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Resolving multiple protein conformers in equilibrium unfolding reactions: A time-resolved emission spectroscopic (TRES) study of Azurin

P. Guptasarma *

Centre for Cellular and Molecular Biology, Hyderabad - 500 007, (A.P) India

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Abstract

Unlike steady-state spectrofluorimetry, time-resolved emission spectroscopy (TRES) can resolve emissions from fluorophores with similar quantum yields and overlapping steady-state emission spectra. Time-resolved emission studies of the protein-intrinsic fluorophore, tryptophan (Trp), can thus potentially be used to examine protein conformational heterogeneity in solution, as well as to investigate the existence of populated intermediate structural states in equilibrium unfolding reactions of single-tryptophan proteins. Here, the single-Trp copper protein, azurin, is examined in various concentrations of guanidine hydrochloride (GdnCl) with its disulphide bond in an intact state. Interestingly, multiple envelopes of Trp emission are observed in all TRES spectra acquired, instead of just two emission envelopes (corresponding to the native and unfolded states) expected from two-state unfolding. These envelopes appear to be centred around the same set of emission wavelengths in different TRES spectra, and only intensities and decay rates vary with the concentration of denaturant used. This suggests that structural states representing different levels of exposure of Trp to the aqueous solvent might, in fact, be populated at equilibrium during the unfolding of azurin by GdnCl. © 1997 Published by Elsevier Science B.V.

Keywords: Fluorescence decay time-resolved emission; Protein conformation; Protein folding; Structural intermediates

1. Introduction

The fluorescence emission spectrum of the indole sidechain of tryptophan (Trp) is sensitive to the polarity of its environment within protein molecules. It shifts to longer wavelengths upon unfolding of proteins in aqueous media, as a consequence of the exposure of Trp to the polar solvent. Thus, for proteins in which Trp residues are buried away within the native structure, emission spectra convey useful information about conformational status [1]. However, steady state fluorescence methods can only be of limited use in any attempt to investigate the mechanisms of folding/unfolding of a protein through equilibrium rather than kinetic studies, since steady-state spectrofluorimetry cannot sufficiently resolve the overlapping, broad-band emissions of Trp from different environments. Even though different structural states [e.g., the native (N), unfolded (U),

* Present address: Institute of Microbial Technology (IMTECH), Sector 39-A, Chandigarh 160 036, India, Tel.: +91 172 690004, Fax: +91 172 690632, e-mail: pg@lion.imtech.ernet.in.

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and possible intermediate (I_x) states] must expose Trp residues to the solvent to different extents, the observed spectrum of a protein unfolded at equilibrium by chemical or thermal means tends to be just a broad structureless emission profile incorporating individual contributions from different structural states. With increase in denaturant concentration, the emission profile gradually shifts to ever longer wavelengths until every molecule in solution is unfolded and the emission spectrum of the population shows a maximum at about 352 nm, corresponding to the wavelength of emission of free Trp in water.

Since at least some of the intermediate protein conformations thought to exist on the kinetic pathways of folding/unfolding, are anticipated to be populated at equilibrium (e.g., the pre-molten globular and molten globular states) [2], it would be useful to devise a fluorescence method which could detect and resolve between multiple protein conformers in solution. Such a method would also allow one to investigate whether the native and unfolded states of proteins comprise homogenous or heterogenous populations of structural states and sub-states. This paper outlines a possible method, and describes its use in detecting multiple conformational states and substates in samples of the single-Trp, copper protein, Azurin, as a function of its exposure to the denaturant GdnCl.

2. Materials and methods

2.1. Choice of method

Time-resolved emission spectroscopy (TRES) is the method of choice for resolving closely-spaced emissions. TRES data is compiled by initially accumulating, at a number of discrete emission wavelengths, information about the fluorescence decay of the sample following very short (nanosecond or picosecond) pulses of excitation, and then combining the numerous decay curves obtained at various wavelengths to create a three-dimensional plot showing the evolution of the emission spectrum of the sample with time.

