Modified Spectrophotometer for Multi-Dimensional Circular Dichroism/ Fluorescence Data Acquisition in Titration Experiments: Application to the pH and Guanidine-HCl Induced Unfolding of Apomyoglobin

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ABSTRACT In a previous paper (Ramsay and Eftink, *Biophys. J.* 66:516–523) we reported the development of a modified spectrophotometer that can make nearly simultaneous circular dichroism (CD) and fluorescence measurements. This arrangement allows multiple data sets to be collected during a single experiment, resulting in a saving of time and material, and improved correlation between the different types of measurements. The usefulness of the instrument was shown by thermal melting experiments on several different protein systems. This CD/fluorometer spectrophotometer has been further modified by interfacing with a syringe pump and a pH meter. This arrangement allows ligand, pH, and chemical denaturation titration experiments to be performed while monitoring changes in the sample's CD, absorbance, fluorescence, and light scattering properties. Our data acquisition program also has an ability to check whether the signals have approached equilibrium before the data is recorded. For performing pH titrations we have developed a procedure which uses the signal from a pH meter in a feedback circuit in order to collect data at evenly spaced pH intervals. We demonstrate the use of this instrument with studies of the unfolding of sperm whale apomyoglobin, as induced by acid pH and by the addition of guanidine-HCl.

INTRODUCTION

One of the most important areas of study in molecular biophysics is the characterization of the cooperative nature of processes involving biomacromolecules. A classical example of cooperativity is ligand binding to proteins possessing multiple sites, such as oxygen to hemoglobin. Another case is that of protein unfolding. However, in both ligand binding and conformational transitions the process may not be perfectly cooperative. In studying the thermodynamics of protein unfolding, e.g., one must consider that a significant concentration of the protein may exist in physical states other than the folded or unfolded states. Unfortunately, intermediate states are not always easy to detect, possibly because of low concentration of the intermediates and/or the similarity of the intermediates' observed signals to those of the initial or final states. Therefore, it is advisable to use more than one type of experimental technique to monitor a conformational transition. Furthermore, these various techniques should measure different features of the system. For example, far ultraviolet circular dichroism (CD) can be used to monitor the secondary structure of proteins during unfolding, whereas fluorescence measurements can follow changes in the microenvironment of tryptophan residues.

Ideally the signals measured by the various techniques will all be well described by the same theoretical model for the transition (e.g., a two- or three-state thermodynamic model for a conformational transition). Furthermore, nonlinear least-squares curve fits of the available data should give the same thermodynamic fitting parameters. However, for many reasons it is possible that significant differences between the fitted parameters can occur. In these cases the researcher may have discovered that the assumed model is inadequate. Alternatively, the model may be valid but there may be experimental errors. For example, when making measurements with various instruments, it is a common practice to prepare a fresh sample for each experiment. Each sample could vary in pH, ionic strength, protein concentration, etc., and these inadvertent variations could result in discrepancies between different types of measurements. Furthermore, random noise and calibration errors (such as in the thermal probes) in the various instruments can result in deviations between measurements. The researcher must decide whether differences between various measurements are due to some new, previously unknown mechanism, or whether the differences are within the experimental errors.

To address this situation we have developed a multi-dimensional spectrophotometer (Fig. 1) that can make CD, fluorescence, relative absorbance, and light scattering (at 90°) measurements (Ramsay and Eftink, 1994a). The advantage of this instrument is that all measurements are made with a sample during a single experiment. This approach reduces the effects of sample preparation and instrument calibration errors, resulting in better correlation between the different types of measurements. Additionally, because all measurements are made during a single experiment there is a saving of time and material. This instrument initially was designed to study thermal transitions of a macromolecule in solution. However, we have recently modified it to also perform titration type experiments by adding a syringe pump and a pH meter. To synchronize the activities of the

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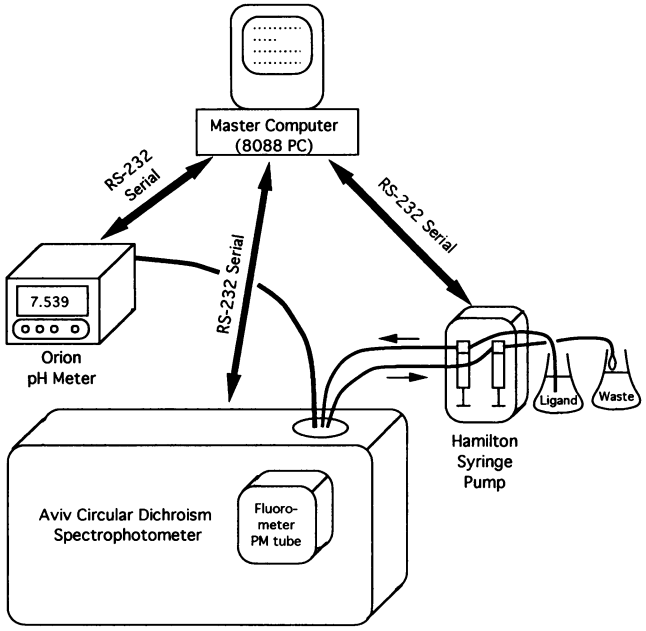

