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Lanthanide Ions Unfolded Im9

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Abstract. Colicins are plasmid encoded antibacterial proteins synthesised by bacteria to kill other bacteria. They have an N-terminal translocation domain, a central receptor recognition domain and a C-terminal cytotoxic domain. This study focused on lanthanide ion binding to the E9 cognate immunity protein (Im9), which neutralises the killing action of colicin E9 in its producing cell. The effects of most of the lanthanide ions on binding to these proteins were investigated. Changes in protein fluorescence emission and luminescence resonance energy transfer to Eu^{3+} and Tb^{3+}, which are the most strongly emitting lanthanide ions, were monitored by using luminescence spectroscopy. Denaturation of Im9 was revealed when lanthanide ion was added as indicated by their tryptophan fluorescence and luminescence resonance energy transfer from tryptophan to Eu^{3+} and Tb^{3+}.

Introduction

Colicins are a diverse family of plasmid-encoded antibacterial proteins[1]. Most of them are synthesised by *Escherichia coli* in the form of a non-covalent, heterodimeric complex with an immunity protein during times of nutrient or environmental stress, or in response to DNA-damaging agents as a means of reducing competition from other microbial populations[2-4]. Colicins have been classified into groups on the basis of the cell surface receptors to which they bind. The E group colicins, of which the colicin E9 studied in this paper belongs, all bind to the product of the *BtuB* gene, which is responsible for vitamin B_{12} transport in *E.coli* and whose crystal structure has been solved by Chimento 2003[5]. Following receptor binding, the toxin translocates into the cell and initiates cell death[6]. Each of these functions is performed by a

particular domain within the structure of the colicin[7]. Thus, the structure of colicins is divided into three domains: (i) translocation domain (N-terminal), (ii) receptor-binding domain (central region), and (iii) cytotoxic domain (C-terminal), (Fig. 1).

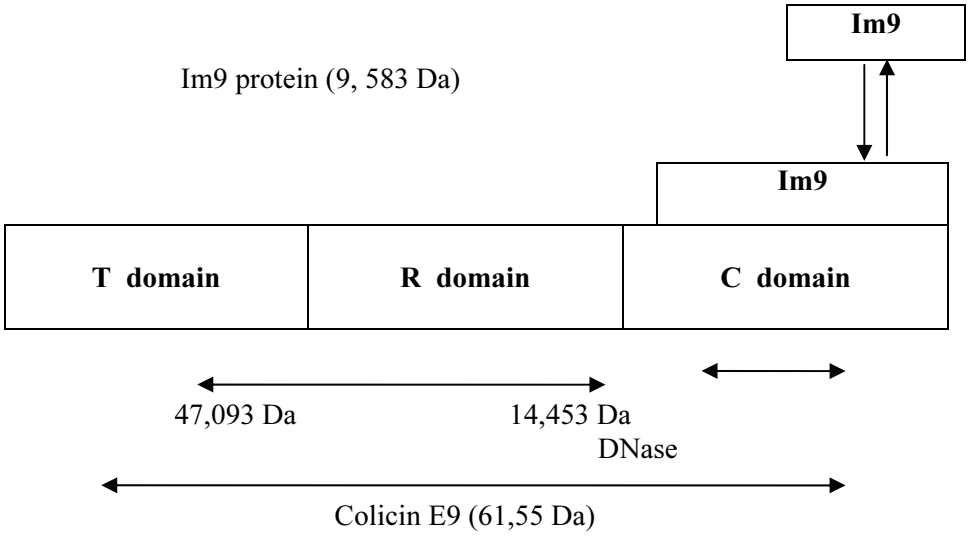

Fig. 1. The general three-domain structure of colicins. The N-terminal domain is associated with translocation of the colicin across membranes, the central region with receptor recognition, and the C-terminal domain with cytotoxic activity. The structure shown is based on the DNase colicin E9.

E group colicins have been subdivided into 9 types, ColE1–ColE9. Colicin E9 is a 60 kD endonuclease [8].

The metal ion in the E9 DNase-Im9 complex is Ni^{2+}[9], while in the related E7 DNase-Im7 complex, prepared by a different procedure, the metal ion was Zn^{2+}[9,10]. The Ni^{2+} of the E9 DNase-Im9 complex was coordinated by three histidine residues (102, 127 and 131) and a phosphate ion located at the putative DNA binding site[11,12]. By contrast, the Zn^{2+} of the E7 DNase-Im7 complex was coordinated by three histidine residues and water even though phosphate was present in the crystallising solution. Recent crystallographic studies for the E9 DNase-Im9 (Fig. 2)[13,14] and E7 DNase-Im7 complexes[15,16] have been reported.

DNase and RNase E group colicin producing organisms protect themselves against the action of the toxin by the coordinate synthesis of an acidic immunity protein that binds to the basic cytotoxic domain of the colicin and neutralises the bacteriocidal activity within the producing host[17].

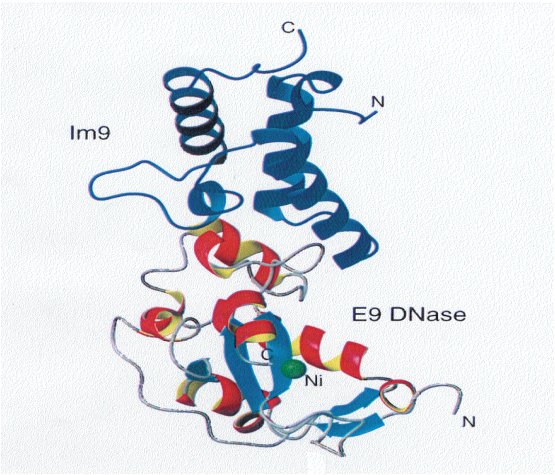

Fig. 2. Ribbon representation of the X-ray structure of colicin E9 DNase bound to Im9. The metal-binding site is shown in green produced from PDB file 1BXI using the program RASMOL[14].