Since fluorescence decays of Trp are generally monitored on the nanosecond timescale – a period of time too short for interconversions between protein conformational states to occur during the actual collection of decay data – one is effectively taking a stroboscopic snapshot of the characteristics of the protein solution in any TRES measurement. Although decay data at each wavelength is acquired by averaging over many exciting pulses to build up the signal/noise ratio of the decay curve, the existence of equilibrium is expected to ensure that each pulse finds the same number of molecules populating any given state, so that any interconversions amongst states occurring in-between pulses are not of consequence. Since, in principle, different conformers of a single-tryptophan protein can contain Trp in different states of exposure to the solvent but still display the same (or similar) quantum yield(s) of fluorescence, TRES turns out to be immensely useful for distinguishing between conformers because it examines both variations in the initial emission intensity, and the rate of fluorescence decay of a fluorophore with variations in the wavelength of monitoring of emission, unlike steady state spectrofluorophorimetry which monitors only variations in quantum yield (i.e., variations in the total area under a decay curve) with wavelength, in a time- and population-averaged manner. Individual emissions from different states can thus be resolved to a much higher degree through TRES [3]. Notably, TRES has recently been used to examine individual emissions from Trp fluorophores in multi-Trp proteins [4,5] – something that could not have been accomplished through steady-state spectrofluorimetry. Also, time-resolved decay data of Trp at different wavelengths [6,7], as well as decay associated spectra [7], have been used to point to indications of heterogeneity in Trp exposure conditions, in native solutions of single-Trp proteins.

2.2. Choice of protein

Azurin, the protein chosen for the studies presented here, is a single-tryptophan protein [8]. The Trp in azurin displays the shortest wavelength of emission known among natural proteins (a 308/315 nm doublet) [9] which is useful because it improves the resolution of the emissions from the N and U states. [In a completely unfolded molecule, Trp is expected to emit with a wavelength maximum of 352 nm [1]; the wavelength at which Trp emits in aqueous environments]. The presence of a single Trp ensures that emission bands can be more or less

safely assigned to individual conformers of azurin, rather than to different Trp fluorophores that are exposed to the solvent to different extents within the same molecule. The advantage of using the copper-bound form of azurin, over the apo form (given that both have similarly blue-shifted emissions) is that the quantum yield of Trp is affected (quenched) by the bound copper. Thus, the rise in quantum yield following the release of bound copper further facilitates the distinguishing of the folded form from unfolded forms in TRES spectra. A further advantage with Azurin is that its single Trp emits with monoexponential fluorescence decay in the apo-protein form and biexponentially in the holo form [6], which is useful for fitting decay data.

2.3. Analytical considerations

Fig. 1 schematically represents what might be expected to happen in an equilibrium unfolding experiment, if a significant number of kinetic intermediate states are capable of being populated at equilibrium. As the concentration of denaturant is raised, molecules in the native state tend to populate more and more unfolded ($I_{1,2..n}$) states, until most molecules end up populating the completely unfolded (U) state at a very high concentration of denaturant. If each of the structural intermediates on the unfolding pathway were to expose Trp to the solvent to different extents, the various conformers would all emit maximally at different emission wavelengths between the 308/315 nm doublet emission (native protein) and the 352 nm emission (unfolded protein). Importantly, while the distribution of the protein population amongst various states would be altered by the denaturant concentration used, the same discrete set of emissions would be observed under all conditions, if the unfolding of all molecules proceeded through the same set of intermediates. In this context, it may be noted that single-Trp proteins do not always show single-exponential decay, and bi-exponential or tri-exponential decays have been attributed to conformational heterogeneity in the ground state [6,7] (with multiple exponentials arising from separate, overlapping Trp emissions centred at nearby wavelengths).

