

# High-Sensitivity Fluorescence Anisotropy Detection of Protein-Folding Events: Application to $\alpha$ -Lactalbumin

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**ABSTRACT** An experimental procedure has been devised to record simultaneously fluorescence intensity and fluorescence anisotropy. A photoelastic modulator on the excitation beam enables the anisotropy signal to be recorded in one pass using a single photomultiplier tube and eliminates the need for a polarizer on the emission path. In conjunction with a stopped-flow mixer, providing a time-resolved capability, this procedure was used to study the refolding of *apo*  $\alpha$ -lactalbumin following dilution from guanidinium chloride. Although the fluorescence intensity does not change detectably, the fluorescence anisotropy was found to resolve the conformational changes occurring between the initial unfolded state and the molten globule state formed either kinetically during refolding at pH 7.0 or at equilibrium at pH 2.0 (A-state). This result provides further evidence that fluorescence anisotropy is a valuable probe of protein structural transitions and that the information it provides concerning the rotational mobility of a fluorophore can be complementary to the information about the local environment provided by fluorescence intensity.

## INTRODUCTION

Studies of the process of refolding of a protein following denaturation have involved the use of many different biophysical techniques, each of them probing the recovery of a different feature of the native state (Plaxco and Dobson, 1996). In the case of those proteins that fold with effective two-state kinetics, the different methods provide similar kinetic parameters as the folding reaction is highly cooperative (see, for example, Kragelund et al., 1995; Van Nuland et al., 1998). Other proteins show more complex folding behavior involving a series of events taking place with different kinetic profiles. In such cases, different biophysical techniques generally respond in different manners to these events and therefore allow dissection of the various steps in the complex transformation that converts an unfolded protein into its native structure. The use of a wider range of techniques is therefore likely to lead to the resolution of greater detail in the folding process (Dobson et al., 1998; Callender et al., 1998). Examples of complex folding processes include those of *apo*-myoglobin, which involves at least three populated intermediates (Jennings and Wright, 1993), and human lysozyme, where multiple pathways are observed, one of which again involves three refolding intermediates (Canet et al., 1999).

The development of biophysical techniques able to reveal new levels of complexity in the behavior of proteins is therefore of considerable importance. We have used a newly developed procedure to record fluorescence anisotropy in a

time-resolved mode. This procedure involves the use of a photoelastic modulator on the excitation beam and allows stopped-flow fluorescence anisotropy experiments to be carried out routinely, very much like stopped-flow fluorescence intensity or stopped-flow circular dichroism experiments. This is in contrast to conventional fluorescence anisotropy methods, which require three polarizers and two detectors (Otto et al., 1994; Lakowicz, 1999a). Using this procedure, fluorescence anisotropy data have been recorded simultaneously with fluorescence intensity during the refolding of *apo*  $\alpha$ -lactalbumin from guanidinium chloride (GuHCl). The results show that fluorescence anisotropy can detect the formation of structure that restricts the motion of tryptophan side chains in the early stages of the folding process before the formation of additional interactions that result in significant changes in fluorescence intensity. This method is particularly helpful for *apo*  $\alpha$ -lactalbumin because it enables a distinction to be made between the initial unfolded state and a burst-phase collapsed state, or molten globule that forms early in the folding reaction.

## MATERIALS AND METHODS

### Materials

Bovine  $\alpha$ -lactalbumin and GuHCl were obtained from Sigma Chemical Co. (Poole, UK). GuHCl concentrations were determined by measurement of the refractive index (Pace, 1986). To sequester any remaining  $\text{Ca}^{2+}$  ions and generate the *apo* form of the protein,  $\alpha$ -lactalbumin was dissolved in a 6 M GuHCl solution containing 1 mM EDTA. The refolding buffer contained 3 mM EDTA to ensure the protein remained free of bound  $\text{Ca}^{2+}$  during the refolding process.

### Stopped-flow procedures

Refolding of *apo*  $\alpha$ -lactalbumin was initiated by mixing 1 vol of protein in 6 M GuHCl with 10 vol of refolding buffer in an SFM-3 stopped-flow mixer (Bio-Logic, Claix, France), except for the traces in Fig. 3 where 1