FIGURE 1 Block diagram of instrumentation. The master computer communicates with the syringe pump, spectrophotometer, and pH meter through serial ports COM1, COM2, and COM3. The pump is equipped with two syringes, which can add or remove solutions from the sample cell.

pump, pH meter, and spectrophotometer, a second PC computer has been added, and new software packages have been developed. In this paper we will describe the hardware and controlling software and we will show examples of how this instrument can monitor pH titrations and chemically induced unfolding reactions.

This is not the first report of a multi-dimensional spectrophotometer capable of fluorescence and CD measurements. Sutherland et al. (1976) reported on a spectrophotometer capable of a large variety of measurements, including absorbance, CD, magnetic CD, fluorescence, fluorescence-detected CD, and fluorescence-detected magnetic CD. However, the different types of measurements required the components of the instrument to be placed in different positions, so simultaneous measurements of all spectroscopic signals were not possible. An automated instrument capable of simultaneous measurements of CD and fluorescence was developed by Wada et al. (1980). This instrument was capable of monitoring the progress of thermally and chemically induced denaturation of proteins. In their case the denaturant concentration was increased by mixing the sample with a concentrated stock. Data analysis was performed by correlation diagrams, where the measured values of different data profiles were plotted against each other.

The instrument presented in this article can make the same types of measurements as the Wada et al. (1980) instrument; however, there are several important differences. First, we use the method of Job to perform chemically induced denaturation experiments, so that the concentration of the macromolecule is constant throughout the experiment. Second, we use a syringe pump with two syringes to maintain a constant cell volume. This approach allows higher denaturant concentrations to be reached. Rather than compare pairs of data with correlation plots, we use global data analysis to fit various thermodynamic models. This fitting procedure performs nonlinear, least-squares curve fitting of multiple types of data, taking into account the noise levels of the different measurements (to give proper weighting to the various data sets). Our acquisition routine also has options for sensing when a signal has reached equilibrium after each addition. Finally, we have a procedure for automatically adding aliquots (i.e., of H^+ ions) to achieve concentrations of titrant that are evenly spaced on a log scale (i.e., to automatically perform titrations at evenly spaced pH values).

METHODS AND MATERIALS

Materials

Wild-type sperm whale apomyoglobin (D122N variant) was provided as a gift from Dr. John Olson, Department of Biochemistry and Cell Biology, Rice University. Samples were prepared by diluting stock protein into 200 mM potassium phosphate buffer to 1–5 μM protein. Staphylococcal nuclease A was isolated from *Escherichia coli* strain AR120, harboring a plasmid containing the gene for the protein, using the procedure described by Shortle and Meeker (1989).

Spectrophotometer

All experiments were done with a modified Aviv 62DS CD spectrophotometer (Aviv and Associates, Inc., Lakewood, NJ). This instrument has a second Hamamatsu (Bridgewater, NJ) 948 photomultiplier tube (PMT) mounted at right angles to the excitation beam of the sample compartment (Ramsay and Eftink, 1994a). For fluorescence measurements a light filter is placed in the emission beam path between the sample and the second PMT. Tryptophan fluorescence is measured by exciting in the range of 280–295 nm and using an interference filter to select the emitted light at 340 nm. An excitation bandwidth of 0.6 nm was used for all measurements unless otherwise stated. Simple light scattering measurements are possible by exciting the sample at wavelengths transmitted by the filter. Relative absorbance changes are detected by recording the in-line dynode voltage of the PMT during CD measurements.

Accessories

To enable various titration experiments, we have connected (via a serial port) a Hamilton series 940 dual-syringe pump to the spectrophotometer. A special cell lid holds the ends of the syringe tubing and a thermistor probe (or a pH electrode) above the beam path (see Fig. 2). The sample compartment has a built-in stirring motor and thermoelectric temperature control device. The two syringes allow a number of experiments. The first syringe delivers the titrant (ligand, acid, or denaturant), and the second syringe can be used to deliver a higher concentration of titrant, to withdraw solution from the cell to avoid overfilling, or to add a second type of titrant. A pH microelectrode (8 mm diameter, connected to an Orion model 720 pH meter) can be inserted into the cell.

