meas} \cdot 10^{(A_{ex}L_{ex} + A_{em}L_{em})} \quad (9)$$

where I_{corr} and I_{meas} are the corrected and measured fluorescence signals, A_{ex} and A_{em} are the 1-cm path absorbances at the excitation and emission wavelengths, respectively, and L_{ex} and L_{em} are the effective excitation and emission path lengths in the cuvette. Because tryptophan has no absorbance at 320 nm (the emission wavelength), the "em" terms can be ignored. Values of A_{ex} were determined using a spectrophotometer and ranged from 0–2.0 (open circles in Fig. 2). The value of L_{ex} is usually assumed to be 0.5 in a typical fluorometer. When $L_{ex} = 0.5$ is used, the corrected signal, I_{corr}, is found to be roughly linear up to an $A_{ex} = 0.5$, but above this absorbance, the corrected florescence value shows an upward curvature, indicative of overcorrection. Therefore, the measured signal was optimized by nonlinear least squares curve fitting to obtain the effective L_{ex} that gives a linear I_{corr} over the span of $A_{ex} = 0$ to 2.0. Shown as the triangles in Fig. 2 is I_{corr} calculated using an effective L_{ex} of 0.35 ± 0.01 cm.

Thus the fluorescence signal responds to changes in the absorbance of a fluorescent substances in an expected manner (except with $L_{ex} = 0.35$ instead of 0.5). In most studies of conformational transitions in a macromolecule, fluorescence changes will be due primarily to intrinsic differences in the fluorescence quantum yield of different states, more so than differences in the absorbance of the states. So the corrections for absorptive screening, using Eq. 9, will not usually be necessary, unless there is a large change in absorbance at the excitation wavelength that accompanies the transition. In the latter cases, Eq. 9 can be used to correct the fluorescence intensity data. In a typical experiment, the absorbance values at the excitation wavelength (i.e., at 280 nm for a protein, where the emission comes from tryptophan residues) will be between 0.1 and 1.0, and the observed fluorescence signals should track the fraction of folded and unfolded states of a protein, provided there are fluorescence differences between the states.

In Fig. 3 is shown the correlation between the dynode voltage measured by the in-line PM tube and the A_{575} of series of neutral density filters. An excellent correlation is observed up to A values of 1, although at higher absorbance the correlation is poorer. Dynode voltage can be measured between 180 and 600 volts (below 180 volts there is too little current for the tube to make an accurate measurement; above 600 volts there is too little light). However, this effective range does not present an experimental obstacle, because the slit width can be adjusted to bring voltage levels with range. Presumably, the dynode voltage could be used to make absorbance measurements. The 62-DS is a single beam instrument and to calculate absorbances, the signal for a reference solution must first be recorded. However, for the purpose of monitoring transitions, the simple dynode voltage signal is sufficient, because Fig. 3 shows that this signal should linearly track the concentration of species in a solution.

The dynode voltage signals may show some quantum noise, when the signals are low. Quantum noise results from

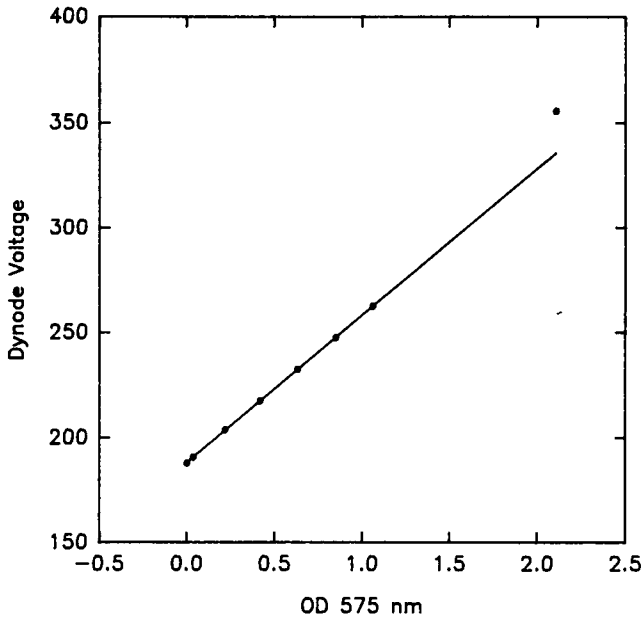

FIGURE 3 Dynode voltage of in-line CD PM tube as a function of A_{575} for a set of neutral density filters. Note the excellent linearity of the dynode voltage for the absorbance range of 0 to 1.0 (correlation coefficient of 0.9999).

the analog to digital signal conversion of the DC component of the dynode voltage. The voltage proceeds in steps of 0.68 volts, which correspond to changes of approximately 0.01 absorbance units. This quantum noise will be insignificant in cases where the absorbance changes are much larger than the above steps, or when there are other, larger sources of noise.