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vol of protein in 6 M GuHCl was mixed with 20 vol of buffer. The refolding buffer was 50 mM sodium phosphate, 3 mM EDTA and contained various concentrations of GuHCl at pH 7.0. The protein concentration in the cell for the scanning experiments was 21  $\mu$ M; in the kinetic experiments it was 12  $\mu$ M, except for the experiment shown on Fig. 3 where it was 32  $\mu$ M. The fluorescence signals were digitized using two time bases, which allowed a large number of data points to be recorded at short times after mixing. The traces shown in Fig. 4 are the result of single stopped-flow shots in each buffer solution. The traces shown in Fig. 3 are an average of four shots. Experiments were also carried out by manual mixing using a Bio-Logic SC-1 standard cuvette holder with a 1 cm  $\times$  1 cm cuvette and a protein concentration of 7  $\mu$ M. Stability parameters calculated from the fits to the anisotropy transition curves are the mean and standard deviations resulting from the two experiments at pH 7.0 conducted in the stopped-flow mode and with the SC-1 cell holder. For refolding experiments at pH 2.0, a 50 mM KCl/HCl buffer was used; all the other parameters were the same as those of the experiments carried out at pH 7.0. The errors quoted for the stability parameters at pH 2.0 are those obtained from the fitting procedure. All experiments were carried out at 25°C, temperature control being achieved by using a water bath connected to the SFM-3 mixer.

### Fluorescence anisotropy and fluorescence intensity measurements

The dimensions of the stopped-flow cell were 10 mm  $\times$  1.5 mm (Bio-Logic TC-100/15), except for the experiment shown in Fig. 3 where a 2 mm  $\times$  2 mm cell was used (Bio-Logic FC-20). The light source was a 150-W mercury-xenon lamp (Hamamatsu, Enfield, UK) followed by a Jobin-Yvon (Longjumeau, France) BH-10UV monochromator. For the anisotropy excitation spectra, the excitation beam had a slit width of 4 nm and the emission spectrum was monitored above 335 nm with a WG 335 cutoff filter (Schott Glaswerke, Mainz, Germany). For kinetic experiments, the excitation wavelength was set at 297 nm with a slit width of 4 nm; the emission signal was monitored above 335 nm.

The experimental setup consisted, along the excitation beam, of a calcite polarizer set for a fixed polarization (vertical or horizontal) followed by a PEM-90 photoelastic modulator (Hinds Instruments, Hillsboro, OR), operating at half-wave retardation. The modulator, installed with its axis at 45° from the polarizer direction, generated excitation light with vertical and horizontal polarizations alternating at a frequency of 100 kHz. The emission was detected at right angles to the excitation axis without a polarizer installed in front of the photomultiplier. Emission intensities corresponding to vertical or horizontal excitation polarization directions were separated by a lock-in amplifier synchronized with the vibration of the photo-elastic modulator (PMS-400 module from Bio-Logic). The resulting two emission signals were digitized in a PC (Fujitsu P166, Nanterre, France). The Bio-Kine software (Bio-Logic) was used to calculate the anisotropy and fluorescence intensity in real time from the two emission signals. Further details of the calculation methods are given below in the Results.

### Global analysis of the kinetic curves

Simultaneous analysis of the kinetic traces of the fluorescence intensity and of the fluorescence anisotropy was performed using Origin software (Microcal, Northampton, MA) using a theoretical approach described previously (Otto et al., 1994). The two kinetic traces were fitted to a reaction scheme involving two states. The two species were characterized by their fluorescence quantum yield ( $q_i$ ) and their anisotropy level ( $r_i$ ). The observed fluorescence intensity was calculated as  $S(t) = \sum_i x_i(t) \times q_i$  where  $x_i(t)$  is the fraction of species in state  $i$  at time  $t$ . The observed fluorescence anisotropy was calculated as  $r(t) = (\sum_i x_i(t) \times r_i \times q_i) / (\sum_i x_i(t) \times q_i)$ . The fitting procedure provides values for the rate constant of the process being

monitored, for the two quantum yields ( $q_i$ ) and for the two anisotropy levels ( $r_i$ ). It is noteworthy that the time dependence of the anisotropy is non-exponential, as it results from a ratio of two terms each having exponential behavior (Otto et al., 1994).

### Analysis of the amplitudes resulting from the kinetic fits as a function of GuHCl

The amplitudes resulting from the global analysis described above ( $q_i$  and  $r_i$ ), carried out under different folding conditions, were plotted as a function of GuHCl. Of the resulting transition curves, those showing the burst phase anisotropy amplitudes were analyzed according to a two-state model. For this purpose, the function:  $r = r_n / (1 + \alpha) + r_u \times \alpha / (1 + \alpha)$  was used, where  $r_n$  and  $r_u$  are the anisotropy levels of the initial and final species in the GuHCl transition curve, and where  $\alpha = \exp((m(\text{GuHCl}) - \Delta G_0)/RT)$ .  $\Delta G_0$  is the Gibbs free energy change in the absence of GuHCl and  $m$  is a cooperativity index (Santoro and Bolen, 1988).