Computer interfacing and software

An 8086 based master computer is used to synchronize the activities of the syringe pump, pH meter, and spectrophotometer (via a second computer that controls the latter). The three devices are connected via serial ports 1,

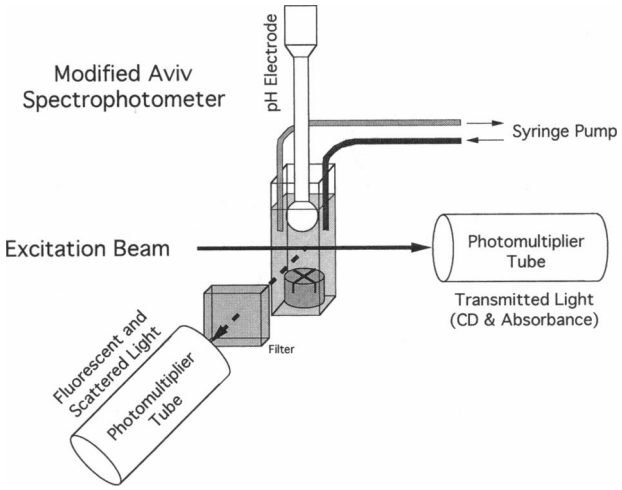

FIGURE 2 Modified Aviv spectrophotometer cell. The pH electrode is placed in the sample just above the excitation beam.

2, and 3 (COM1, COM2, and COM3) of the master computer. Because PC computers running DOS have poor support of COM3, we used a special software library to allow communications with COM3.

The schematic in Fig. 3 is a flow chart for the control and data acquisition routines. If a pH (selective ion) electrode measurement is being made, then the right-most route is used. If a denaturant (or other ligand) titration experiment is being performed, then the inner loop is used.

The program that runs on the master computer and controls all of the devices is called AAP. AAP first initializes the pump's syringes and then prompts the user for several experimental parameters (i.e., the sizes of the syringes, the volume of the injection, the sample volume, the total volume of the cell; these volumes are needed to enable AAP to avoid overflowing the cell). AAP controls communication with the pH meter and uses a TITRATE file to control the pump's actions. TITRATE is a file (written in the pump's custom language) that lists a series of commands to make injections and/or withdrawals from either of the syringes. The schedule of injections need not be evenly spaced volumes, and the two syringes can be controlled independently. For example, to maintain a constant concentration change with each injection the user can list increasing injection volumes. Alternatively, if there is a particular concentration range of interest, the user can list smaller injection volumes in the region of interest to give greater detail. Finally, it is possible to place two different concentrations of ligand (or even two different ligands) in the two syringes. With this arrangement the experiment can start with one syringe, then switch later to the other syringe at any time during the titration.

During an experiment the master computer commands (via connected COM2 ports) the Aviv computer to run an Aviv macro program for data collection. This macro can include any combination of data types at any set of wavelengths, so many measurements (e.g., CD and fluorescence at various wavelengths) can be made. The macro collects data as brief "kinetics" experiments (see Ramsay and Eftink, 1994a; "kinetics" refers to the collection of data as a function of time) and saves the average and standard deviation of the signals. AAP then commands the pump to make an injection. As described in Appendix I, the AAP program has three optional procedures for assuring that the signals have equilibrated after the titrant has been injected. The simplest of these procedures uses a specified pause between measurements; the more sophisticated procedures measure the drift in a signal and compare this drift to either a predetermined slope tolerance or to the standard deviation (noise) of the experimental signal. After equilibrium is reached, the AAP software commands the Aviv computer to make the next set of measurements. This routine continues until the titration experiment is complete. If the pH option has been turned on, then pH measurements are made before spectroscopic measurements. AAP continues to measure the pH until two consecutive values are within a particular tolerance of each other and then saves the last measured pH value. An optional procedure is the automatic pH titration algorithm described in Appendix II, which uses pH measurements in a feedback

