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Biophysical Chemistry 107 (2004) 197–211

Biophysical Chemistry

www.elsevier.com/locate/bpc

A fast and accurate procedure to collect and analyze unfolding fluorescence signal: the case of dystroglycan domains☆

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Received 25 August 2003; received in revised form 15 September 2003; accepted 15 September 2003

Abstract

Monitoring the fluorescence signal upon unfolding often represents a very effective method to rapidly retrieve the first preliminary structural information on a protein domain. The relationship between intrinsic fluorescence signals and unfolding of proteins are discussed, including several practical considerations for properly setting fluorescence experiments and the phenomenological equations required to analyze the spectra. In particular, a fast and accurate method which allows to minimize the deleterious effect of photobleaching is provided. A number of unfolding reactions relative to immunoglobulins (IgG and IgM) and to the different domains of the adhesion molecule dystroglycan are presented. Special attention is dedicated to a α-dystroglycan immunoglobulin-like domain showing a 'reverse' behavior of the fluorescence signal as a function of the denaturing agent concentration.

Keywords: Fluorescence; Immunoglobulins; Proteins; Dystroglycan; Protein unfolding

☆ First author would like to dedicate this paper to everyone who is running away: 'In omnibus requiem quaesivi et nusquam inveni nisi in angulo cum libro.' (Thomas a Kempis 1379–1471)

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doi:10.1016/j.bpc.2003.09.005

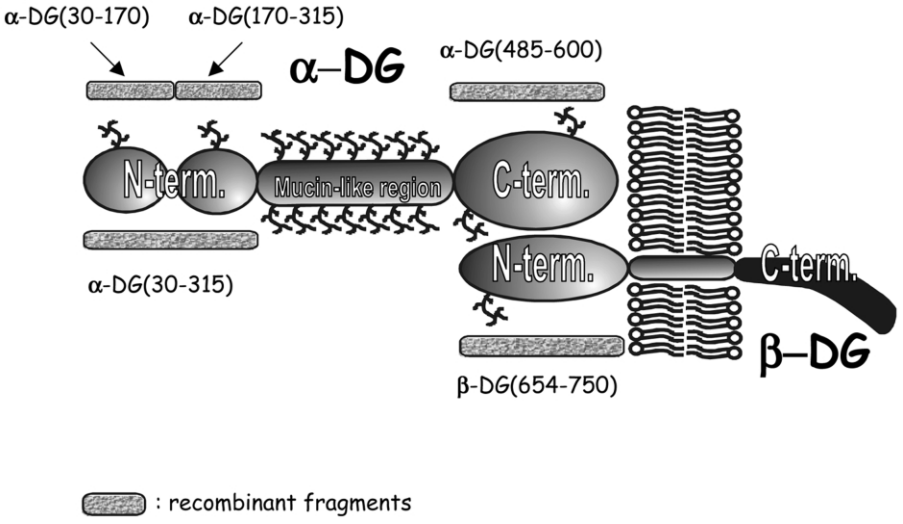

Fig. 1. Scheme of dystroglycan domain organization. The dystroglycan adhesion complex is formed by two subunits, α and β, which interacts non-covalently. The recombinant fragments under analysis, all belonging to the extracellular side of the complex, are reported.

1. Introduction

Among biophysical techniques, fluorescence spectroscopy is one of the more sensitive, making it possible to perform experiments on protein solutions at very low concentration. Often, it may be important to work at micromolar (or lower) concentrations to minimize the amount of molecule to use in order to solve problems associated with its aggregation and low solubility and, especially in the case of wild-type or recombinant mutated proteins, costs related to their expression and/or purification. One of the practical use of fluorescence technique is to obtain thermodynamic and kinetic information about conformational transitions of macromolecules, such as protein folding reactions, collecting knowledge on their structure with the limitation that many fluorescence signal changes cannot be directly or unequivocally related to molecular details. When recombinant mutated proteins are available, or as in this work different dystroglycan domains, it is often of interest to study their unfolding behavior and stability [1,2].

The first aim of this article is to consider the application of intrinsic fluorescence, with particular attention to some practical and technical aspects, to study the unfolding transition in a series of proteins and how this information help to retrieve preliminary structural details making use of a fast, but accurate, and easy setup. In studies with proteins the fluorophores can be either intrinsic (such as tryptophan, tyrosine and phenylalanine residues) or extrinsic probes. Tryptophan residues are particularly valuable probes, since the indole ring is very sensitive to its local environment and since there are often only a few tryptophan residues in a protein molecule making it possible an easy assignment of their specific contributions. In this work, we analyzed proteins harboring intrinsic fluorophores, with special attention to dystroglycan domains (Fig. 1).

Dystroglycan is an adhesion complex formed by two subunits, α and β [3]. It is involved in a wide number of biological functions and its biomedical interest is constantly increasing [4]. In muscle, dystroglycan forms a continuous link between the extracellular matrix (ECM), via α-dystroglycan binding to laminin-2, and the cytoskeleton, via β-dystroglycan binding to dystrophin, which is considered crucial for muscle stability [5]. Moreover, dystroglycan participates in (i) post-synaptic elements maturation and stabilization at the neuro-

muscular junction as well as within the central and peripheral nervous system; (ii) early embriogenesis and (iii) infectious diseases such as leprosy and arenaviruses infections [6,7]. As for other ECM molecules or receptors [7], dystroglycan functional versatility is largely originating from its modular structure. A primary strategy in order to fully understand its structural–functional aspects, is the recombinant dissection into separate domains which can often be easier to be analyzed using standard biochemical and biophysical techniques [8–11].

First of all, the experimental approach to conformational transitions and the relationship between fluorescence signal changes and unfolding of proteins will be discussed, moreover several practical considerations for fluorescence experiments and phenomenological equations to analyze fluorescence spectra of proteins, minimizing sample exposure to high-intensity light beam, will be described. Finally, examples of unfolding reactions of different proteins, in particular dystroglycan domains, will be presented.