Thermal unfolding of ribonuclease A

Fig. 4 shows the application of our multidimensional spectroscopic thermal unfolding method to the protein ribonuclease A. It is well known that this protein undergoes a two-state thermal unfolding transition (Biringer and Fink, 1982; Brandts et al., 1989). In the presence of 50% methanol, however, the thermal unfolding of ribonuclease is a multistate process (Brandts et al., 1989).

Panel A of Fig. 4 shows the change in the aromatic CD signal at 280 nm with increasing temperature for ribonuclease A in 50% methanol at pH 2.2. Likewise, panels B, C, and D show the changes in the far UV CD (at 222 nm and 230 nm), the light scattering, and the fluorescence signals with an increase in temperature from 0°C to 70°C. The individual data sets were analyzed in terms of the two-state unfolding model (solid lines) and a multistate unfolding model (dashed lines); listed in Table 1 are the resulting fitting parameters. Also, the 5 data sets were globally analyzed in terms of a multistate model. The dotted lines through the points is this global fit, with the parameters given in Table 1.

Notice that the two-state model gives excellent fits to the near UV CD and light scattering signals, whereas the fluorescence signals shows clear evidence for the existence of one or more intermediate states. For the latter data, and the

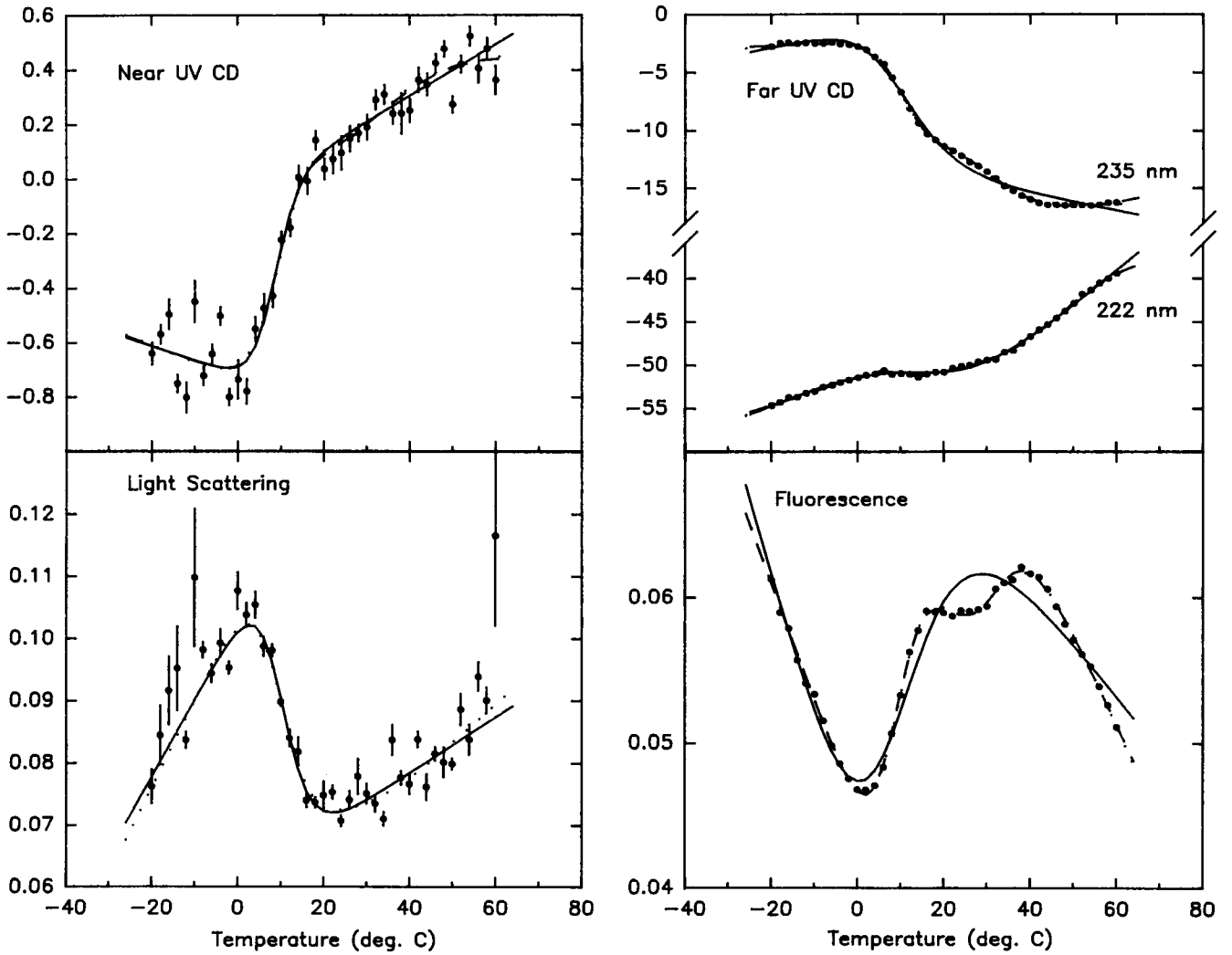

FIGURE 4 Thermal unfolding transition for ribonuclease A in 50% methanol at pH 2.2, as monitored with the multidimensional spectrophotometer. Signals monitored include: the CD_{280} (near UV CD, upper left quadrant, using 0.5 nm slit); the CD_{235} and CD_{222} (far UV CD, upper right, using 1 and 2 nm slits, respectively); light scattering at 320 nm (lower left, using 1.3 nm slit); the fluorescence at 280 nm (emission observed at 320 nm, lower right, using 1.3 nm slit), and the dynode voltage at 222 nm (see Fig. 6, using 1 nm slit). A standard 1 × 1 cm fluorescence cuvette was used and the protein sample had an A_{280} of 0.2. All measurements are the average of 16 data points collected at 1-s intervals. The error bars are the calculated standard deviations for the data points (when not shown, the standard deviations are smaller than the points on the graphs). The solid lines (— —)are the best possible fits of each data set with a two-state unfolding model. The dotted and dashed lines (- · -) are best fits of each data set with a three-state unfolding model. The dotted lines (···) are a global fit of the combined data sets (including the dynode voltage data, not shown) to a single set of thermodynamic parameters. This figure was reproduced from Ramsay and Eftink (1994) with permission from Academic Press, Inc.