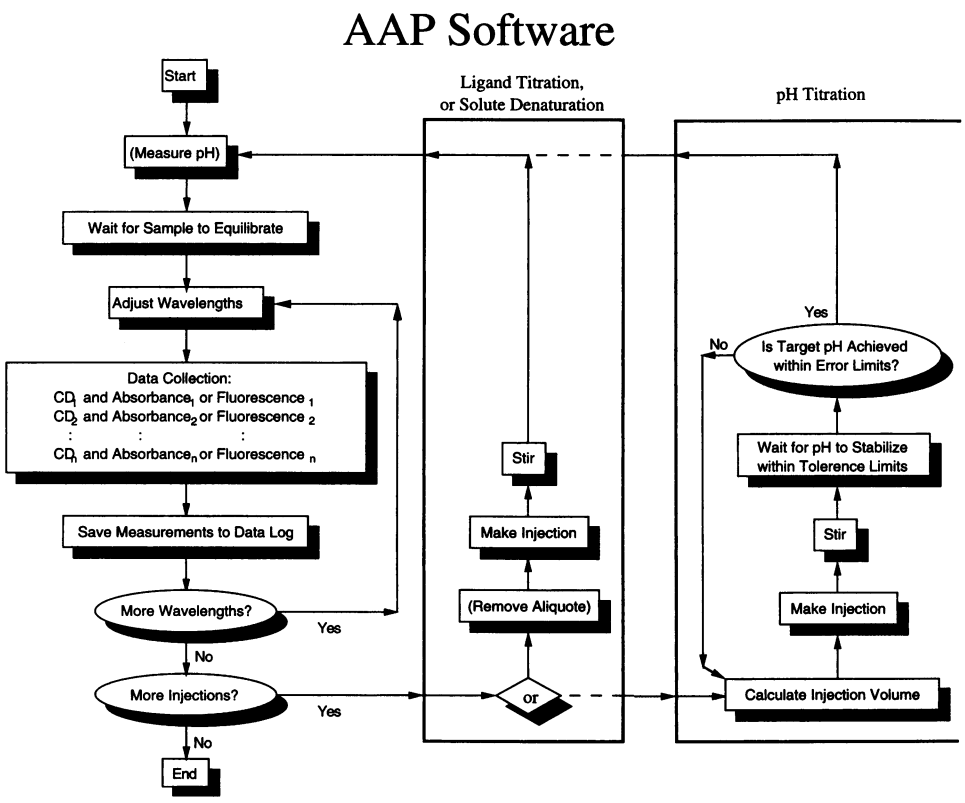

FIGURE 3 Schematic for the instrument control and data acquisition of our multi-dimensional spectrophotometer titration system. The route shown on the right is followed for pH (specific ion) titrations. The route shown in the middle is followed for denaturant (or other ligand) titrations.

routine to control the volume of aliquot to be added to reach the desired pH increments (i.e., to collect data at evenly spaced pH values).

File manipulation and data analysis

After the experiment is finished, the user must extract the different types of measurements from the data log file and merge the pH values (if made) with the corresponding spectroscopic data. This is done with a program called EXTRACT, which calculates the titrant concentrations and saves the individual data types into separate files in a format suitable for use with the nonlinear least-squares fitting program NONLIN (Johnson and Fraiser, 1985; Ramsay and Eftink, 1994b).

RESULTS

The combination of a multi-dimensional spectrophotometer and titration pump that we have assembled is designed so that a variety of experiments can be performed. As a demonstration of the usefulness of this instrument, two different types of experiments are shown. First, the titration of apomyoglobin with HCl is shown as an example of a pH titration. For these experiments it is advantageous to be able to cover several orders of magnitude of ligand concentration (H^+ concentration in this case), and, in the case of pH titrations, it is desirable to collect data at relatively evenly spaced pH values. By systematically increasing the injection volumes during the titration, the accessible concentration range can be increased. We will also show a procedure that uses the reading from the pH meter in a feedback routine that enables us to perform titrations over evenly spaced pH increments.

A second type of experiment involves chemical denaturation of a protein; these studies present a different set of problems. Here the challenge is to complete the experiment without overflowing the cell and to mix well the sample and the concentrated denaturant. The first problem is solved by using the two-syringe pump to maintain a constant cell volume. However, the concentrated urea and guanidine-HCl solutions have higher densities than the buffer solutions, so the tendency is for the denaturant to sink to the bottom of the cuvette and not to mix. We have found that with a sample volume of 1.8 ml (in a standard 1 × 1 cm cuvette) there is sufficient sample for measurements, and complete mixing is achieved with a stirring bar. Larger volumes are troublesome; none of the stirring bars that we have used can perform adequate mixing of urea and guanidine-HCl solutions in a 1 × 1 cm cuvette for volumes above 1.8 ml.

pH titration

The results for the pH titration of apomyoglobin are shown in Fig. 4. This titration was accomplished using the feedback procedure described in Appendix II to automatically achieve evenly spaced pH values. An alternative strategy (which can be used for titration of other ligands) is to create a schedule of injection/withdrawal volumes in a TITRATE file (used to control the syringe pump). In either case, for pH titrations starting at neutral pH it is useful to have some buffer in the solution so that the addition of the first few aliquots of acid (or base) do not cause too large a change in pH.

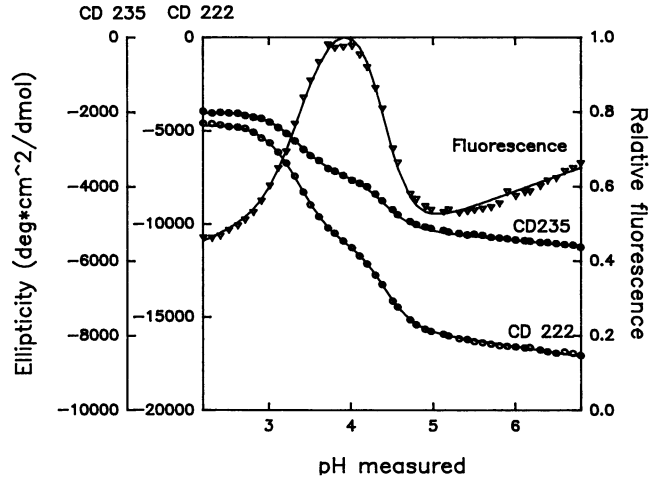

FIGURE 4 pH titration of apomyoglobin. The relative fluorescence and CD at two wavelengths were measured. Fluorescence was measured by exciting at 295 nm and monitoring the emission with a 340 nm interference filter. The initial sample was prepared in 0.2 M sodium phosphate buffer, 25°C. Titration was performed with 1 N HCl. The solid lines through the three data sets are for a global fit of a model described by Scheme 1, as shown in the text.