2. Materials and methods

2.1. Proteins and reagents

Bovine serum albumin and lysozime were from Sigma (St. Louis, MO). Immunoglobulins IgM, purified from human myeloma serum and IgG from pooled human serum were from ICN Biomedicals Inc. (Aurora, OH). DNA constructs encoding for the murine dystroglycan domains under analysis were amplified using standard RT-PCR protocols and the recombinant proteins were expressed in the *E. coli* BL21(DE3) strain and purified using nickel nitrilotriacetate affinity chromatography as described elsewhere [8,9,11]. Guanidinium hydrochloride (Gdn/HCl) was purchased from Fluka (Buchs, Switzerland) and used without further purification.

2.2. Fluorescence analysis

Proteins were dissolved in 100 mM sodium chloride (NaCl), 5 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), pH 7.4, or 100 mM phosphate buffer saline, pH 7.4. Steady-state fluorescence titration measurements were carried out at 25 °C in a 1-cm quartz cell, using a Spex (Edison, USA) FluoroMax spectrofluorimeter as described [12,13]. Protein concentration used in this study varied within the nanomolar–micromolar range, depending on the intensity of the specific fluorescence signal of each protein. Fluorescence emission spectra from 300 to 450 nm were recorded using an excitation wavelength (Ex^{λ}) at 260, 280 and 295 nm. After each addiction of denaturing agent we waited 10 min before spectra acquisition. Some proteins may require hours to reach full equilibrium in unfolding reaction [14]. Control experiments, to check if the equilibrium in the unfolding reaction was reached during the titration, were performed preparing a series of samples for each protein at different denaturant concentration, allowing these to equilibrate overnight, then measuring the fluorescence signal and comparing results in the two conditions. Data obtained from samples allowed to reach equilibrium overnight, did not show significant differences confirming that the equilibrium in the unfolding reaction was reached during the titration.

3. Results

3.1. Spectra analysis and practical considerations

The most frequently used fluorophore in studies of protein unfolding are the intrinsic tryptophan residues, that are extremely sensitive to modification of their microenvironment. The fluorescence emission maximum of proteins ranges from $\approx$310 nm, for buried, to $\approx$350, for fully exposed tryptophan residues [15,16] and is dependent upon several factors affecting their exposure to the solvent phase. In most proteins, also phenylalanine and tyrosine residues can contribute to fluorescence. However, the quantum yield of phenylalanine in proteins is small, so that the emission from this residue is rarely observed. In spite of its high absorption at 280 nm, and its high quantum yield in aqueous solution, the tyrosine emission of most proteins is small and frequently undetectable. Tyrosine, compared to tryptophan, is a weaker emitter, but one might expect it to contribute significantly

Table 1
Decrease of fluorescence signal in proteins exposed to successive spectra acquisition in the different experimental condition A, B and C (indicated by *)

Instrument set up and experimental condition	A	B	C
Number of studied proteins	9	9	9
Excitation wavelength (nm)	280	280	280
Emission wavelength (nm)	310–450	310–450	310–450
Integration time for point (s)*	0.5	0.2	0.05
Number of points (nm)	1	1	1
Total acquisition time for 1 spectra (s)*	70	28	7
Number of acquired spectra	10	10	10
Total acquisition time (s)*	700	280	70
Average fluorescence decrease (%) (after 10 scans)	14.2	6.2	1.0
Maximum fluorescence decrease (%) (α-DG(30–170)	16.5	7.3	1.2
Minimum fluorescence decrease (%) (IgG)	12.8	5.9	0.8
AWP (average wavelength peak) (nm)	–	±0.2	±0.4
AIP (average intensity peak) (%)	–	±0.7%	±1.1%

The instrument was setup with a bandpass of 4 nm. AIP (average intensity peak) is the percentage difference of intensity and AWP (average wavelength peak) is the peak shift in nanometers obtained comparing spectra acquired in experimental condition B and C, to experimental condition A. The intensity and peaks in condition B and C were derived by fitting the spectra with Eq. (1) or Eq. (2) as described in the text.

because it is present in larger amounts. However, tyrosine is quenched by any nearby groups on the peptide chain and because of energy-transfer to tryptophan residues; consequently the absence of tyrosine emission observed in most proteins is largely dependent upon their particular 3D structure. Therefore, the selective excitation of an amino acid may be conveniently used to discriminate the specific contribution of each residue in a protein structure (i.e. $Ex^{\lambda}=260$ nm simultaneous excitation of phenylalanine, tyrosine and tryptophan, $Ex^{\lambda}=280$ emission from both tyrosine and tryptophan, $Ex^{\lambda}=295$ selectively excitation of tryptophan).

Fluorescence spectra of proteins were recorded as a function of guanidine hydrochloride concentration. Any background signal (fluorescence or scattered light) from the buffer was subtracted and the fluorescence intensity was corrected for the progressive protein dilution [15,17–19]. In the protein concentration range taken into consideration the fluorescence signal was linear with the concentration of the protein. A decrease in fluorescence signal, usually defined as 'bleaching effect', due to successive exposure of sample to high-intensity light beam [15–18], was found to be characteristic of each protein and dependent on its concentration and on the instrument and experimental set up (bandpass, acquisition time, number of acquisition points, etc.) and although not linear with exposure time, was generally well described by a simple parabolic equation (data not shown). However, an experimental evaluation of this phenomenon should be performed every time fluorescence measurements are carried out. Fast fluorescence readings at fixed wavelength, generally, restrict this drawback to less than 5% of the initial intensity. When the fluorescence spectra need to be recorded, the previous effect could become relevant (5–15%) due to long sample exposure to high-intensity light beam (Table 1). A good strategy to overcome the problem is to acquire a fast fluorescence scan followed by a mathematical interpolation of data. In this case, at constant protein concentration and spectral bandpass of monochromators, the integration time for each scan point and/or the number of acquired points can be strongly reduced (Fig. 2a–c, Table 1).