## RESULTS

### Experimental procedures

In the conventional fluorescence anisotropy experiment, fluorescence emitted from a sample is detected at right angles to the excitation beam, and these two beams define the scattering plane. By means of a polarizer the excitation light is linearly polarized in a direction perpendicular to the scattering plane (first index  $v$ ). For rapid fluorescence anisotropy measurements, usually two photomultiplier tubes (PMTs) are employed in a T-shaped configuration to measure simultaneously the emitted fluorescence light under the two respective relative polarizations, parallel (second index  $v$ ) and perpendicular (second index  $h$ ) to the polarization of the incoming beam (Otto et al., 1994). The fluorescence intensity is:

$$S = I_{vv} + 2 \times G \times I_{vh} \quad (1)$$

The anisotropy  $R$  is defined as the normalized fluorescence intensity difference between the two channels after vertically polarized excitation:

$$R = (I_{vv} - G \times I_{vh}) / (I_{vv} + 2 \times G \times I_{vh}) \quad (2)$$

The factor of 2 in these formulae originates from the cylindrical symmetry of the arrangement around the  $z$ -axis; after vertically polarized excitation, two orthogonal horizontal directions exist that are equivalent to each other (Lakowicz, 1999a). For example in Fig. 1 A,  $\vec{\mu}$  is the dipole moment that has been excited by the vertical excitation beam and that has subsequently reoriented before emission takes place. The emitted intensity associated with the  $z$ -component of the dipole moment ( $\mu_z$ ) gives rise to the  $I_{vv}$  intensity. The two orthogonal horizontal components of the dipole moment are  $\mu_x$  and  $\mu_y$ , and these components are equivalent for a randomly orienting sample. Thus, only one polarization direction needs to be measured, and in the case of Fig. 1 A it is  $\mu_x$ .

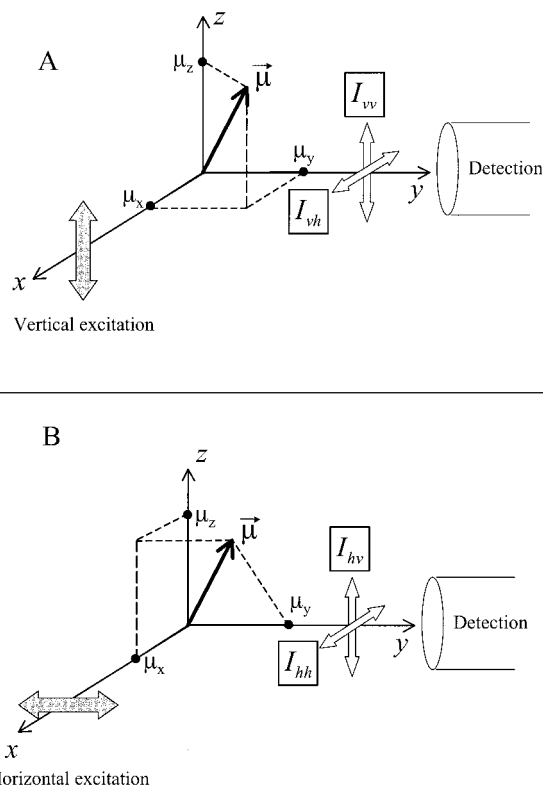


FIGURE 1 Schematic view of the experimental procedure to measure fluorescence anisotropy. (A) The excitation polarization is vertical, and detection without a polarizer gives  $I_{vh} + I_{vv}$ . (B) The excitation polarization is horizontal, and detection gives  $I_{hh} + I_{hv}$ . The latter has been shown to be equal to  $2 \times I_{vh}$  if it is assumed that the sample is randomly orienting, as explained in the text.

A correction factor  $G$  is introduced into Eqs. 1 and 2 above to take into account the differences in sensitivity of the detection system in the two polarizing directions  $I_{vv}$  and  $I_{vh}$ . With conventional fluorescence anisotropy detection systems these differences in sensitivity are unavoidable, in particular because of differences in sensitivity of the two photomultipliers used for measuring fluorescence in the two polarization directions. The  $G$  factor can be evaluated by turning the excitation polarizer to a horizontal position (first index  $h$ ). As the two emission polarizers are now at right angles to the excitation direction, equal intensities must be recorded by the two emission channels. The correction factor is thus defined as:

$$G = I_{hv}/I_{hh} \quad (3)$$

This correction is not, however, fully satisfactory and introduces potential new errors into the measurements. The main problem resides in the physical rotation of the excitation polarizer, which can introduce displacements of the excitation beam that in turn may change the balance between the two measurement directions.