For the acid titration of apomyoglobin in Fig. 4, it is clear that both the CD and fluorescence signals observe a multistep process, which can be minimally described by the equilibria

$$H_mMH_n^{(m+n)+} \rightleftharpoons mH^+ + MH_n^{n+} \rightleftharpoons nH^+ + M$$

Scheme I

where there are m protons that dissociate with $K_{D,m} = [MH_n^{n+}][H^+]^m/[H_mMH_n^{(m+n)+}]$ and n protons that dissociate with $K_{D,n} = [M][H^+]^n/[MH_n^{n+}]$. Starting at neutral pH (where the M state predominates), the fluorescence increases as the protein is converted to the MH_n^{n+} state. With further addition of acid, the fluorescence decreases as the protein is converted to the fully protonated $H_mMH_n^{(m+n)+}$ state. The CD signals also show the stepwise transition as a plateau region near pH ~ 4. The solid lines in Fig. 4 are a global analysis of the three data sets to the above thermodynamic model with $K_{D,m} = 1.30 \times 10^{-7}$ M^m, $m = 2.05$ protons, $K_{D,n} = 4.52 \times 10^{-10}$ M^n, and $n = 2.12$ protons. This model is based on two assumptions: first, that there are only three different macromolecule species, which differ in the degree of protonation, and, second, that each of these species has a characteristic CD and fluorescence signal. This is a simpler model than has been used by Barrick and Baldwin (1993) to describe the combined pH and urea induced unfolding of apomyoglobin (monitored by CD data). Because we have not varied the concentration of denaturant in the data in Fig. 4, we have not included an unfolding equilibrium constant as is contained in the model proposed by Barrick and Baldwin (1993). The multi-phasic pH titration data that we have presented in Fig. 4 are very similar in appearance to the

data of Barrick and Baldwin (1993), but we have simultaneously obtained and analyzed the fluorescence and CD changes for the pH titration of this protein.

Denaturant titrations

As an example of the use of our multi-dimensional spectrophotometer to study the denaturant-induced unfolding of proteins, we have published data for the urea-induced unfolding of *trp* aporepressor from *E. coli* (Eftink et al., 1994). In our previous study we found that a three-state unfolding model is required to describe the data at pH 4–6.5, whereas a two-state model is adequate at other pHs. As a second example we show here data for the guanidine-HCl unfolding of apomyoglobin. The unfolding of apomyoglobin has been reported to be multi-state (Balestrieri et al., 1976; Irace et al., 1981; Barrick et al., 1994; DeYoung et al., 1993), with an intermediate identified as a molten globule state (see Hargrove et al., 1994 for further reference). To confirm that the three-state model is adequate, we have monitored the GuHCl-induced unfolding of wild-type apomyoglobin at neutral pH with simultaneous measurements of CD signals at two wavelengths and tryptophan fluorescence (see Fig. 5). Our observations are similar in character to that in previous work. However, we detect a multi-phasic denaturation mechanism in the CD signal (Fig. 5 and Table 1), which has not been previously observed at neutral pH. This is in part because our apparatus allows us to easily make many measurements in the transition region and thus to reveal the details. The individual data sets were fitted using nonlinear least squares to a three-state model (assuming a linear dependence of the free energy of unfolding on denaturant concentration).

$$N \rightleftharpoons I \rightleftharpoons U \tag{1}$$

$$K_{I/N} = [I]/[N]; \quad K_{U/I} = [U]/[I] \tag{2}$$

where N, I and U are native, intermediate, and unfolded states of the protein, respectively. These fits are shown as the dashed lines in Fig. 5; see also the residual patterns for these fits in Fig. 6 and the fitting parameters in Table 1. Interestingly, the midpoints of the transitions (here defined as the GuHCl concentration where $K_{I/N} = 1$ or $K_{U/I} = 1$) for the first transition do not agree between the CD and fluorescence measurements, whereas good agreement is seen for the second transition. Global analysis with a three-state model, where all of the CD and fluorescence data from a single experiment are fitted simultaneously, has been performed (see the solid lines in Fig. 5 and the residuals in Fig. 6). The global fit is not quite as good as the individual fits of the three data sets. There are several possible reasons for this. First, the CD and fluorescence baselines of the native and unfolded states are assumed to be linear, and the baseline of the intermediate is assumed to be the average of the native and unfolded states. Second, the free energy changes of the transitions are assumed to be linear functions of the guanidine-HCl concentration. If any of these assumptions are incorrect then the analysis could give poor fits, despite the three-state model being valid. Alternatively, the three-state model could give a poor fit because more than one equilibrium folding intermediate exists. We are now performing further experiments to better characterize the thermodynamics of the unfolding of apomyoglobin.