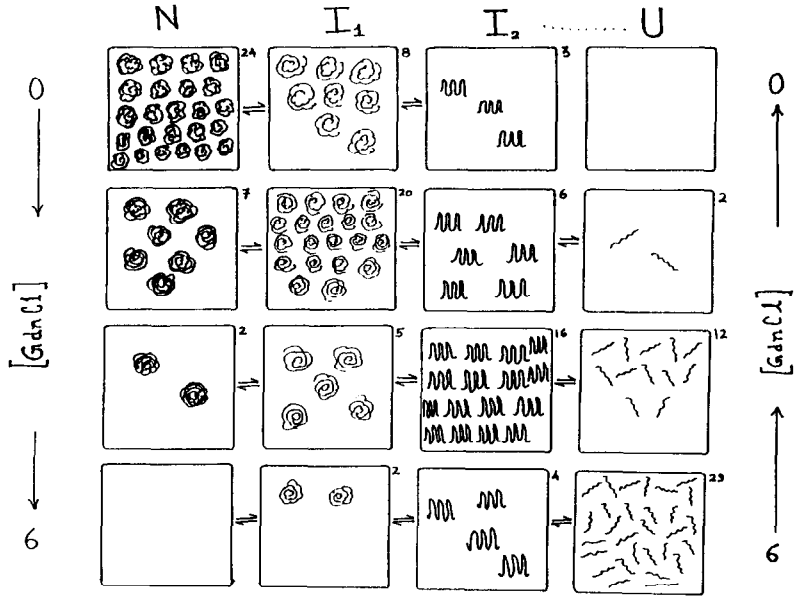


Fig. 1. A schematic diagram showing likely changes in the distribution of a protein population among various conformers (folding intermediates), as a function of the denaturing conditions extant in equilibrium unfolding reactions. Although the population of each intermediate state is altered as a consequence of change in denaturant concentration, the same discrete set of conformers might be expected to exist under all conditions.

2.4. Materials

Copper-bound azurin from the organism *Pseudomonas aeruginosa* was obtained from Sigma (USA). Guanidine hydrochloride (GdnCl) was obtained from Aldrich (USA) and recrystallized prior to use. The concentrations of GdnCl stocks were estimated through refractive index measurements as per Nozaki [10]. Azurin solutions containing GdnCl were prepared in phosphate buffer (0.1 M, pH 7.6) to various concentrations of GdnCl. These solutions were incubated at 20 deg C for at least 10 hours prior to use.

2.5. Fluorescence measurements and TRES spectra

A Hitachi F-4000 spectrofluorimeter was used for steady-state fluorescence studies of azurin unfolding. Spectra were acquired through excitation at 280 nm and 296 nm, using excitation and emission bandpasses of 5 and 3 nm, respectively. A PTI (Photon Technology International) LS-100 nanosecond fluorescence spectrometer equipped with a thyratron-gated nitrogen lamp functioning as the pulsed light source, was used for time-resolved fluorescence studies. Fluorescence decays were acquired through excitation at 296 nm, since that was the lowest excitation line available for Trp excitation in the nitrogen lamp spectrum. The nominal excitation bandpass used was 5 nm, while an emission bandpass of 4 nm was used. For each concentration of the denaturant (GdnCl), individual fluorescence decays were collected through accumulation and averaging of over a hundred two-nanosecond pulses, at 21 wavelengths ranging from 304 nm to 364 nm at intervals of 3 nm. After deconvolution of the lamp signal from the decay (performed automatically), decays were fitted with a single exponential, or two exponentials, and normalized with respect to each other by the software controlling the LS-100 system. This software was also used to transform the set of 21 decays (each plotting emission intensity vs – time) into a three-dimensional, time-resolved plot of emission spectra; with time (in nanoseconds), emission wavelength (in nm) and emission intensity (arbitrary units) plotted along the three axes. [For earlier uses of this instrument and software, please see [4,11,12]. Thus each plot for a particular concentration of denaturant was effectively a representation of the evolution of the emission spectrum of the protein with time, on a time-scale of nanoseconds.

3. Results and discussions

Fig. 2 shows the steady-state emission spectra of azurin in the presence of various concentrations of GdnCl, collected through excitation at 280 nm (the wavelength generally used for steady-state fluorescence monitoring of protein unfolding; Fig. 1a), and 296 nm (a wavelength that selectively excites tryptophan, and not tyrosine; Fig. 1b), respectively. Some differences are observed in the spectral features characterizing the native and denatured states as elicited by the two excitation wavelengths, presumably due to a differential photoselection of Trp states arising from the bound copper atom and/or due to contributions from tyrosine fluorescence arising from dual excitation of tyrosine and tryptophan at 280 nm. Since only Trp is selectively excited by 296 nm light in a protein containing both tyrosine and tryptophan, and since, furthermore, 296 nm is the lowest suitable wavelength that could be used in the pulsed-excitation studies due to the limitations relating to the N_2