We used two phenomenological equations to analyze emission spectra. The first is a Gaussian curve, generally used to simulate excitation and

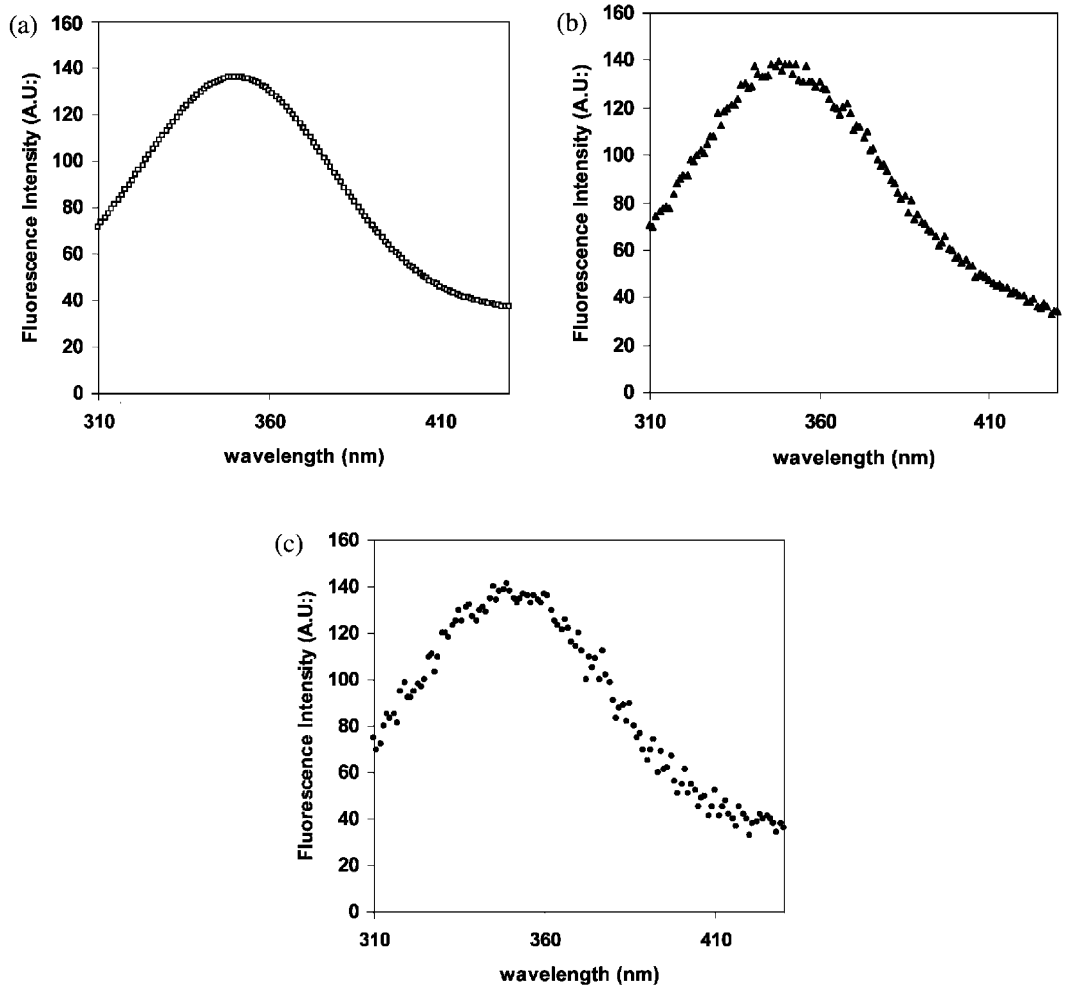

Fig. 2. Fluorescence spectra of unfolded bovine albumin at 6 M guanidine hydrochloride, acquired at the different setup conditions, described in Table 1, A (panel a—□), B (panel b—▲) and C (panel c—●).

emission spectra for folded and unfolded protein [20]

$$F = a\frac{\exp\left[-\frac{\nu_i - \nu}{2\sigma}\right]^2}{\sigma\sqrt{2\pi}} + b \quad (1)$$

where F is the fluorescence intensity, ν is the wavenumber ($=1/$wavelength in cm^{-1}), ν_i is the wavenumber for the center of the Gaussian curve, σ is the width of the curve in cm^{-1} and a and b are fitting parameters.

The former equation fails to correctly fit data for spectra with peak at wavelengths lower than 340 nm (i.e. folded proteins) (Fig. 2a and b). In this case, the best fit is obtained with the following one

$$F = a(\lambda_{em} - \lambda_{ex})^b \exp\left[-\frac{\lambda_{em} - \lambda_{ex}}{c}\right] \quad (2)$$

where a, b and c are fitting parameters, λ_{ex} is the fixed excitation wavelength, λ_{em} is the emission

wavelength. From analytical study of the curves can be derived the maximum fluorescence intensity, the peak wavelength and any intensity at different wavelength. In all the experiments spectra from 310 to 430/450 nm were fitted according to Eqs. (1) and (2).

3.2. Denaturant induced unfolding

The thermodynamic approach used to describe the unfolding reaction of each protein was based on a two-state model, where N is the native state and U is the unfolded state of the molecule and K_{un} is the unfolding equilibrium constant [20]; the free energy change for unfolding can be described by the following equation

$$\Delta G^0_{un} = -RT \ln K_{un} = \Delta H^0_{un} - T\Delta S^0_{un} \quad (3)$$

ΔH^0_{un} and ΔS^0_{un} are the enthalpy change and entropy change for the unfolding transition. If the protein unfolding is induced by the addiction of a chemical denaturant [*d*], the following linear relationship is generally accepted to describe the thermodynamics of the system [21,22]

$$\Delta G^0_{un} = \Delta G^0_{0,un} - m[d] \quad (4)$$

here $\Delta G^0_{0,un}$ is the free energy change for the unfolding reaction at the reference condition in the absence of denaturant and *m* is an empiric parameter indicating the denaturant susceptibility (i.e. $m = -\delta G^0_{un}/\delta[d]$, the dependence of δG^0_{un} on denaturant concentration). The above assumption can be made when the unfolding reaction is a single step (two-state) process. If there is one or more intermediates in the unfolding process, then the thermodynamic models become more complex [20]. However, as for various proteins analyzed in this paper, if the fluorescence properties of the intermediate states are similar to U or N states, the transition may appear to be a simpler two-state.

