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Perturbation of Conformational Dynamics of *ASCUT-1* from *Ascaris lumbricoides* by Temperature and Sodium Dodecyl Sulfate

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Abstract

ASCUT-1 is a protein found in cuticlin, the insoluble residue of the cuticles of the nematode *Ascaris lumbricoides*. It contains the CUT-1-like domain which is shared by members of a novel family of components of extracellular matrices. The monomeric form of *ASCUT-1* contains a single tryptophan residue. An understanding of the structure-function relationship of the protein under different chemical-physical conditions is of fundamental importance for an understanding of its structure and function in cuticles. In this paper we report the effect of the temperature and sodium dodecyl sulfate on the structural stability of this protein. The structure of the protein was studied in the temperature range 25–85°C in the absence and in the presence of sodium dodecyl sulfate by frequency-domain measurements of the intrinsic fluorescence intensity and anisotropy decays. The time-resolved fluorescence data in the absence of SDS indicated that the tryptophanyl emission decays were well described by a bimodal lifetime distribution, and that the temperature increases resulted in the sharpening and in the shortening of the tryptophanyl lifetime distribution. In the presence of SDS an unimodal fluorescence lifetime distribution as well as a marked decrease in the anisotropy decay values were observed.

Keywords

Cuticlin; frequency-domain fluorometry; protein stability; anisotropy decays; *Ascaris lumbricoides*

INTRODUCTION

Proteins are generally stable mechanical constructs whose biological function depends on the internal motion allowed by their structure. Such internal motions may be subtle and may involve complex correlation between atomic motions, but their nature is inherent to the

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structure and to the interactions within the molecule [1]. In fact, the protein macromolecules even in their native state are not in a unique structural state, but fluctuate among a large number of conformations which differ in small structural details [2,3], that can influence the emitting properties of tryptophanyl residues [4]. Tryptophanyl residues in proteins are often used as spectroscopically active probes to monitor the structural properties of the macromolecules in solutions [5,6].

Frequency-domain fluorometry is commonly used to elucidate the structural and dynamical aspects of tryptophan containing proteins [4]. The emission decay of proteins containing a single tryptophan residue can often be represented by a quasi-continuous distribution of lifetimes. The center of the lifetime distribution is indicative of the average microenvironments surrounding the indolic residue, while the distribution width is related to the number of subconformations that the protein macromolecule can assume [7,8].

The cuticle is an important part of the nematode's body structure, functioning as an exoskeleton and protecting the animal from the environment under a variety of conditions. The cuticle contributes much of the body shape of nematodes; the major components of this structure (up to 90%) are cross-linked collagen-like proteins. Beside collagen, the other major structural component of the cuticle is a mixture of proteins that are found as a residue that is resistant to collagenase and insoluble in strong detergents and in disulfide reducing agents such as β -mercaptoethanol [9]. In the literature this residue is referred to as cuticlin. We have previously identified two genes, *cut-1* and *cut-2*, coding for components of the insoluble cuticlin residue of the free living nematode *Caenorhabditis elegans* [10, 11]. The cloned genes have provided the opportunity to obtain recombinant cuticlin proteins. In particular, it has been possible for the first time to study some aspects of the mechanism of CUT-2 cross-linking [11]. In addition, specific antibodies have been raised against the recombinant proteins and have been used to localize the corresponding proteins within the cuticle layers [12,13]. While CUT-2 is a component of the cuticle of all stages of *C. elegans* [11], CUT-1 is present only in the cuticle of dauer larvae where it forms two ribbons running the whole length of the worm, underneath the lateral alae [10].

The two genes define two different families of cuticlin components. CUT-2 shares homology with a group of insect proteins, which also participate in the formation of extracellular protective structures such as the eggshell-layers [10]. CUT-1 and CUT-2 have no sequence similarity except for a short stretch of CUT-1 (~ 40 aa long) which is rich in alanine and proline residues and resembles the motif repeated several times in CUT-2. CUT-1 defines a new family of proteins which we have named CUT-1-like. Homologues of *cut-1* have, for the moment, been identified only in nematodes: in *Ascaris lumbricoides* at least three genes [14] and one gene in *Meloidogyne artiella* [15]. In addition to *cut-1*, the *C. elegans* Genome Sequencing Project [16] has revealed the presence in this organism of more than 20 genes coding for proteins that contain a 262 amino acid CUT-1-like region. Given its novelty and its success at least within the nematode phylum it is of interest to understand more about the structure of this class of proteins. To this end we have produced, in *Escherichia coli*, the mature version of CUT-1 from *Ascaris lumbricoides* (*ASCUT-1*). We have recently reported studies with circular dichroism and Fourier Transform Infrared spectroscopy on this recombinant protein [17] that indicate that the protein structure is uncommonly stable at

high temperatures and that this stability is increased by the addition of the detergent SDS. Here we report conformational dynamics studies on *ASCUT-1* in the presence and in the absence of SDS using frequency-domain fluorometry and anisotropy decays which may be particularly informative since the protein contains a single tryptophan residue.