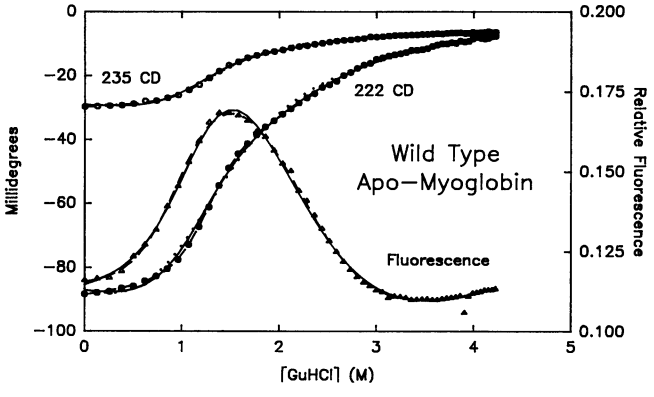

FIGURE 5 Guanidine-HCl titration of apomyoglobin. (●) CD measurements at 222 nm; (○) CD measurements at 235 nm. (△) Fluorescence measurements, with excitation at 285 nm, and emission followed by a 340 nm interference filter. (·····) Fits of the individual CD profiles with the two-state model; the fluorescence data cannot be fitted by a two-state model. (-------) Fits of the individual CD and fluorescence profiles with the three-state model. (——) Fits using global analysis of the CD and fluorescence with the three-state model. Fitting results are shown in Table 1. Linear baselines were used for the native and unfolded states, while the baseline slope of the intermediate state was assumed to be an average of the native and unfolded states.

CONCLUSIONS

The merits of having a multi-dimensional spectrophotometer such as ours have been previously shown with thermal melt experiments (Ramsay and Eftink, 1994a,b). Here we have expanded its usefulness by adding a computer-controlled titrator. The advantages of this setup are that pH/ligand titration and solute denaturation experiments can run automatically, freeing the experimenter from this chore. A typical experiment takes 2–3 h, and several spectroscopic data sets can be collected in the same run. All measurements are made with one sample, so there is no sample variation; consequently, there can be better agreement between the different types of measurements. This also allows subtle differences between the measurements to be more easily revealed (e.g., the case of the first transition of apomyoglobin). The ability to make multiple measurements does not eliminate the need to show reproducibility of the experiment.

The AAP software pauses for a predetermined delay between the injection and the data measurements to allow the sample to equilibrate. We have also recently modified the software to perform periodic spectroscopic measurements to determine whether the signal is stabilized (see Appendix I). The advantage of this approach is that slowly

TABLE 1 Thermodynamic parameters for the guanidine-hydrochloride-induced unfolding of wild-type apomyoglobin, pH 7.0

Signal	$\Delta G^\circ_{o,I/N}$	$m_{I/N}$	$[GuHCl]_{1/2,I/N}$	$\Delta G^\circ_{o,U/I}$	$m_{U/I}$	$[GuHCl]_{1/2,U/I}$
222 CD	3.6 ± 0.4	3.0 ± 0.2	1.2	3.1 ± 0.6	1.3 ± 0.3	2.4
235 CD	3.7 ± 0.3	3.0 ± 0.1	1.1	3.5 ± 0.7	1.4 ± 0.2	2.5
285 Flu	2.0 ± 0.6	2.9 ± 0.3	0.7	4.1 ± 2.4	1.7 ± 0.9	2.4

Units are kcal/mol protein for three energy changes, kcal/(mol protein·mol/l GuHCl) for m values, and mole/liter GuHCl for $[GuHCl]_{1/2}$. The ΔG°_o values are the free energy change for a transition in the absence of guanidine-HCl; the m values are the dependence of the apparent free energy change for unfolding on the concentration of guanidine-HCl (i.e., for the linear free energy relationship, $\Delta G^\circ = \Delta G^\circ_o - m\cdot[GuHCl]$). The errors shown are the standard deviations for three independent titration experiments.

reacting samples will be automatically recognized and be given the chance to equilibrate.

The use of a pH electrode (or another ion-specific electrode) allows these free ligand concentrations to be measured along with the spectroscopic data. The interface with the pH meter also presents the opportunity to create a feedback loop. Rather than use predetermined volumes to make the injections, the syringe pump, pH meter, and computer can potentially operate like a titrator and scale the injection volumes to obtain evenly spaced concentration changes (see Appendix II).

APPENDIX I

Frequently a conformational transition of a macromolecule does not rapidly come to equilibrium after a perturbation such as a change in temperature, an increase in denaturant concentration, or a pH jump. This is troublesome because accurate thermodynamic parameters for the transition can only be calculated when the sample is at equilibrium. Frequently the criterion for equilibrium is that the observed signal is stable over time. When an experiment is done manually it is easy for the researcher to wait for it to stabilize before recording the values. However, automatic data collection systems usually do not have any criteria for judging whether the sample is equilibrated and are therefore susceptible to misleading measurements.