far UV CD data, the multistate model[1] gives superior fits. The ability of the global analysis, in terms of the multistate unfolding model, to simultaneously describe all of the fitted data profiles demonstrates the power of this multidimensional method. Some of the data profiles are insensitive to the second unfolding transition; if only one such observable were used to monitor the transition, its complexity could be missed. Besides the advantage of global analysis of multiple data sets in discriminating between models, such an analysis is also beneficial because the fitted parameters should be more accurately determined, than those obtained by analysis of individual data profiles. This is because global analysis employs a larger total data set, which minimizes the effect of random noise in individual data points. Furthermore, the fitted parameters have reduced cross correlations, as compared to the analysis of individual signal profiles (see Ramsay and Eftink, 1994).

Summary and possible future developments

We have developed a multidimensional spectrometer and controlling software that will simultaneously measure circular dichroism, steady-state fluorescence (or light scattering), and absorbance data. We have applied this instrument, along with a thermoelectric cell holder, to automatically monitor the thermal unfolding of a protein. We combine the

TABLE 1 Fitting Parameters for the Thermal Unfolding of RNase A in 50% Methanol, 50 mM Glycine, pH 2.2

	Two-State Model			Four-State Model				
Data Type	Tm °C	ΔH kcal/mole	Variance	Tm_1 °C	ΔH_1 kcal/mole	Tm_2 °C	ΔH_2 kcal/mole	Variance
280 nm	8.3	56	4.63	9.1	57	33.5	17	4.79‡
CD*	6.0–10.5	29–99		7.2–10.9	32–112	26.0–41.4	10–26	
235 nm	10.2	27	152	11.6	47	28.0	26	4.59§
CD*	8.1–12.2	22–33		11.3–11.9	45–49	26.7–29.2	24–29	
222 nm	20.2	20	11.6	8.6	95	47.7	31	3.54
CD*	16.8–23.6	17–24		6.9–10.3	59–155	45.0–50.1	26–36	
222 nm	8.3	49	1070	N/D	N/D	N/D	N/D	N/D
Absorb.	7.0–9.3	36–59						
320 nm	10.2	50	4.81	N/D	N/D	N/D	N/D	N/D
Fluor.*,**	8.5–11.8	33–68						
280 nm	9.3	24	719	9.1	45	32.2	45	26.2
Fluor.*	6.5–12.1	19–29		8.2–10.2	41–48	30.2–33.7	37–55	
Global	N/D	N/D	N/D	9.8	44	31.0	40	10.6
				9.0–10.8	41–46	29.1–32.7	34–46	

Baseline parameters were also fitted, but are not shown here.

* Signifies that the global analysis included these data profiles.

‡ Confidence intervals for intermediate and final state baseline slopes could not be determined.

§ Confidence interval for intermediate state could not be determined.

‖ N/D signifies that the parameter was not determined.

** The "320 nm Fluor." data are actually scattered light measurements, rather than fluorescence.

multidimensional data with a global analysis strategy to obtain thermodynamic data for the unfolding process and to test the validity of the two-state unfolding model.

Our present application is to study thermally induced unfolding reactions. In the future we plan to add a computer-controlled syringe pump to enable various titration studies (i.e., of a protein with ligand, chemical denaturant, or acid/base). Other future developments may include the addition of a stopped flow mixer for multidimensional kinetic studies, an emission monochromater (or filter wheel) to enable variation in emission wavelength, and attempts to eliminate the quantum noise in the dynode voltage readings.

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