MATERIALS AND METHODS

Reagents

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ was obtained from J. T. Baker Chemicals (New York, NY, U.S.A.). All other chemicals were commercial samples of the purest quality.

Protein Assay

The protein concentration was determined by the method of Bradford [18], with bovine serum albumin as standard.

Production of *ASCUT-1*

To produce the recombinant protein, total RNA from the appropriate nematode life-cycle stage was reverse transcribed and the resulting cDNA was amplified with two primers. Primer 1 corresponds to the amino acids IPVDNG which immediately follows the signal peptide and has at its 5' end an NdeI restriction site which was used for cloning and which provides the initiator ATG. Primer 2 is complementary to an untranslated region at the 3' end of the mRNA and also has, at its 5' end, a restriction site (*Bam*HI). The product of the amplification reaction was cut with NdeI and BamHI, gel purified, and legated in plasmid Bluescript (Stratagene). Several positive clones were analyzed by restriction digestion and sequenced. One clone, which had no amplification errors, was used to prepare a fragment that was cloned in pT7.7. Positive transformants were analyzed by restriction digestion. In vector pT7.7 the gene to be expressed, in this case *ascut-1*, is under the T7 phage RNA polymerase promoter. The T7 polymerase gene, in turn, is under the control of the lac-Z promoter. In normal growth conditions there is no T7 polymerase and *ascut-1* is not transcribed. Upon addition of isopropylthio- β -galactoside, the lac operon inducer, the T7 polymerase gene is transcribed and its product transcribes the gene of interest and at the same time shuts off almost all other transcriptions.

For a standard preparation, 400 ml of broth was inoculated with *E. coli* strain harboring the plasmid with the insert. When the culture reached an absorbance of 0.5 at 560 nm, isopropyl- β -thiogalactoside was added to 1.0 mM final concentration and the culture incubated with shaking for 3 hours. Cells were harvested by centrifugation, washed in H_2O and resuspended in 1/20th vol. of H_2O containing 1% Triton X-100. The cells were disrupted by sonication while kept in an ice bath. The broken cells were centrifuged at 10,000g and the pellet was resuspended again in 1% Triton X-100, incubated for 10 min at 37°C and centrifuged again. The pellet containing the inclusion bodies was resuspended in 4 M urea and incubated for 20 min at 37°C. After centrifugation, the resulting pellet was resuspended in H_2O . A typical preparation gave 2–4 mg of *ASCUT-1* which was more than 95% pure, as revealed by SDS-PAGE.

Preparation of the Samples for Spectroscopic Analysis

For fluorescence measurements the inclusion bodies were washed several times in H₂O and the protein was solubilized in 7.0 M urea. Urea was substituted with 10 mM Tris/HCl pH 7.0 using an ultrafiltration apparatus equipped with PM-10 membranes (Amicon); a final clear solution, as shown by light scattering measurements, containing 0.5 mg protein/ml buffer was obtained. This protein solution was the starting material for the fluorescence analysis.

Fluorescence Spectroscopy

Emission spectra were obtained with a Jasco FP777 spectrofluorometer, at a protein concentration 0.05 mg/ml in 10 mM TRIS-HCl buffer, pH 7.4, plus the specified amounts of SDS. The excitation was set at 295 nm in order to exclude the tyrosine contribution to the overall fluorescence emission.

Frequency domain data were obtained with a frequency domain fluorometer operating between 2 and 200 MHz [19–22]. The modulated excitation was provided by the harmonic content of a laser pulse train with a repetition rate of 3.75 MHz and a pulse width of 5 ps, from synchronously pumped and cavity dumped rhodamine 6G dye laser. The dye laser was pumped with a mode-locked argon ion laser (Coherent, Innova 400). The dye laser output was frequency doubled to 295 nm for tryptophan excitation. For intensity decay measurements, magic angle polarizer orientations were used. The emitted light was observed through an interference filter at 340 nm. The frequency-domain intensity data were fit the time-resolved expression

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i} \quad (1)$$

where α_i are the pre-exponential factors, t , the decay times, and $\sum \alpha_i = 1.0$.