The modified procedure described in this paper avoids this problem and uses a simplified detection system that uses only one PMT without any polarizer. Here, the polarization of the excitation light is altered between  $v$  and  $h$  by means of a photoelastic modulator. On the emission side both polarization directions are detected simultaneously. As shown in Fig. 1 A, when the excitation light is vertically polarized, the sum of the emitted intensities  $I_{vv}$  and  $I_{vh}$  associated with the  $\mu_z$  and  $\mu_x$  components, respectively, are detected. Hence, this measurement yields:

$$A_v = I_{vh} + I_{vv} \quad (4)$$

The horizontally polarized excitation situation is shown in Fig. 1 B. The PMT signal now represents the  $I_{hv}$  and  $I_{hh}$  value. These emitted intensities again are associated with the  $\mu_z$  and  $\mu_x$  components of the dipole moment, respectively, which are now both orthogonal to the excitation direction. For a randomly orienting sample, the two intensities are equivalent to each other; due to the right-angled geometry, they are also equivalent to the orthogonal component  $I_{vh}$  created by  $\mu_x$  in Fig. 1 A. Therefore, the signal detected for horizontally polarized excitation yields:

$$A_h = 2 \times I_{vh} \quad (5)$$

And the anisotropy is calculated from  $I_{vv}$  and  $I_{vh}$  using the resulting expression:

$$R = (I_{vv} - I_{vh})/(I_{vv} + 2 \times I_{vh}) \\ = (A_v - A_h)/(A_v + 0.5 \times A_h) \quad (6)$$

This method was tested by measuring the anisotropy of the light scattered by a dilute suspension of colloidal silica in water. This measurement should yield an anisotropy of  $R = 1.00$  as the signal arises from scattering (Lakowicz, 1999a). Without any additional correction, we reproducibly obtained values above 0.95 using the method described above. This was considered satisfactory for kinetic measurements, and no correction equivalent to the value of the  $G$  factor was introduced into the measurement procedure. Work in progress to improve the performance of electronic components of the equipment used in the experiment should permit a value of above 0.99 to be achieved.

### Refolding of $\alpha$ -lactalbumin followed by fluorescence anisotropy measurements

The anisotropy excitation spectra of *apo*  $\alpha$ -lactalbumin in its native state at pH 7.0, in a partially folded state (the molten globule or A-state) at pH 2.0, and highly unfolded in 6 M GuHCl at pH 7.0 are shown in Fig. 2. The spectra are in good agreement with earlier anisotropy spectra of proteins and other tryptophan-containing compounds (Lakowicz et al., 1983; Teale and Badley, 1970). The anisotropy spectra of all three states show a maximum close to 265 nm,

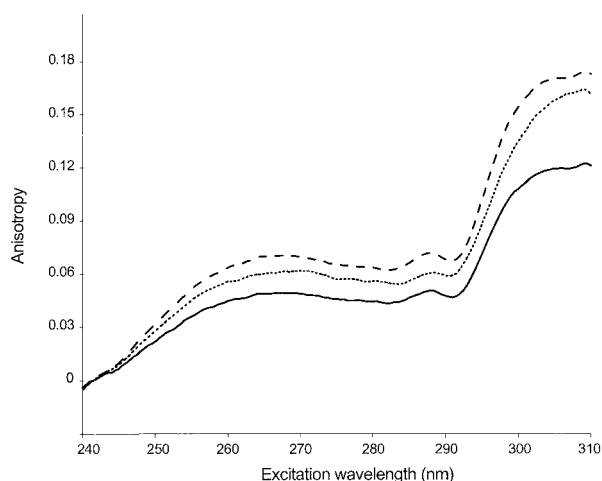


FIGURE 2 Excitation spectra of *apo*  $\alpha$ -lactalbumin fluorescence anisotropy under different conditions. These are denatured state in 6 M GuHCl at pH 7.0 (—), the A state formed at pH 2.0 (···) and the native state at pH 7.0 (---). Emission was measured at wavelengths above 335 nm.

two minima between 280 and 290 nm, and a maximum above 295 nm. The shape of the spectra is due to the intrinsic properties of the electronic transitions of tryptophan (Lakowicz, 1999b). To obtain the best signal-to-noise ratio in the stopped-flow refolding experiments, we chose an excitation wavelength of 297 nm, where the anisotropy difference between the different states is substantial, where the fluorescence intensity is still high, and where the output of the source lamp is high. The anisotropy of the A-state is located between the values for the native and highly unfolded states, which is consistent with an intermediate degree of structural organization.