Measurement of fluorescence vs. [*guanidine*] or [*urea*] is a standard method for studying the stability of proteins. The following equation describes such data [20]

$$F = \frac{F_{0N} + s_N[d] + (F_{0U} + s_U[d])e^{(-\Delta G^0_{0,un} + m[d])/RT}}{1 + e^{(-\Delta G^0_{0,un} + m[d])/RT}} \quad (5)$$

In this equation F_{0N} and F_{0U} are the intensities of native and unfolded states in the absence of denaturant, s_N and s_U are the baseline slopes for the native and unfolded regions and $\Delta G^0_{0,un}$ and *m* are the same as before. A useful parameter linked to the stability of the protein is the $Gdn_{50\%}$ defined as the ratio of $\Delta G^0_{0,un}$ and *m* indicating the denaturant concentration inducing the 50% of fluorescence intensity change (e.g. the mid-point of the curve) [14].

The fluorescence peak change as a function of guanidine concentration was fitted using a phenomenological equation to calculate the guanidine concentration inducing the 50% ($Gdn_{50\%}$) effect on the peak shift. The following equation was used

$$F_{peak} = a + \frac{(b-a)}{1 + \left(\frac{c}{[Gdn]}\right)^d} \quad (6)$$

In this equation *c* represents the guanidine concentration at the mid-point of the curve and *a* and *b* are fitting parameters.

3.3. Experimental set up and minimizations

In Table 1 are showed the different experimental conditions and instrument set ups (reported as A, B and C) used in our measures and the average value of decrease in fluorescence signal after ten acquisition scans on the various proteins under analysis. Each protein or protein domain, depending on its concentration and its amino acid composition, shows a different decrement in fluorescence intensity. However, differences are in the order of few percentage points with respect of the average value; the most and the least 'bleaching' resistant protein are immunoglobulins (IgG) and α-DG(30–170), respectively.

Fluorescence spectra of unfolded bovine albumin (at 6 M guanidine hydrochloride), acquired at the different setup conditions A, B and C, are

reported in Fig. 2a–c, respectively. As expected, decreasing acquisition integration time for each point, from 0.5 s (A) to 0.05 s (C), leads to loss in the curve resolution. On the other hand, the 'bleaching effect' on fluorescence at the end of the titration, when working in condition C, is only $\approx 1\%$ compared to $\approx 15\%$ in condition A (Table 1).

In fluorescence unfolding measurements, where the fluorescence change range is broad (from ≈ -50 to $\approx +110\%$ see Table 2), the previous described 'bleaching effect' could become relevant. The use of a mathematical function to describe fluorescence spectra of protein, acquired as fast scan (i.e. instrument set up C), allows minimization of the decrease in fluorescence signal due to prolonged exposure of sample to a high-intensity light beam. Interpolating spectral data for the correct resolution of the fluorescence intensity and peak could be critical. We used X^2 ratio values (X^2 of Eq. (1) divided by X^2 of Eq. (2)) to compare the accuracy of the two equations in fitting fluorescence data recorded between 310 and 450 nm with the different excitation wavelength of 260, 280, 295. X^2 ratio values listed in Table 2 show that the classical Gaussian equation (Eq. (1)), generally used to simulate fluorescence spectra of proteins [20,22], fails to follow the shape of folded protein spectra. Moreover, in Table 1 are reported the differences in the maximum fluorescence intensity and peak obtained comparing their values derived by fitting the spectra acquired in experimental condition B and C, to the value measured in experimental condition A.

Fig. 3a and b show, respectively, fluorescence spectra of folded α-DG(30–315) dystroglycan domain, unfolded bovine serum albumin and the best fit curve for Eq. (1) (dotted line) and Eq. (2) (continuous line). The curve in Fig. 3a is a typical spectrum of a native protein, showing a fluorescence peak in the spectral region of buried tryptophan residues. On the other hand, in Fig. 3b is shown a fluorescence spectrum typical of an unfolded protein or of proteins having aromatic residues exposed to solvent with their fluorescence peaks red shifted. In Fig. 3 the X^2 values of each fit curve calculated using the non-linear least square method are listed [23].

In Table 2 are shown the X^2 ratio values of different proteins in folded, unfolded and at $Gdn_{50\%}$ concentration conditions, their fluorescence peaks at excitation wavelengths of 260, 280 and 295 nm, the percentage of fluorescence change upon unfolding transition, the number of aromatic residues (Trp, Phe, Tyr) for each protein, the $Gdn_{50\%}$ value and the *dG* of the reactions calculated by Eq. (5).

3.4. A typical unfolding behavior: decrease of fluorescence intensity

Figs. 4–6 show the change in fluorescence intensity (panel A) and in the maximum emission peak (panel B) as a function of guanidine hydrochloride concentration for all the proteins under analysis. In Fig. 4a are reported bovine serum albumin, used as a control, and dystroglycan domains α-DG(30–315) and α-DG(170–315). These proteins all show a clear decrease of fluorescence intensity upon unfolding. In this case the exposure of buried amino acids to the aqueous phase causes a typical and strong fluorescence quenching of tryptophans. α-DG(30–315) and α-DG(170–315) have similar $Gdn_{50\%}$ values. A similar behavior is observed in Fig. 4b where the wavelength of the fluorescence emission maximum is reported. All proteins start with a wavelength maximum peak value typical of folded proteins, i.e. tryptophans not exposed to solvent, ranging from 330 to 337 nm. After the addiction of the denaturing agent, they completely unfold, exposing tryptophans to solvent as showed by a wavelength peak shift towards values higher than 350 nm. The $Gdn_{50\%}$ values extrapolated from fitting the unfolding curves for intensity and wavelength according to Eqs. (5) and (6), respectively, are similar.