The frequency-domain anisotropy data were fit to

$$r(t) = r_0 \sum_i g_i e^{-t/\theta_i} \quad (2)$$

where $r_i = r_0 g_i$ is the amplitude of the component with the rotational correlation time θ_i . The parameters were recovered by non-linear least squares using the theory and software described elsewhere [23].

The standard deviation errors for phase and modulation were 0.3 and 0.005 degrees, respectively. The mean lifetime values were calculated according to the equation

$$\tau = \sum_i f_i \tau_i \quad (3)$$

where f_i is the fractional intensity of each lifetime component. The values of f_i are given as

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \quad (4)$$

RESULTS AND DISCUSSION

Figure 1 shows the fluorescence emission spectra of *ASCUT-1* in the absence and in the presence of 3% SDS, at 25°C and 85°C. Figure 1 also shows the normalized *ASCUT-1* fluorescence spectra at 25°C and 85°C both in the absence and in the presence of 3% SDS. The emission spectra of the protein in the absence and in the presence of the detergent at 25°C show a maximum at 330 and 337 nm, respectively. In both cases, the position of the emission maximum is blue-shifted with respect to the emission maximum of N-acetyltryptophanamide (NATA) which is centered at 352 nm [4], indicating that the tryptophan residue of *ASCUT-1* is embedded in buried and/or unrelaxed microenvironments [4]. Fluorescence emission spectra of *ASCUT-1* in the absence of SDS at 85°C resulted in a 7 nm red shift of the fluorescence emission maximum as well as in a marked quenching of the fluorescence intensity. On the other hand, only a 2 nm red shift of the emission maximum was observed for the protein in the presence of 3% SDS when the temperature was raised to 85°C. Moreover, the quenching of the fluorescence intensity of the protein in the presence of SDS is much less marked than in its absence. These data suggest that the protein structure of *ASCUT-1* is stabilized by the addition of the detergent. In our opinion this is conceivable since, at least in part, the stability of the protein structure, upon addition of SDS, may be due to the stabilizing effect of the detergent molecules binding the highly hydrophobic trans-membrane domain at the carboxy terminus of the protein.

In order to gain information on the *ASCUT-1* conformational dynamics, we investigated the protein fluorescence properties by frequency-domain fluorometry and anisotropy decays. The data were analyzed in terms of the multi-exponential model. Table I shows the multi-exponential analysis of *ASCUT-1* in the absence and in the presence of 3% SDS, in the range of temperature 25–85°C. Figure 2 shows the frequency-domain data of *ASCUT-1* in the absence and in the presence of 3% SDS. The best fits were obtained by using the two exponential model, characterized by chi-square values that were much lower than those obtained with a single decay time. Figure 3 shows the mean lifetimes of *ASCUT-1* in the absence and in the presence of 3% SDS and in temperature range from 25 to 85°C. The protein in the absence of the detergent shows a mean lifetime of 4.0 ns at 25°C. Increasing the temperature results in a strong decrease of the mean lifetime, which is 1.35 ns at 85°C. On the other hand, *ASCUT-1* in the presence of 3% SDS exhibits a mean lifetime of 3.0 ns at 25°C. Increasing the temperature up to 85°C, results in only a 1.2 ns shortening of the lifetime, which at 85°C is of 1.8 ns. These data suggest that *ASCUT-1* in the presence of 3% SDS at 25°C has a more dynamic yet protected conformation than in its absence.

In an attempt to visualize the conformational dynamics of *ASCUT-1* in the absence and in the presence of SDS we analyzed the protein fluorescence lifetimes by the distribution analysis [7–8]. The best fits were obtained from a bimodal distribution with Lorentian shape. It is our opinion that the interpretation of the emission decays in terms of continuous

distribution is more informative than that obtained by means of discrete components. The use of a lifetime distribution allows one to visualize the various decay time components and their amplitudes more readily than examination of the α_1 and τ_1 values from equation (1).