Fig. 3 shows typical kinetic traces of the fluorescence intensity and the fluorescence anisotropy corresponding to the refolding of *apo*  $\alpha$ -lactalbumin in 0.3 M GuHCl at pH

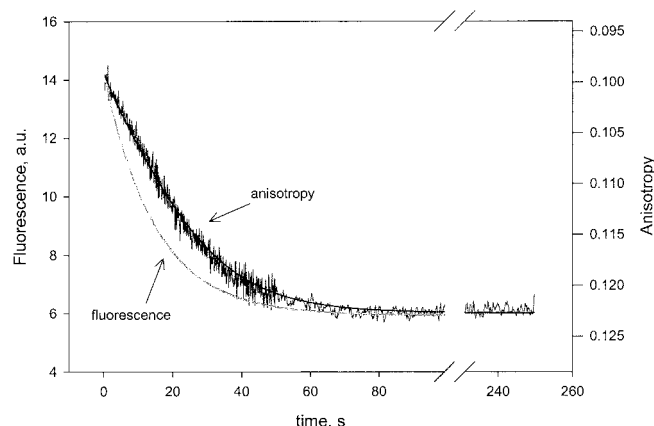


FIGURE 3 Fluorescence and anisotropy kinetic traces of the refolding of *apo*  $\alpha$ -lactalbumin at pH 7.0 in 0.3 M GuHCl. The fits resulting from global analysis (see text) are also displayed. Note that the anisotropy change lags behind the fluorescence change (see text).

7.0. The anisotropy has been re-scaled and inverted in sign to overlay graphically the initial and end points of the two traces, and fits resulting from the global analysis of the data (see Materials and Methods) are shown. As expected from theoretical considerations (Otto et al., 1994) the anisotropy trace lags behind the fluorescence intensity, which follows a pure single-exponential decay, because the fluorescence intensity is decaying during the reaction. If the fluorescence intensity were to increase, the anisotropy intensity would be expected to develop more rapidly than the exponential growth of the fluorescence intensity (Otto et al., 1994).

Fig. 4 shows the time dependence of the fluorescence intensity and the fluorescence anisotropy resulting from the refolding of *apo*  $\alpha$ -lactalbumin at pH 7.0 at a series of different GuHCl concentrations after dilution from a 6 M solution. A well defined slow kinetic phase is observed only at GuHCl concentrations below 2 M. At higher GuHCl concentrations there is no change in either the fluorescence intensity or the fluorescence anisotropy observable during

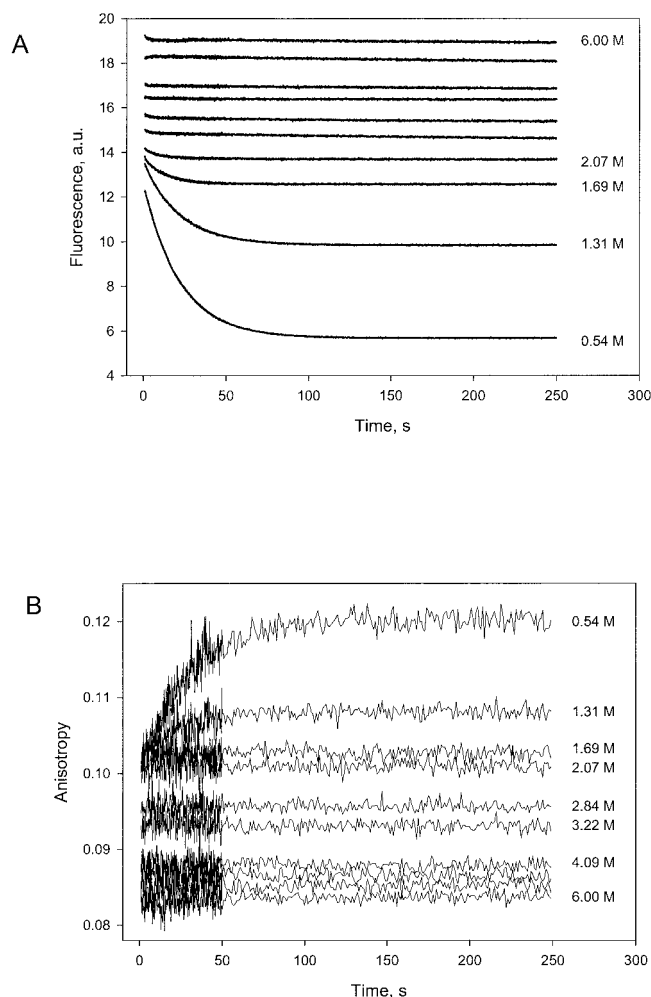


FIGURE 4 Kinetic refolding data for *apo*  $\alpha$ -lactalbumin at pH 7.0 at different GuHCl concentrations. (A) Fluorescence intensity; (B) Fluorescence anisotropy.



the course of the stopped-flow experiment; all changes occur in the dead-time of the experiment. The slow kinetic phase is characterized by a simultaneous decrease in the fluorescence intensity and an increase in the anisotropy values. The rate constant determined from the global analysis of these measurements at 0.54 M GuHCl is  $0.046 \text{ s}^{-1}$ , a value in very good agreement with previously published results for the rate of folding of *apo*  $\alpha$ -lactalbumin under similar experimental conditions ( $0.041 \text{ s}^{-1}$  at 0.4 M GuHCl (Arai and Kuwajima, 1996)). The changes observed in the fluorescence intensity and in the fluorescence anisotropy therefore correspond to the formation of the fully native state. This state is destabilized above 2 M GuHCl (Arai and Kuwajima, 1996), and at higher concentrations of GuHCl both fluorescence intensity and anisotropy changes are observed only in the burst phase.