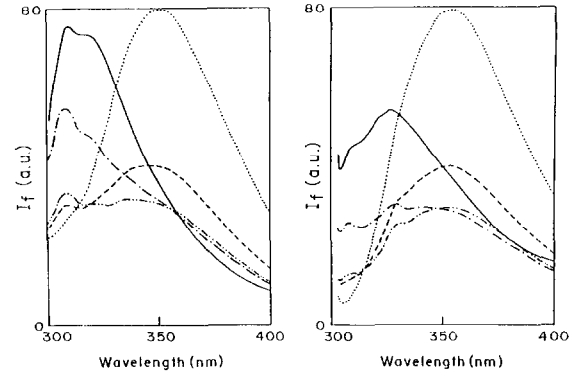
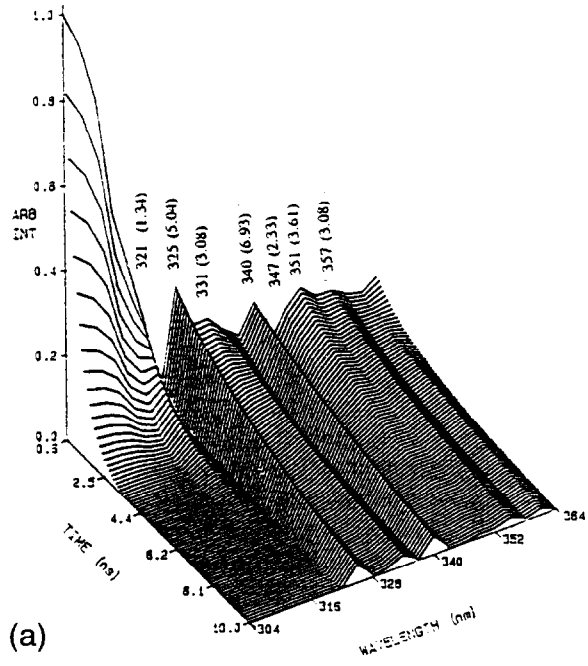


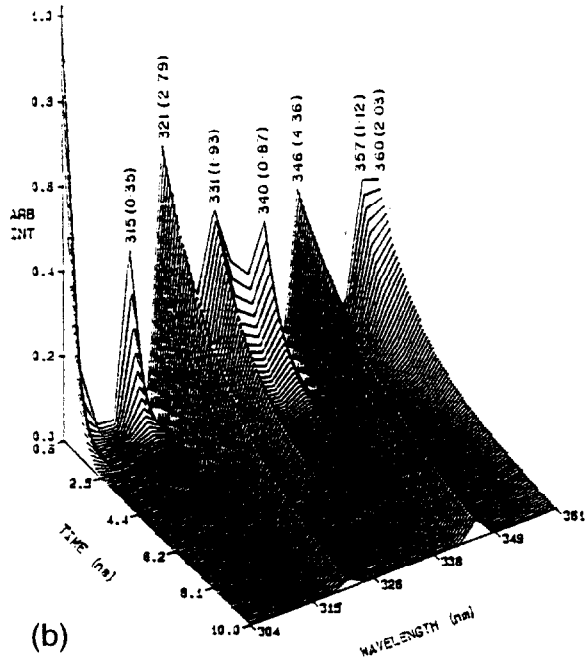
Fig. 2. Steady-state fluorescence emission spectra of azurin in aqueous solution at certain representative concentrations of the denaturant GdnCl. Spectra were acquired through excitation at 280 nm (1A) and 296 nm (1B), with excitation and emission bandpasses set at 5 nm and 3 nm, respectively (—————) 0.0 M; (—·—·—) 0.96 M; (—··—) 1.35 M; (– – –) 1.48 M; (····) 3.0 M GdnCl. The increase in quantum yield upon complete denaturation may be explained by the observation that the copper-bound protein has only 60% of the quantum yield of the apoprotein [13].

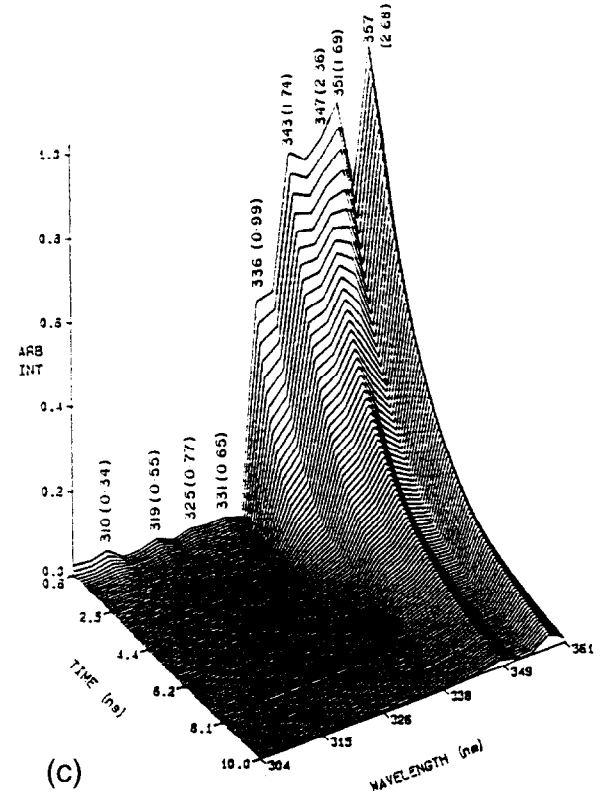
light source, we shall concentrate essentially on data from 296 nm excitation which displays an emission max of 315/321 nm. Under conditions of selective Trp excitation, the fluorescence quantum yield of the protein is enhanced greatly upon unfolding; the quantum yield of Trp in the holoprotein is only 60% of that in the apoprotein [13], presumably due to quenching by the bound copper and possibly also due to quenching by the protein's disulphide bond. The holo form of azurin was used in these experiments to further aid resolution of native (and native-like) states from partially unfolded or completely unfolded states by using the differences in lifetime arising from the differential quenching of Trp in different structural states.