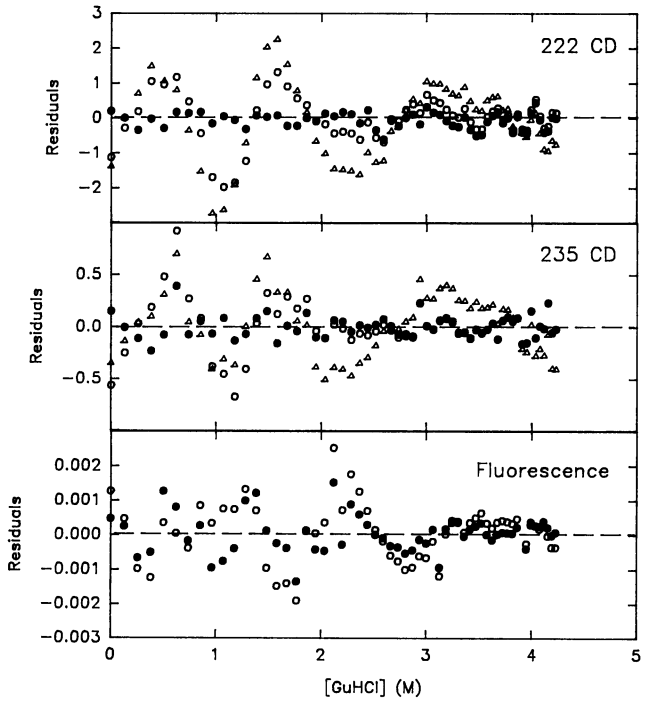

FIGURE 6 Residuals of the fits for the apomyoglobin data shown in Fig. 5. (△) Residuals of fits of the individual CD profiles with the two-state model (triangles not shown for the fluorescence data, because a two-state model will not describe these data). (●) Three-state fits of the individual profiles. (○) Global analysis of all of the CD and fluorescence data.

The AAP software that we have developed has three criteria for judging when the time is appropriate to make spectroscopic measurements. The first is to simply wait a fixed time (user-specified) after an injection. This approach is reasonable when the reaction kinetics are known to be rapid and when the only concern is to allow for complete mixing of the sample.

The second criterion is to wait a fixed period of time and then to monitor for changes in the spectroscopic signal. This is done by repeatedly measuring the absolute signal and waiting for a period of time (user-specified) between measurements. If the signal for the two most recent measurements differs by less than a specified tolerance, then the sample is judged to be equilibrated, and the AAP program triggers the Aviv instrument to begin data acquisition. If the signal change is larger than the specified amount, then AAP pauses for the waiting period and repeats the equilibration checking procedure.

The third means of checking if the sample is equilibrated is done in the same manner as the second, except that the criterion is not whether the signal has changed more than a fixed amount. Rather, the signal is compared with the measured noise in the actual spectroscopic signal. The standard deviation of the signal is measured and it is multiplied by a constant that the user has entered. If this scaled standard deviation is larger than the observed change in the absolute signal (from checks made after a waiting period), then the signal is considered to be stable. A change in the signal that is larger than the scaled standard deviation is considered unstable. The advantage of this approach over the second is that the criterion for equilibration is automatically scaled to the ability of the instrument to detect significant changes in the signal. The disadvantage is that the criterion is constantly changing (with changes in the noise of the data).

Another option to initiate collection of spectroscopic data at a particular condition is by a user override command.

There is no certain mechanism for automatically determining when a sample is fully equilibrated. The above options all require that the user have some prior knowledge of the system's behavior, so that the appropriate parameters (tolerated signal change, time intervals, scaling constant) are used. The best possible approach is for the user to perform separate kinetics experiments to determine the system's relaxation time for an induced conformational transition.

As a test of the "approach to equilibration" routines, we have performed urea-induced unfolding studies with staphylococcal nuclease A at 5°C, as shown in Fig. 7. This protein and temperature were chosen because the relaxation time for the unfolding reaction is ~15 min at the $[Urea]_{1/2}$ (Sugawara et al., 1991). This relaxation time is longer than the time needed for the acquisition of a data point at a given concentration of denaturant in a study such as that in Fig. 5. Apomyoglobin, on the other hand, unfolds so rapidly that equilibration is quickly reached and thus would not be a useful protein to demonstrate the utility of this acquisition routine.