In addition to the slow kinetic phase observed at GuHCl concentrations below 2 M, the fits to the fluorescence traces also reveal a small amplitude fast phase that is present at all GuHCl concentrations. This additional fast phase, having a rate constant of  $0.48 \pm 0.05 \text{ s}^{-1}$  has not, to our knowledge, been reported in previous refolding studies of *apo*  $\alpha$ -lactalbumin. This rapid phase is highly reproducible and was also detected in control experiments carried out by manual mixing in a Bio-Logic SC-1 cell holder. The origin of this phase is unknown, but as it is less than 5% of the amplitude of the major kinetic phases it is neglected in the remainder of the analysis described in this paper.

Fig. 5 shows the amplitudes of the fluorescence intensity and fluorescence anisotropy as a function of GuHCl concentration. In Fig. 5 *A* are shown the amplitudes of the fluorescence intensity and of the fluorescence anisotropy in the slow kinetic phase. The data reveal a transition monitored simultaneously by both measurements, which reflects the destabilization of the native state with increasing concentrations of GuHCl (Arai and Kuwajima, 1996). The transition midpoint is at  $1.33 \pm 0.06 \text{ M}$  GuHCl. This kinetic denaturation curve is very similar to the equilibrium unfolding transition curve monitored by tryptophan fluorescence shown elsewhere (Arai and Kuwajima, 1996).

Fig. 5 *B* depicts the magnitudes of the burst-phase amplitudes of the fluorescence intensity and the fluorescence anisotropy as a function of GuHCl concentration. The burst phase amplitude of the fluorescence intensity increases linearly with the denaturant concentration; this can be attributed simply to a solvent effect of the increasing amounts of GuHCl on the fluorescence intensity of the tryptophan residues. This phenomenon has been observed previously with small peptides (Itzhaki et al., 1994; Matagne et al., 1998) and is usually described as a post-transition baseline in the literature (Arai and Kuwajima, 1996). Thus, the fluorescence intensity does not reveal any conformational changes during this burst phase.

The burst-phase amplitude of the anisotropy signal, also shown in Fig. 5 *B*, is, however, more informative. The data

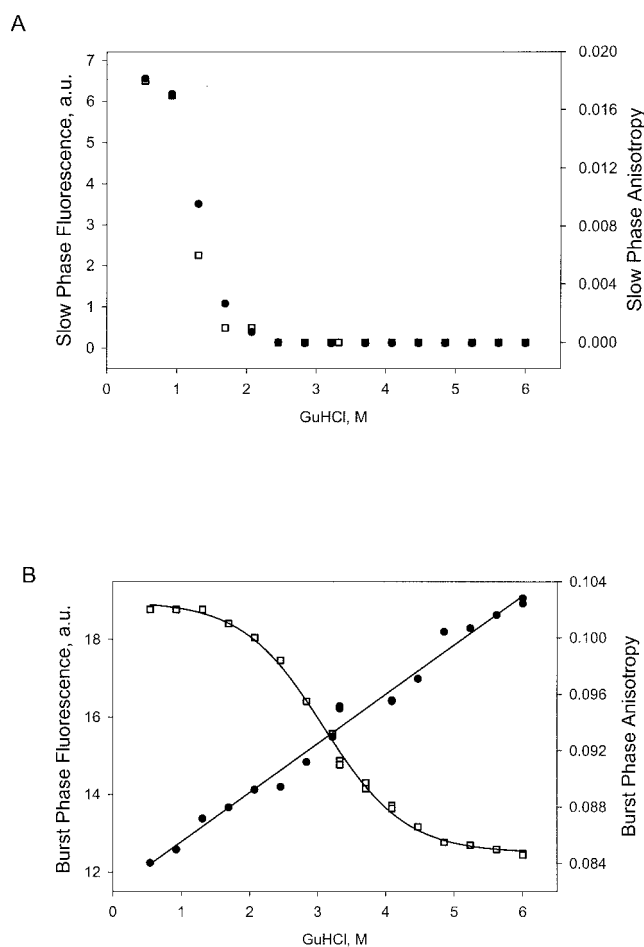


FIGURE 5 Amplitudes of the slow phases (*A*) and burst phases (*B*) detected in the experiment shown in Fig. 4 using fluorescence intensity (●) and anisotropy (□). The burst phase amplitudes were fitted to a linear equation (for fluorescence intensity) and to a two-state transition (for fluorescence anisotropy). The parameters resulting from these fits are given in the text.