Fig. 3 shows the time-resolved emission spectra of the protein at three representative denaturant concentrations of 0.0 M, 0.7 M and 6 M GdnCl. Notably, multiple emissions at the same set of wavelengths are observed in all three plots, with the relative initial emission intensities and decay constants of these emissions varying from one GdnCl concentration to another. [The lifetimes marked at different wavelength positions are those obtained from the best fit of decay data to a single-exponential, or the lifetime of the larger contributing component in case of best-fit to double exponential. These lifetimes are just provided to give an idea of the overall lengthening of decay time upon release of Trp quenching due to unfolding]. As can be observed in all three TRES spectra, the emission profile exhibits several discrete maxima at 310, 315, 319–321, 325, 331, 340, 346–347, 351 and 357 nm, respectively, with -an additional component at 336 nm that is not always distinguishable from nearby emissions. [As outlined in the materials and methods section, any discrete set of structural forms that is populated by the protein as it unfolds from the native to the unfolded state, should show up in time-resolved emission spectra as a fixed set of emission bands at every GdnCl concentration, representing conformations that are populated to different extents at different denaturant concentrations]. Most emission envelopes observed in the native protein [0 M GdnCl] are clustered in the region of wavelengths characterizing steady-state emission from this sample when it is in the native state (310, 315, 319–321 nm), while emission bands in the denatured protein [6.0 M GdnCl] are seen to be located mostly in the region of wavelengths characterizing steady-state emission in the denatured state (346–347, 351, 357 nm). What is remarkable is that multiple emission envelopes are actually observed, indicative of conformational heterogeneity [notably, conformational heterogeneity in native holoazurin has been reported by other investigators [6]]. Since azurin is a single-tryptophan protein, a single well-defined native conformation should have given rise to a single emission band, even in the time-resolved emission spectrum. Multiple bands therefore indicate either that (i) the native and denatured protein populations are themselves heterogenous mixtures of structural forms (each exposing Trp to the solvent to a different extent) which are not necessarily intermediates on the unfolding/folding pathways, or (ii) that some of the longer wavelength emissions observed in the native sample as well as some of the shorter wavelength emissions observed in the denatured sample might represent folding intermediate states.

If the first of the two possibilities outlined above were to apply exclusively, all the emission bands constituting the native cluster could be expected to together decrease in prominence with increasing GdnCl concentration, while all the bands representing the denatured cluster could be expected to become more prominent: i.e., a two-state situation, in which each state comprises numerous sub-states. On the other hand, if the second possibility were also partly correct, the emissions in the middle of the range would tend to be more prominent than the main bands in the native and denatured clusters at intermediate concentrations of GdnCl, consistent with unfolding occurring through structural intermediates populated in the equilibrium unfolding reaction. The TRES data for 0.7 M GdnCl shows that the bands in the middle of the range are indeed the most prominent, rather than the bands from the extremes of the range representing the N and U states. Thus, it would seem that at least some of the emissions (319–321, 325, 331, 336 nm) represent structural intermediates, while the others might represent either structural intermediates or conformers related to the native and denatured states. If so, it is possible that these states are partially populated at both 0 M GdnCl and 6.0 M GdnCl, and indeed this is what is observed. Interestingly, such a coexistence of rare partially folded


ARB INT
321 (1.34)
325 (5.04)
331 (3.08)
340 (6.93)
347 (2.33)
351 (3.61)
357 (3.08)
TIME (ns)
WAVELENGTH (nm)
(a)