Fig. 7 shows the unfolding profile for nuclease A (as measured by fluorescence and CD) when only a short pause of 30 s is allowed between injection and data acquisition (Fig. 7, *open symbols*). Also shown are data obtained using the third option described above for approaching equilibration, in which

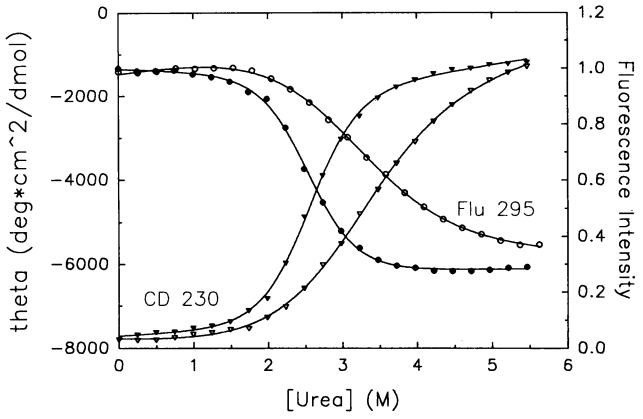

FIGURE 7 Urea-induced unfolding of staphylococcal nuclease A at 5°C (pH 7, 0.05 M Tris buffer). The open symbols are data acquired using only a 30-s pause before each data acquisition. The closed symbols are data acquired using option 3 for determining the approach to equilibrium. This method compares the drift in the CD signal with the noise in the signal. When the drift is less than 2.0 times the noise of the signal, the acquisition is started. The solid lines are a fit to a two-state unfolding model. With the approach to equilibrium option used (●, ▼), the global fit of the two data sets gives $\Delta G^{\circ}_{o,U/N} = 4.31 \pm 0.05$ kcal/mol and $m = 1.69 \pm 0.02$ kcal/(mol·M of urea); for the 30-s pause option (○, ▽), the global fit gives $\Delta G^{\circ}_{o,\ U/N} = 2.78 \pm 0.12$ kcal/mol and $m = 0.93 \pm 0.06$ kcal/(mol·M urea).

the drift in the signal after each addition is compared with the actual noise in the signal (Fig. 7, *closed symbols*). As can be seen, the latter equilibration routine produces a sharper unfolding curve (and a larger $\Delta G^{\circ}_{o,N/U}$ and $m_{N/U}$) than that obtained with the short pauses. This is what is expected if the unfolding transition has not reached equilibrium. An indication that the approach to equilibrium routine works is that one needs more than 600 s before achieving the equilibration condition and acquiring data in the transition range. We have not thoroughly tested this acquisition feature, but it is clear that it will be important, in cases where the unfolding equilibrium is reached slowly, to use one of the above equilibrium-checking options to be assured of obtaining valid thermodynamic parameters.

APPENDIX II

The AAP software also includes a procedure for performing automatic pH (or specific ion) titrations in combination with an interfaced pH meter. For these experiments the left syringe of the syringe pump is loaded with acid or base (or specific ion solution). The user is prompted for the final desired pH, pH increment, pH tolerance, and the acceptable pH error. The pH increment (e.g., 0.1 pH units) represents the desired steps for the titration; it is used to calculate the target pH, pH_T, from the prior pH, pH_P, according to $pH_T = pH_P$ − increment (or + increment, if an upward pH scan is desired). The pH tolerance (i.e., 0.001 pH units) is the amount of change in successive pH signals that is considered acceptable for a pH value to be measured (i.e., for mixing and electrode equilibration). If this tolerance is not achieved within a few seconds, then the AAP software continues waiting. The pH error is the value above and below the target pH that is considered acceptable to begin spectroscopic data acquisition. For example, if the target pH_T is 6.5 and the pH error is 0.03, then a measured pH between 6.47 and 6.53 is considered to be adequately close to the pH_T. After calculating a set of target pH values, the AAP software begins by injecting an initial test volume, v_i, into the cell and then measures the current pH, pH_C (once the pH measurement is within the tolerance). The $(pH_C - pH_P)/v_i$ ratio is the rate of change of pH with volume, $\Delta pH/v_i$. After this test injection, the AAP program calculates the next injection volume, v_j, by using a linear extrapolation relationship. That is, the following equation is used to approximate the volume, v_j, needed to reach the target pH: $pH_T = pH_C + (\Delta pH/v_i) * v_j$. Solving for v_j, a second (and successive) injection(s) is made until the current pH is within the error range of pH_T.

Using this algorithm, we have performed automatic pH titrations in combination with the multi-dimensional spectrophotometer, as shown by the example in Fig. 4. In this example, we were able to perform a titration from pH 7 to 2, with measurement of CD and fluorescence signals and 30 0.1 pH steps, over a time of about 1 h. By using a 1.0 N HCl solution in the syringe and 2.5 ml of sample in the 1 × 1 cm cell, we achieved this pH titration by adding a total volume, Σv, of HCl of 0.15 ml, which is only about 5% of the initial volume of the sample. Because a small dilution of the sample occurs, the CD and fluorescence signals have been corrected for dilution. We find that the titrations work best when the sample has some buffering capacity at the initial pH (assuming that a titration is started near neutral pH and proceeds to either extreme pH).

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