show a nonlinear change that can be fitted to an equilibrium two-state model in which the initial unfolded state converts into a partially folded state. This analysis yields a  $\Delta G_0$  value for unfolding of the species formed in the burst phase of  $14.3 \pm 2.4 \text{ kJ mol}^{-1}$  and an  $m$  value of  $4.7 \pm 0.8 \text{ kJ mol}^{-1} \text{ M}^{-1}$ . The midpoint is at  $3.0 \pm 0.5 \text{ M}$  GuHCl. It is noteworthy that no baseline corrections were used in this fit, which implies that, in contrast to fluorescence intensity measurements (Pace, 1986), the dependence of the fluorescence anisotropy on the bulk GuHCl concentration in the absence of conformational changes is negligible. Such a dependence could, for example, have resulted from changes in viscosity, which will affect the motional rate of the probes. Apparently such effects are comparatively very small.

Thermodynamic parameters have been determined in a previous kinetic study of the folding of *apo*  $\alpha$ -lactalbumin from the transition obtained by following the far UV ellip-

ticity at 222 nm. Under similar conditions these were found to be  $\Delta G_0 = 7.9 \text{ kJ mol}^{-1}$  and  $m = 3.6 \text{ kJ mol}^{-1} \text{ M}^{-1}$  (Arai and Kuwajima, 1996). This transition was attributed to the unfolding of the kinetic molten globule state formed in the refolding reaction (Arai and Kuwajima, 1996). The transition monitored by fluorescence anisotropy in the present study is likely also to reflect the unfolding of the kinetic molten globule state. The differences in the thermodynamic parameters obtained by the two techniques (e.g., the midpoint is 3.0 M for the anisotropy measurement and at 2.2 M GuHCl for the circular dichroism (CD) measurement) can be attributed to the fact that this transition is not fully cooperative. This characteristic of a partially folded state of  $\alpha$ -lactalbumin has also been shown by a residue-specific NMR study of the pH 2.0 state of the homologous human protein (Schulman et al., 1997). A more recent NMR and CD study of bovine  $\alpha$ -lactalbumin under similar conditions shows that the CD signal at 222 nm is lost before all the residues involved in  $\alpha$ -domain helices, which contains three of the four tryptophan residues of the protein, become fully unstructured (Wijesinha-Bettoni, 2000). This provides an explanation of the fact that the probes monitored by fluorescence anisotropy acquire the rotational mobility characteristic of the unfolded state only at GuHCl concentrations greater than these at which the CD signal at 222 nm is lost.

The stopped-flow experiment was repeated by diluting  $\alpha$ -lactalbumin in 6 M GuHCl into a pH 2.0 buffer. No slow phase is observed in these experiments, consistent with the fact that under these conditions the native state of the protein is not stable even in the absence of GuHCl (Kuwajima, 1996). The species formed in this experiment corresponds to the partially folded A-state that is stable under equilibrium conditions. The burst-phase amplitudes of the fluorescence intensity and anisotropy are shown in Fig. 6 as a function of GuHCl concentration. As found at pH 7.0, a transition is observed in the anisotropy data, whereas the

fluorescence intensity shows only linear changes. The fit of the pH 2.0 anisotropy data to the same function as used to analyze the pH 7.0 data yields values of  $\Delta G_0 = 15.0 \pm 0.6 \text{ kJ mol}^{-1}$  and  $m = 4.0 \pm 0.2 \text{ kJ mol}^{-1} \text{ M}^{-1}$ . These values are close to those found at pH 7.0. This supports strongly the identification of the burst phase at pH 7.0 to the transition from the highly unfolded state in 6 M GuHCl to a partially folded molten globule state.

There is a general shift in the anisotropy values between the two conditions, the transition at pH 7.0 representing a change between 0.103 and 0.085 anisotropy units, whereas the transition at pH 2.0 represents a change from 0.118 to 0.100 anisotropy units.

## DISCUSSION

We have introduced a new technical solution for the simultaneous detection of intrinsic protein fluorescence intensity and fluorescence anisotropy in a stopped-flow experiment. By using a photoelastic modulator in the excitation beam, we show that it is possible to simplify the detection system, such that it involves only one photomultiplier tube and does not require a polarizer. We have applied this new procedure to study the folding of *apo*  $\alpha$ -lactalbumin, a process explored in detail by other methods (Arai and Kuwajima, 1996; Schulman et al., 1997; Forge et al., 1999; Troullier et al., 2000).