ARB INT
315 (0.35)
321 (2.79)
331 (1.93)
340 (0.87)
346 (4.36)
357 (1.12)
360 (2.03)
TIME (ns)
WAVELENGTH (nm)
(b)


ARB INT
310 (0.34)
319 (0.55)
325 (0.77)
331 (0.65)
336 (0.99)
343 (1.74)
347 (2.36)
351 (1.69)
357 (2.68)
TIME (ns)
WAVELENGTH (nm)
(c)

states with the native state has been recently reported for RNaseH [15]. On the whole, it is expected that a longer wavelength of emission would correlate with a greater degree of unfolding, although it must be stressed that this does not necessarily have to be the case in each instance.

In solutions containing GdnCl it is expected that an increasing proportion of the population would lose bound ligands with increasing GdnCl concentration. The loss of bound ligand probably explains the enhancement of fluorescent intensity at higher GdnCl concentrations, as seen in the steady-state emission spectra. Azurin contains a disulphide bond [13] which was not reduced in these studies – so as not to introduce additional complexities arising from oxidation/reduction reactions, especially involving cysteines that do not participate in the native disulphide bond. The maintenance of the protein in the oxidized form also presumably allowed the protein to interconvert between the N and U states efficiently in the presence of denaturant. Most proteins can refold spontaneously from 6 M GdnCl if disulphide bonds have not been reduced, whereas non-native disulfide species forming adventitiously can complicate the folding reaction and both slow it down and reduce the efficiency of refolding in proteins refolding from a reduced state. Reduction of the disulphide bond in these studies could thus affect the relative rates of forward and backward interconversion of states by greatly slowing down the conversion of unfolded species to folded species, such that all reduced protein molecules would be completely unfolded. Intermediate states would then be poorly populated. With regard to azurin, it is now known that the disulphide bond is crucial to the stabilization of the native structure [14]; hence, it was perhaps best to leave the disulphide bond intact in these studies to facilitate the population of any relatively stable intermediate structures, by not pushing the equilibrium towards maintaining all molecules in the completely reduced and unfolded form. In principle, in the disulphide-intact form of the protein exposed to 6 M GdnCl, there could still be some structure remaining which keeps the population from converting completely to the unfolded state, and this is consistent with the observation of multiple, discrete emissions from azurin in 6 M GdnCl.

There is a negative feature about the method that keeps one from making this study a more rigorous examination of folding intermediates or conformational heterogeneity, in that there is no necessary correlation between the quantum yield of Trp and its exposure to the solvent. Quantitation of the numbers and relative proportions of molecules populating different states is therefore not possible, and one needs to remain content with a qualitative analysis. [In this connection, it is useful to remember that it is not the height of each envelope that provides one with this qualitative indication of the number of molecules populating different states, but rather the volume under each emission envelope]. Another seeming caveat is that the method can only be used reliably to study single-tryptophan proteins, or perhaps at most proteins containing two tryptophans. If appropriate, site-directed mutagenesis could be used to reduce the number of tryptophan moieties in multi-tryptophan proteins, by replacing Trp with tyrosine or phenylalanine without altering stability overmuch, such that studies of other proteins could become feasible.

To the best of my knowledge, this is the first application of TRES to the study of protein unfolding, and towards detection of multiple protein conformers in equilibrium unfolding situations, although tryptophan fluorescence decays collected at one or more selected wavelengths have been used in some instances to examine conformational heterogeneity in apoazurin [6], and decay associated spectra and time-resolved decay data have been used to examine conformational heterogeneity in at least one other system [7]. The discussions and data presented here

Fig. 3. Time resolved emission spectra of azurin in aqueous solutions with GdnCl present at concentrations of 0.0 M (3a), 0.7 M (3b) and 6.0 M (3c). With reference to Fig. 1, it is relevant to point out that the emission bands seemingly resolved in each time-resolved emission spectrum have the same (or very similar) maxima, just as one would expect (see Section 2). All three plots show bands with emission maxima at 310, 315, 319–321, 325, 331, 340, 346–347, 351 and 357 nm respectively. The most prominent bands are marked on the figures, with fluorescent lifetimes (in nanoseconds) mentioned in brackets. The population of each species emitting at a particular wavelength changes in the expected manner with GdnCl concentration.

point the way forward, to more rigorous and detailed studies that would surely be required before the question of whether structural folding intermediates are populated at equilibrium is settled.

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