Figure 4 shows the *ASCUT-1* lifetime distributions at different temperatures in the absence and in the presence of 3% SDS. Table II shows the values of *ASCUT-1* fluorescence lifetime distribution analysis at 25°C in the absence and in the presence of 3% SDS. The fluorescence lifetimes in the absence of SDS appear distributed in two well distinct peaks, suggesting that *ASCUT-1* emissive properties arise from different tryptophanyl microenvironments during the lifetime of the excited state [4], which are subject to different degrees of tertiary constraints. At 25°C and in the absence of the detergent (Fig. 4), two components appear in the lifetime distribution: one centered at 0.65 ns and the other at 4.5 ns. The short component (0.65 ns) is very broad, showing a width of 2.5 ns. The long component (4.5 ns) is sharper, with width of 1.1 ns. Increasing the temperature to 45°C results in the shortening of both the distribution centers, with the short and the long component centered at 0.8 and 3.5 ns, respectively. Moreover, the width of the both components becomes narrower (0.5 ns and 0.07 ns, respectively), suggesting that the tryptophanyl microenvironment are more homogeneous and/or that the interconversion between different protein sub-conformational states is faster at 45°C than at 25°C. When the temperature is raised to 65°C the long component appears centered at 3.1 and the width of the short component becomes narrow (0.32 ns). Finally, at 85°C the centers of the both components are shortened, with centers of the short- and long-components at 0.2 and 1.0 ns, respectively. Moreover, the short component becomes very sharp (0.08 ns) while the width of the long component decreases to 0.27 ns.

In Fig. 4 is also shown the effect of SDS on the protein structure at different temperatures. The addition of SDS strongly affects *ASCUT-1* conformational dynamics. In particular, the *ASCUT-1* frequency-domain data in the presence of the detergent are well represented by an unimodal distribution with Lorentian shape. At 25°C (Fig. 4) the *ASCUT-1* lifetime distribution is centered at 1.5 ns and appears to be very broad, with a width of 1.7 ns. When the temperature is raised to 45°C the distribution width becomes narrower than at 25°C, while the center remains at 1.5 ns. Further temperature increases just resulted in the sharpening of the distribution width (the width 0.072 ns at 85°C), with the distribution center fixed at 1.5 ns up to 85°C. The shape and the center of the distribution suggest the stabilization of an *ASCUT-1* conformation by SDS addition. Moreover, the temperature increases do not result in any shift of the distribution center, suggesting that the presence of SDS increases the protein stability. These data are in good agreement with our previous investigation on the stability of *ASCUT-1* by using circular dichroism and infrared spectroscopy [17]. In particular, we showed that *ASCUT-1* protein structure was quite stable up to 85°C and that its stability was enhanced by the presence of 3% SDS [17].

The frequency-domain anisotropy decays of *ASCUT-1* in the absence and in the presence of 3% SDS at 25°C and 85°C are shown in Fig. 5 and Table III. We utilized the fixed values of the intrinsic tryptophan fluorescence from Table I in order to analyze the frequency-domain anisotropy decays. The best fits of anisotropy decay parameters were obtained using the two-correlation time model (equation 2), characterized by chi-square values that were much

lower than those obtained with a single correlation time. The short correlation time (520 ps at 25°C) can be associated with the local freedom of the tryptophanyl residue, as described in several studies of anisotropy decays on proteins [24,25]. The longer correlation time (22 ns at 25°C) can be associated to the overall rotation of the protein [24,25]. In the absence of SDS the two observed correlation times indicate both the local motions of *ASCUT-1* tryptophanyl residue and the overall rotation of the macromolecule. The addition of 3% SDS to the protein solution (at 25°C) results in a decrease of the overall rotational diffusion component r_{0g2} from 0.134 ns to 0.03 ns. This result suggests that the presence of the detergent induces a greater depolarizing contribution from either local motion and/or a greatly accelerated global motion of *ASCUT-1* (for the largest percentage of *ASCUT-1* that is now being averaged into the θ_1 parameter earlier ascribed to the local motion). In Fig. 5 are depicted the anisotropy decays of *ASCUT-1* in the absence and in the presence of 3% SDS, at 25°C and 85°C. From the differential phase data (upper panel in Fig. 5) we observe that at 25°C and in the absence of the detergent *ASCUT-1* shows a higher long-component differential phase value than in the presence of SDS. Temperature increases result in a very low differential phase value at 20 MHz both in the absence and in the presence of 3% SDS (Fig. 5). In the lower panels of Fig. 5 are shown the *ASCUT-1* modulated anisotropy values at 25°C and 85°C in the absence and in the presence of 3% SDS. The addition of SDS to the protein solution at 25°C causes a marked decrease in the *ASCUT-1* anisotropy. At high temperature we observe a further decrease of the anisotropy values both in the absence and in the presence of SDS. According to the Perrin-Webber equation [4] the ratio $(\theta_2 \text{ } ASCUT-1 \text{ no SDS})/(\theta_2 \text{ } ASCUT-1 + 3\% \text{ SDS})$ is proportional to the ratio $(ASCUT-1 \text{ Volume no SDS})/(ASCUT-1 \text{ Volume} + 3\% \text{ SDS})$. We calculated (from the values shown in Table III) that this ratio is 1.15 and 10 at 25°C and 65°C, respectively. In our opinion, the addition of SDS to the protein solution could prevent the formation of inter-molecular interactions between *ASCUT-1* molecules, thus resulting in a faster overall protein rotation. In fact, it is well known that the hydrophobic interactions among CUT-1 molecules play an important role in the assembly of the nematode cuticle [9] and the presence of SDS could avoid the formation of these hydrophobic interactions. Moreover, the presence of SDS could confer a large negative charge to the protein molecule and in turn prevents aggregation phenomena among *ASCUT-1* molecules.