The sensitivity of our system allows information to be obtained with amounts of protein significantly smaller than those usually required for stopped-flow CD. As an example, each point on the transition curve shown in Fig. 5 B was obtained with a single stopped-flow shot and a final protein concentration of 12  $\mu\text{M}$ . By contrast, 20 shots and a protein concentration of 27  $\mu\text{M}$  were used in one recently reported CD transition curve (Arai and Kuwajima, 1996), and 30 shots and a protein concentration of 13  $\mu\text{M}$  were employed in a recent kinetic study using CD spectroscopy (Forge et al., 1999). This improvement is due at least in part to the better signal-to-noise-ratio expected for fluorescence anisotropy measurements for a given quantity of protein when compared with CD measurements. In addition, the fluorescence anisotropy is a ratiometric measurement; i.e., it is independent of the protein concentration because it is defined as a ratio of two components both of which are themselves proportional to the concentration. Fluorescence intensity, like absorbance or CD measurements, is by contrast proportional to the protein concentration. This ratiometric property is particularly important in stopped-flow experiments, as any injection device has errors in reproducing the protein concentration from one shot to another. The anisotropy measurements are not sensitive to these changes, a fact that is consistent with the smaller deviations from the fits in Fig. 5 B for the fluorescence anisotropy data as compared with the fluorescence intensity data. Therefore,

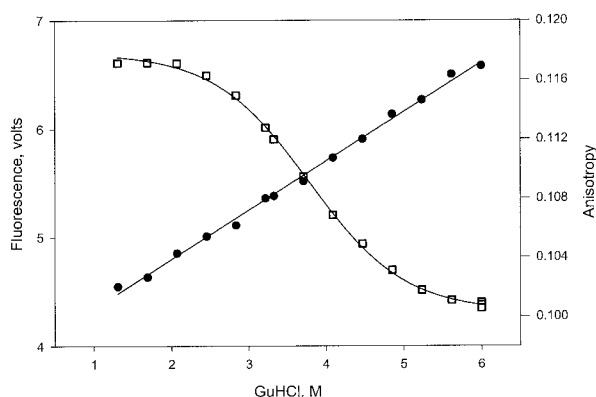


FIGURE 6 : Kinetics of unfolding of the A-state of  $\alpha$ -lactalbumin at pH 2.0 by increasing amounts of GuHCl followed by fluorescence intensity (●) and anisotropy (□). The amplitudes were fitted to a linear equation (for fluorescence intensity) and to a two-state transition (for fluorescence anisotropy). The parameters resulting from these fits are given in the text.

by using fluorescence anisotropy, one source of systematic deviation inherent in most stopped-flow data is eliminated.

Our results show that fluorescence anisotropy provides important information about species that may not be detected by the fluorescence intensity alone. We have demonstrated in particular that fluorescence anisotropy can detect the transition of *apo*  $\alpha$ -lactalbumin from its fully unfolded form to its molten globule state. The measurements can be carried out under conditions both where the molten globule state is transiently populated during the refolding to the native state (burst-phase intermediate) and where it is stable under equilibrium conditions. Very similar data characterizing the stability of the molten globule states were obtained under these two sets of conditions, in good agreement with the assumption that the burst-phase intermediate in  $\alpha$ -lactalbumin refolding is closely similar to the equilibrium molten globule state (Arai and Kuwajima, 1996; Forge et al., 1999).

It is surprising at first sight that the fluorescence intensity is not significantly perturbed between the highly unfolded state of  $\alpha$ -lactalbumin in GuHCl and the compact molten globule state formed at low pH in the absence of GuHCl. It has been reported previously that the intrinsic fluorescence of the native state is internally quenched by a mechanism involving resonant energy transfer from Trp26 and Trp104 to Trp60 and subsequently to two adjacent disulphide bonds (Sommers et al., 1973; Sommers and Kronman, 1980). Resonant energy transfer is, however, dependent on the sixth power of the distance separating the fluorophores (Forster, 1948), and hence only very modest changes in the protein structure will be sufficient to prevent this quenching mechanism. This could be the origin of the fact that the molten globule state, which is slightly expanded relative to the native state, does not show a detectably reduced fluorescence intensity relative to that of the fully unfolded state. Fluorescence anisotropy, by contrast, reports the relative immobilization of the tryptophan residues, and this is shown here to occur to a significant degree in the molten globule state.

In the case of  $\alpha$ -lactalbumin, the presence of four tryptophan residues limits the interpretation of the data in structural terms, as an intermediate level of anisotropy between the unfolded and the native state reflects only an average over the mobilities of all four tryptophan residues. More detailed interpretation of anisotropy data, as with other fluorescence methods (Beechem et al., 1995), is, however, viable with single tryptophan-containing proteins. In such a study, the detection of both fluorescence intensity and fluorescence anisotropy should provide residue-specific information on the environment of the local probe (as detected by the level of fluorescence intensity) and on the degree of mobility of the probe (as detected by fluorescence anisotropy). The present study suggests that it should be possible to obtain such information routinely in kinetic studies of

protein folding in particular and of protein conformational changes more generally.

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