3.5. The N-terminal domain of α-dystroglycan-(30–170), shows a behavior similar to what observed in immunoglobulins

In Fig. 5a is shown the fluorescence intensity change of α-DG(30–170), α-DG(485–600) and β-DG(654–750) dystroglycan domains as a func-

Table 2

Protein	Fluorescence change (%)	Number of Wrp	Number of Tyr	Number of Phe	Gdn/2 [M]	ΔG (kcal/mol)	Excitation wavelength (nm)	Folded		Gdn/2		Unfolded	
								Peak	χ^2 ratio	Peak	χ^2 ratio	Peak	χ^2 ratio
Bovine serum albumin	−45	3	21	30	2.4±0.2	8.8±1.0	260	343	1.1	347	0.2	350	0.1
	−50						280	342	1.5	348	0.3	351	0.4
	−45						295	343	3.4	348	0.4	355	1.0
IgM immunoglobulin	+135	>100	>100	>100	2.4±0.2	7.0±0.9	260	335	3.2	343	0.8	352	0.1
	+130						280	335	3.6	344	0.7	353	0.2
	+125						295	336	3.1	344	0.7	353	0.1
IgG immunoglobulin	+125	>30	>50	>50	3.0±0.2	1.2±0.1	260	332	3.0	341	0.9	349	0.3
	+118						280	333	2.9	341	0.7	350	0.2
	+115						295	333	3.0	342	0.7	352	0.2
α-DG(30–315)	−40	5	3	6	1.3±0.1	1.6±0.2	260	331	4.5	343	1.0	354	0.4
	−43						280	332	3.2	345	1.1	355	0.3
	−41						295	332	4.3	345	0.9	355	0.5
α-DG(170–315)	−45	2	1	3	1.1±0.1	1.1±0.1	260	329	3.2	342	1.2	355	0.2
	−48						280	330	8.1	342	2.1	354	0.6
	−48						295	329	8.4	343	1.3	353	0.2
α-DG(30–170)	0	3	2	3	0.7*±0.1	2.5±0.3	260	340	0.6	347	0.8	354	0.4
	0				0.8±0.1		280	339	2.0	346	0.8	352	0.8
	+30						295	340	1.3	346	1.0	352	1.0
α-DG(485–600)	0	2	4	5	1.4*±0.1	–	260	343	1.1	348	0.7	352	0.2
	0						280	344	1.5	349	0.6	353	0.1
	0						295	344	1.2	349	0.5	354	0.1
β-DG(654–750)	0	1	1	3	1.7*±0.1	–	260	348	0.4	351	0.3	352	0.2
	0						280	349	0.2	351	0.3	353	0.1
	0						295	350	0.3	351	0.6	353	0.1

* From peak shift.

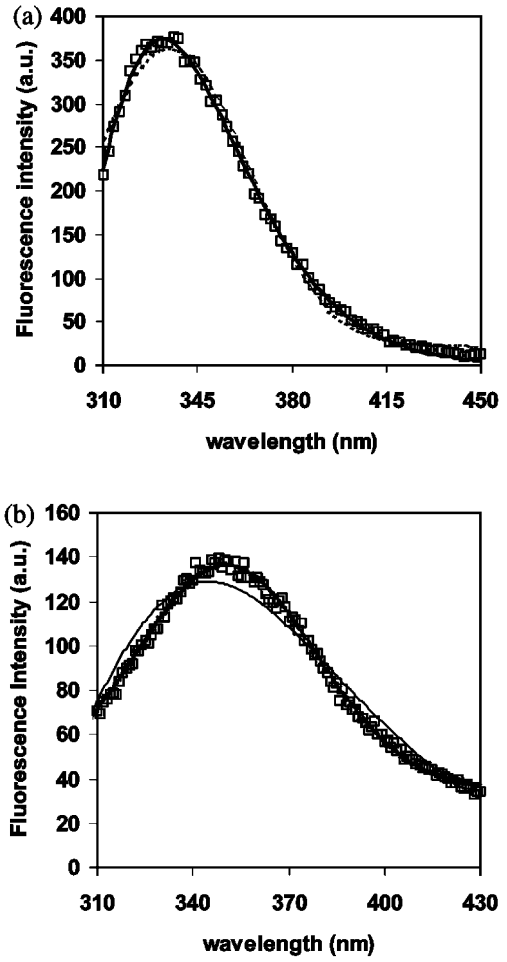

Fig. 3. Fluorescence spectra of folded α-DG(30–315) dystroglycan domain (panel a), unfolded bovine serum albumin (panel b) acquired under experimental condition C (Table 1) and the best fit curve for Eq. (1) (dotted line) and Eq. (2) (continuous line). The X^2 value of each fitted curve, accomplished using non-linear least square method (Bard [23]), are: panel a—Eq. (1) $X^2=127.4$, Eq. (2) $X^2=24.4$, ratio = 5.2. Panel b—Eq. (1) $X^2=5.4$, Eq. (2) $X^2=44.7$, ratio = 0.1. The peak and intensity derived from the two equation compared with the acquisition under experimental condition A are: panel a: α-DG(30–315)—experiment A: peak = 332 nm, Maximum intensity (M.I.) = 374 A.U.; Eq. (1): peak = 334 nm, M.I. = −2.86%; Eq. (2): peak = 332 nm, M.I. = +0.03% Panel b: Bovine serum albumin—experiment A: peak = 351 nm, M.I. = 136.4 A.U.; Eq. (1): peak = 351 nm, M.I. = −0.15%; Eq. (2): peak = 345 nm, M.I. = −5.50%.