Taken together, the lifetime and anisotropy decays data suggest that the protein retains its structural organization over a wide range of temperatures, and that the presence of SDS induces the stabilization of an *ASCUT-1* conformation. Moreover the detergent addition seems to stabilize the protein structure to a large extent, and to prevent the formation of intermolecular aggregation. The structural features of this protein may be correlated with different its natural functions, such as the mechanical resistance and elasticity of the extra-cellular matrices of the nematode cuticle. Finally, the unusual structural properties of this protein make it also interesting in terms of potential biotechnological application.

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Fig. 1. Steady-state fluorescence emission spectra of *ASCUT-1* in the absence and in the presence of 3% SDS at 25°C and 85°C. The excitation was at 295 nm and a 1.0 cm path length quartz cuvette was used. The absorbance of the protein solution was below 0.1 at the excitation wavelength. Protein concentration was 0.05 mg/ml.

Fig. 2. Frequency-dependence of the phase shift and demodulation factors of *ASCUT-1* fluorescence in the absence and in the presence of 3% SDS at the indicated temperatures. Excitation was at 295 nm and the absorbance of the protein solution was below 0.1 at the excitation wavelength. Protein concentration was 0.05 mg/ml.

Fig. 3. Effect of the temperature on the *ASCUT-1* fluorescence mean lifetime in the absence and in the presence of 3% SDS.

Fig. 4. Tryptophanyl-lifetime distribution pattern of *ASCUT-1* in the absence and in the presence of 3% SDS at the indicated temperatures.

Fig. 5. Anisotropy decays of *ASCUT-1* in the absence and in the presence of 3% SDS at the indicated temperatures. The differential phases and modulated anisotropies are shown in the upper and lower panels, respectively.

Table I.

Multi-Exponential Analysis of the Fluorescence Intensity Decay of *ASCUT-1* in the Absence and Presence of 3% SDS

<i>ASCUT-1</i>	τ (ns) ^a	α_1	τ_1 (ns)	α_2	τ_2 (ns)	χ^2_R
No SDS						
25°C	3.98	0.725	1.29	0.275	5.62	2.3
45°C	2.77	0.691	0.93	0.309	3.79	1.8
65°C	2.08	0.836	1.00	0.164	3.60	2.5
85°C	1.35	0.826	0.53	0.174	2.27	2.9
3% SDS						
25°C	2.96	0.890	1.58	0.110	5.93	2.3
45°C	2.30	0.905	1.49	0.095	4.72	2.5
65°C	1.96	0.950	1.50	0.050	4.76	1.8
85°C	1.82	0.994	1.65	0.006	5.47	1.7

^a τ = mean lifetime was calculated according to Eq. 3.

Table II.Fluorescence Lifetime Distribution of *ASCUT-I* in the Absence and Presence of 3% SDS at 25°C

<i>ASCUT-I</i>	τ_1 (ns)	τ_2 (ns)	hw ₁	hw ₂	χ^2_R
No SDS	0.65	4.5	2.5	1.1	1.1
+3% SDS	1.5	—	1.7	—	1.2

Table III.
Anisotropy Decay Analysis of *ASCUT-I* in the Absence and in the Presence of 3% SDS

T (°C)	<i>ASCUT-I</i>						<i>ASCUT-I</i> + 3% SDS					
	r_{0g1}	r_{0g2}	θ_1 (ns)	θ_2 (ns)	χ^2_R		r_{0g1}	r_{0g2}	θ_1 (ns)	θ_2 (ns)	χ^2_R	
25	0.156	0.134	0.52	22.1	1.0	0.278	0.03	0.36	19.2	0.8		
45	0.194	0.101	0.33	16.4	0.9	0.277	0.02	0.31	8.6	1.2		
65	0.220	0.077	0.28	12.0	0.9	0.254	0.01	0.26	1.2	1.0		
85	0.255	0.046	0.28	8.3	0.9	0.276	—	0.17	—	0.9		