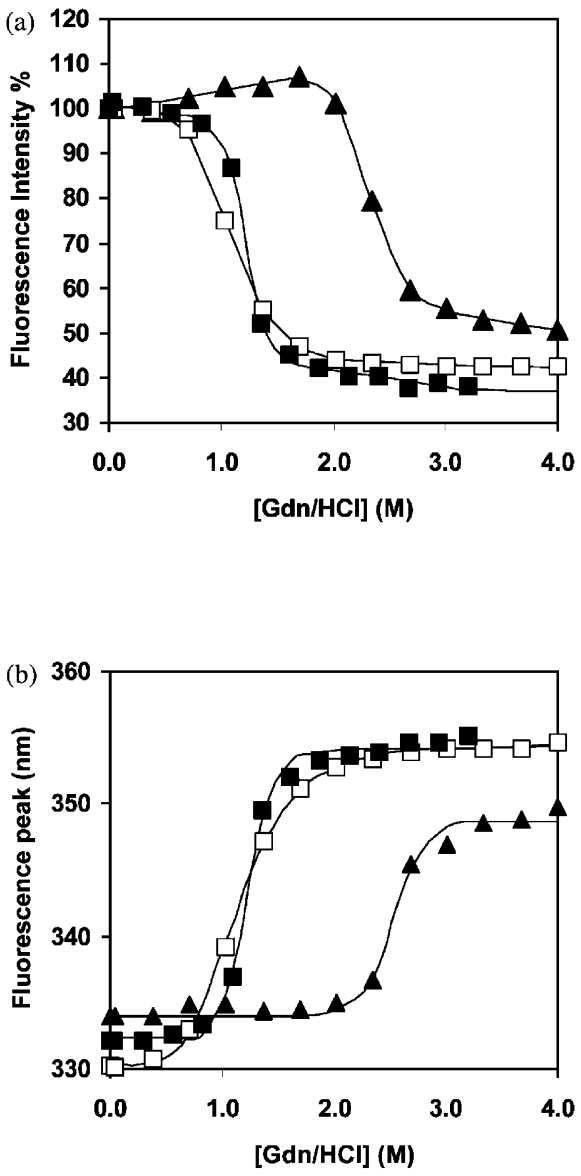

Fig. 4. Fluorescence intensity (panel A) and maximum emission peak (panel B) changes as a function of guanidine hydrochloride concentration of bovine serum albumin (▲), dystroglycan domains α-DG(30–315) (■) and α-DG(170–315) (□). These proteins all show a clear decrease of fluorescence intensity upon unfolding due to the exposure of buried amino acids to the aqueous phase causing a strong fluorescence quenching of tryptophans. Excitation wavelength was 280 nm.

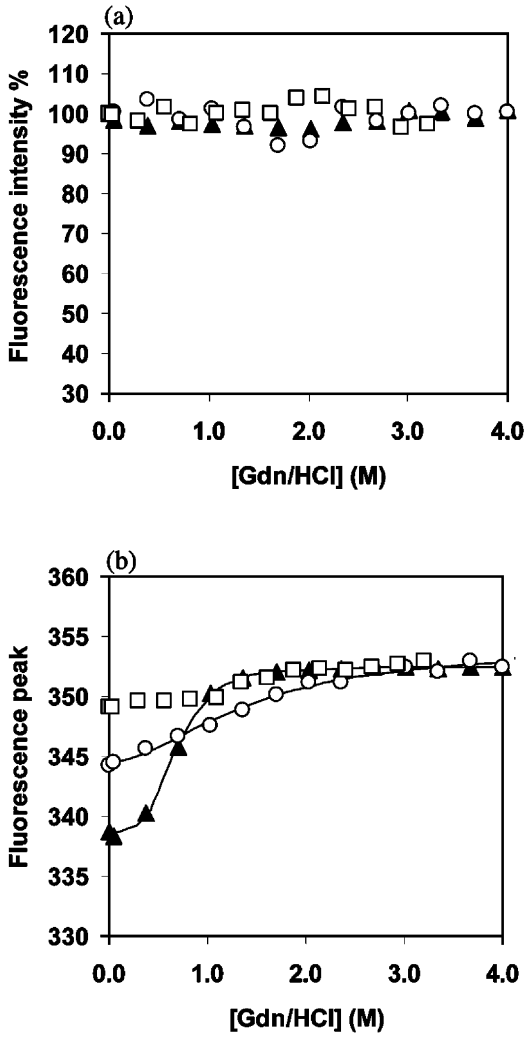

Fig. 5. Fluorescence intensity (panel A) and the maximum emission peak (panel B) as a function of guanidine hydrochloride concentration of α-DG(30–170) (▲), α-DG(485–600) (○) and β-DG(654–750) (□) dystroglycan domains as a function of guanidine concentration. Excitation wavelength was 280 nm. No significant fluorescence change is observed. In the case of β-DG(654–750), the fluorescence maximum dependence on denaturant concentration shows that no transition involving tryptophans occurs. On the other hand, both for α-DG(30–170) and α-DG(485–600) the fluorescence maximum shifts from 337 nm for α-DG(30–170) and 344 nm for α-DG(485–600) to 353 nm, characteristic of totally exposed tryptophan, clearly indicating the presence of an unfolding process.

tion of guanidine concentration at 280-nm excitation wavelength. Interestingly, no significant fluorescence change is observed. In the case of β-DG(654–750), a domain which has already been catalogued inside the natively-unfolded protein

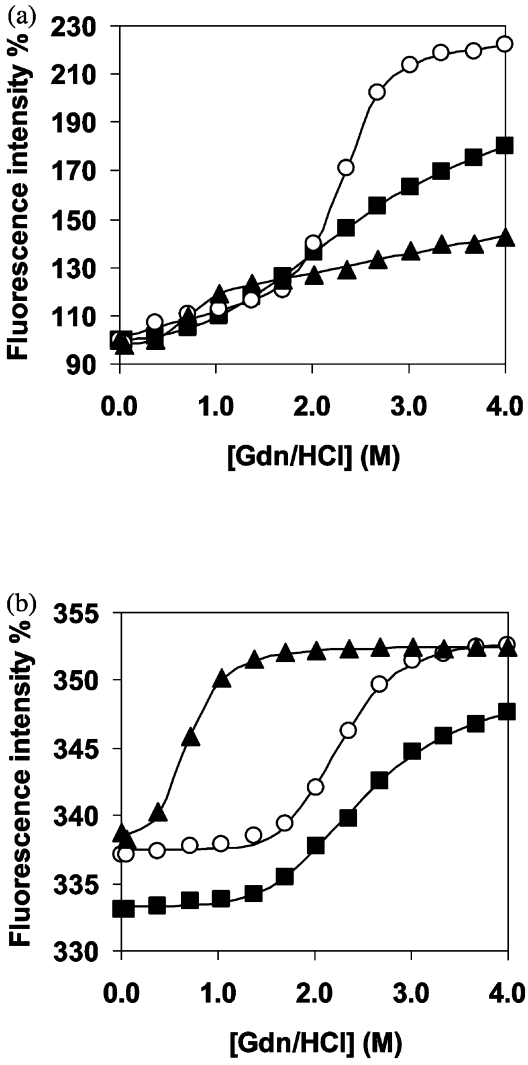

Fig. 6. Fluorescence intensity (panel A) and maximum emission peak (panel B) changes as a function of guanidine hydrochloride concentration of immunoglobulins IgG (n), IgM (○) and α-DG(30–170) (▲) dystroglycan domain. Excitation wavelength was 280 nm for immunoglobulins and 295 nm for α-DG(30–170) thus selectively exciting tryptophan residues. For all these proteins is observed an increase in the emission quantum yield upon unfolding.

family [24], the fluorescence maximum dependence on denaturant concentration (Fig. 5b) shows that no transition involving tryptophans occurs (i.e. minimal peak red shift from ≈349 to ≈352), confirming previous observations [11]. On the other hand, both for α-DG(30–170) and α-DG(485–600) the fluorescence maximum shifts from a value characteristic of buried aromatics, 337 nm for α-DG(30–170) and 344 nm for α-DG(485–600) to the one typically measured for a totally exposed tryptophan (353 nm), clearly indicating the presence of an unfolding process.

In Fig. 6a are reported the denaturation curves of immunoglobulins IgG, IgM and α-DG(30–170) domain as a function of guanidine concentration. It should be noted that in this case the titration curve of α-DG(30–170) is the one obtained using an excitation wavelength of 295 nm, thus selectively exciting thryptophan residues (see discussion for details). For all these proteins is observed an increase in the emission quantum yield upon unfolding (*reverse* behaviour). Moreover, the α-DG(30–170) domain shows a behavior characteristic of a relatively unstable proteins [20] undergoing a complete transition at relatively low concentrations of denaturing agent.

4. Discussion

4.1. Learning from fluorescence unfolding signal

The determination of thermodynamic parameters for the unfolding of proteins has been a topic of fundamental interest for many years. Recently, such studies became important for biotechnological reasons, since it is often necessary to characterize the stability of recombinant or engineered proteins [14]. Monitoring the unfolding behavior of a protein, from an ordered (native) to a disordered (unfolded) state, can provide key thermodynamic information about its stability. The static fluorescence signals of the native and unfolded state may also provide some precious information on the microenvironment of the fluorophores [17,18]. The easiest way to follow protein unfolding is registering steady-state fluorescence intensity, measured at fixed excitation and emission wavelengths However, more detailed information on the unfolding transition can be obtained by following the entire fluorescence spectral changes of the protein as a function of the denaturant concentration. In fact, in this case at least two parameters can be derived. The first is the fluorescence intensity at different wavelengths, the second is the wavelength of the emission maximum. Analyzing the dependence of such parameters by the denaturant concentration leads to the thermodynamic information describing the stability of the protein [17,18,20].

Typically, two different experimental procedures can be applied to follow protein unfolding reactions. In the first, a denaturant agent is progressively added to a protein sample and, after equilibration at every step, the fluorescence spectra are recorded. The second procedure implies the preparation of multiple protein samples at different denaturant concentration; after equilibrium is reached, a fluorescence spectrum for each sample is acquired [17,18]. The major and basic advantage of the first method is that it requires just small amounts of protein. On the other hand, the acquisition of multiple spectra of the same protein sample, each at a different denaturant concentration, implies long exposition to high-intensity light beam, causing a ‘bleaching effect’ on the fluorescence intensity of the sample. This decrease in fluorescence signal, depending on the instrument setup, on protein concentration and on the total time the sample is exposed to light beam, can be determined with appropriate measurements (Section 3 and Table 1).

4.2. Minimizing the ‘bleaching effect’

In this study, we analyzed the ‘bleaching effect’ measured on nine different proteins using three different instrument setups (Table 1). In the experimental condition A, where good resolution of intensity fluorescence and peak was obtained, the ‘bleaching effect’ accounted for 15% of the signal (Fig. 2a and Table 1). In many cases, the global fluorescence change upon unfolding can be heavily affected by this event, resulting in an erroneous estimation of the thermodynamic parameters of the transition. We show that a correct mathematical analysis of spectra can help in reducing the ‘bleaching effect’ to approximately 1%. We have

proposed two different equations (Eqs. (1) and (2)) in order to correctly describe the fluorescence spectra of proteins acquired with a rather low integration time for point, leading to a sensible decrement of the total acquisition time needed to monitor the unfolding reaction, without loosing accuracy on the fluorescence intensity and maximum peak determination. As shown in Table 1, the differences between the measured (condition A) and extrapolated (condition C) intensity are approximately 1% and the peak resolution is within ± 0.4 nm.

4.3. Immunoglobulins display a 'reverse' fluorescence signal change upon unfolding

The fluorescence properties of proteins are generally dominated by tryptophan residues, highly sensitive to solvent effects. There has been much interest in trying to develop rules for interpreting the fluorescence patterns for tryptophan residues in proteins and relating these data to structural details [25,26]. The most widely accepted interpretation is that the bluer emitting tryptophan residues are those with the indole ring surrounded by other apolar amino acid side chains and that interactions between excited state and polar solvent molecules or other polar functional groups cause redder fluorescence.

Typically, the exposition to solvent of aromatic amino acids leads to a decrease in their fluorescence intensity due to water quenching effect [19]. However, in some proteins, such as immunoglobulins or immunoglobulins fragments as observed by Sumi and Hamaguchi [27], the interactions between tryptophan and tyrosine residues in buried region of the folded molecule lead to their mutual quenching, determining an unchanged or increased fluorescence intensity after unfolding [19]. It is often useful the recording of maximum emission wavelength that is typical higher than 350 nm for tryptophan exposed to solvent and lower for buried residues and that can give evidence of the occurring transition. The emission maximum of a tryptophan residue in a protein is thought to be directly related to the polarity of its microenvironment. An interesting suggestion has been made [14,20] that the emission maximum can also be related to the degree of electrostatic stabilization and destabilization of the excited indole ring and charged groups in its microenvironment. Intrinsic tryptophan residues will usually have the environmental sensitivity to track the unfolding of a protein. There are also cases where this is not possible. For example, if a tryptophan is on a unstructured region of the protein both in the native and unfolded state [17,18,20].

In our study, both unchanged and increased fluorescence intensity are recorded (Fig. 5a and b Fig. 6 and a and b). In the former case, a peak shift is observed, proving the exposure of aromatics to solvent; on the latter the increase of intensity could be explained if the mutual quenching of aromatic residues in a folded close moieties of the molecule could be stronger than the quenching effect of the solvent. When both the fluorescence intensity and the emission maximum wavelength remain unchanged, it could mean that: (i) the protein is highly unstructured (i.e. already unfolded), (ii) there are no aromatic residues in the protein undergoing the unfolding transition, (iii) the aromatics already occupy, in the structured molecule, a natively disordered region (Fig. 5a and b).

4.4. Fluorescence unfolding signal help identifying immunoglobulin-like domains in α-dystroglycan

Dystroglycan is an adhesion complex involved in a variety of biological functions [6,28]. It is composed of two protein subunits, α, highly glycosylated and extracellular and β, transmembrane, which form a tight but non-covalent complex [11]. No 3D data are available on dystroglycan subunits so far, although in the last years a particular effort has been made to collect the first structural–functional details mostly based on recombinantly expressed domains [8–11,29]. In Fig. 1 is reported the molecular organization of dystroglycan and the various domains that have been analyzed are indicated. It is noteworthy that all the dystroglycan domains analyzed show ΔG values for the unfolding process (measured from fluorescence intensity change and/or peak shift) that confirm their substantial stability as isolated units (Table 2).

The N-terminal region of α-dystroglycan should be considered as a main functional 'hot-spot' within the whole adhesion complex. In fact, it is likely that its first domain directly contributes to build the high-affinity interaction with LNS domains-containing molecules such as laminins, agrin or perlecan [8]. Moreover, the second N-terminal domain has been proposed to harbor the target site for arenaviruses [30]. Therefore, a detailed structural investigation of these domains would give an invaluable help to design strategies to prevent muscular dystrophies and viral infections.

Our fluorescence data confirm the notion that the N-terminal region of α-dystroglycan (30–170) represents an Ig-like domain [31], which are indeed very common within ECM molecules and their receptors [32]. In the case of α-DG(30–170), when exciting at 280 nm, no fluorescence intensity change is recorded although a maximum peak shift from 338 to 352 nm gives evidence of the unfolding transition. The effect could be attributed to some mutual quenching between tyrosine and tryptophan residues both absorbing at 280 nm and sufficiently close in space, in the unfolded state, to undergo a reciprocal Foster's energy transfer. When selectively exciting tryptophan residues at 295 nm the emission maximum wavelength shift is conserved, while an increase of 30% of fluorescence intensity is recorded. This increase of fluorescence upon unfolding is another proof that the aromatics are close to each other in the folded protein, as also observed with IgG and IgM. The presence in α-dystroglycan of such an Ig-like domain was previously identified based on sequence similarities with the immunoglobulin light chains of the k family [31]. It is worth noting that the fluorescence unfolding behavior of α-DG(30–315) (which shows an overall decrease in the fluorescence intensity) is dominated by the contribution of α-DG(170–315), as also observed with this isolated domain (Fig. 4a), which is likely to be arranged in a different structural fashion (e.g. resembling the ribosomal protein S6) [8].

According to our analysis, the C-terminal domain of α-dystroglycan (485–651) displays a fluorescence unfolding behavior which may as well suggest the presence an Ig-like structural arrangement. However, the fluorescence intensity of α-DG(485–600) does not change as a function of the denaturing agent concentration. This domain harbours the binding epitope for the ectodomain of β-dystroglycan [9,11,29]. Interestingly, it has been proposed based on sequence alignment that also this domain might have an Ig-like 'cadherin-like' structure [33]. Although no further evidence have been collected on the proposed structural similarity to cadherin domains, our fluorescence data could support the idea that the C-terminal region of α-dystroglycan would display an overall Ig-like fold.

5. Concluding remarks

Fluorescence analysis of unfolding processes represents a useful tool to analyze protein domains structurally and thermodynamically. In the post-genomic era, it emerges as an easy and rapid technique to collect the first structural details on a novel protein domain. The *reverse* increase of fluorescence that we have measured for IgG, IgM and α-DG(30–170) fully confirms the Ig-like structural arrangement of the first N-terminal domain of α-dystroglycan.

According to fluorescence unfolding data, the proteins and protein domains that we have analyzed can be classified in the following general categories:

1. Proteins undergoing a change in fluorescence intensity (decrease) and maximum emission peak (increase): albumin, α-DG(30–315), α-DG(170–315).
2. Proteins undergoing a change in fluorescence intensity (increase) and maximum emission peak (increase): IgG, IgM, α-DG(30–170) when excited at 295 nm.
3. Proteins undergoing a change only in the emission peak (increase), with relatively stable fluorescence intensity: α-DG(485–600) and α-DG(30–170) when excited at 260 and 280 nm.
4. Proteins with no change both in fluorescence intensity and emission wavelength (generally already higher than 350 nm in the native protein): β-DG(654–750).

Acknowledgments

We would like to thank Prof. Bruno Giardina (Rome) for his constant advice and support and Maria Giulia Bigotti (Bristol) for critical reading of the manuscript. The Telethon grant (no. 1267) to A.B. is gratefully acknowledged.

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