Im9 is an eighty-six amino acid protein with a molecular weight of 9.5 kDa. The sequence identity in the family of immunity proteins is ~50%[18]. NMR has been used to determine the structure of both Im9-free (Fig. 3) and bound with DNase (Fig. 2)[19-21]. The aim of this work was to use this protein to develop a procedure for binding lanthanide ions, in order to employ lanthanide ions as spectroscopic probes of protein structure and intermolecular interactions.

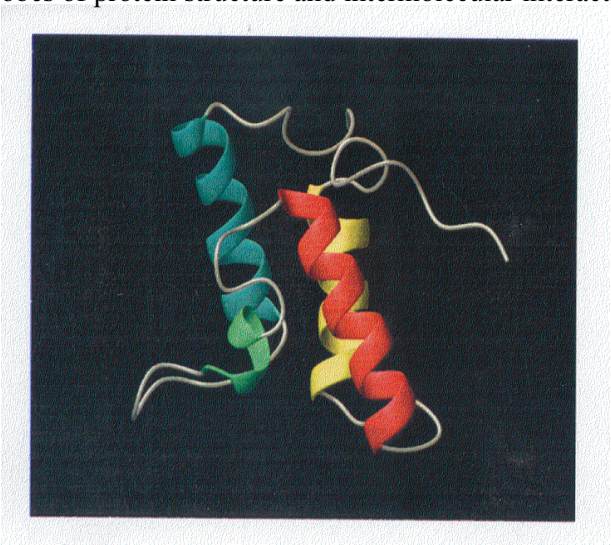

Fig. 3. Ribbon diagram representation of the structure of Im9. The four helices are colour coded, helix I is shown in yellow, helix II in red, helix III in green and helix IV in blue. Produced from PDB file 1IMQ using the program RASMOL[20].

Materials and Methods

Preparation of Lanthanide Solutions

Solutions of each of the lanthanide ions used for the fluorimetric studies were prepared with Analar water. Solid $LnCl_36H_2O$ obtained from Aldrich, were stored in desiccators before use. Stock solutions of 5 mM Ln^{3+} were prepared volumetrically by weighing out the calculated amount of hydrated metal chloride and then dissolved in the appropriate volume of Analar water. The stock solution was diluted to the desired concentrations. The actual concentrations of lanthanide ions were determined by titrations against standard solutions of 0.1 M ethylenediaminetetraacetic acid (EDTA) in the presence of xylenol orange (0.1% w/v) as an indicator. 100 mM sodium acetate buffer pH 5.0 (adjusted by concentrated acetic acid) was used as solute and the absorbance at 570 nm monitored through out the titrations[22].

Titration is carried as in literature[27]. A titration curve was plotted of the absorbance as a function of the number of moles EDTA added. Straight lines were drawn through the transition and post-transition areas of the curve. A point of intersection of lines is equal to the number of moles of EDTA required to bind all the metal.

Assuming 1:1 binding, the number of moles of EDTA added to take the titration to its end-point is equal to the number of moles of lanthanide ion present in the cuvette.

Protein-Lanthanide Titrations

Im9-Lanthanide Fluorescence Measurements

Measurement of the change of tryptophan fluorescence emission at 330 nm upon binding lanthanide ions (La^{3+}, Eu^{3+}, Tb^{3+}, Lu^{3+}) to Im9 was used, with each metal titration was repeated at least twice. A Perkin Elmer LS55 luminescence spectrometer was used for all luminescence experiments. Emission spectra were run over 300-500 nm, with an excitation wavelength of 295 nm to ensure only tryptophan excitation. The excitation and emission slits were set at 5 nm, and a scan speed of 300 nm/min was applied. 1 µl aliquots of 5 mM $LnCl_3$ were added to 2 ml of a 1 µM Im9 in 50 mM TEA buffer (pH 7.1) in an acid washed quartz cuvette, using a P2 Gilson pipette with the disposable tip. Microliter™ Hamilton syringes were also used for some experiments as these were generally more reliable than Gilson pipettes. However, after several titrations, the needles of syringes were contaminated by traces of lanthanide ion. As a result, aliquots contained a mixture of metals, giving Tb^{+3} emission in all experiments. Because of this error a P2 Gilson pipette was used with the

disposable tip. At the end of each titration, measurement of the pH of the solutions was recorded to ensure that no change of the original (pH 7.1).

Im9-Lanthanide CD Measurements

The ellipticity (θ) of Im9 CD spectra was measured on binding Eu^{3+}, the experiment was repeated twice. CD spectra of a 200 µl of the sample was recorded on an Applied Photophysics π^* spectropolarimeter with a path length of 1.0 mm. An average of 20 successive scans for each sample was collected using a computer connected to the spectropolarimeter. 1 µl aliquots of 5 mM $EuCl_3$ were added to 0.2 mg/ml Im9 (20 µM) in 50 mM TEA buffer (pH 7.1) in an acid washed quartz cuvette, using a P2 Gilson pipette with fine disposable tip.

Im9 Binding Ca^{2+} and Mg^{2+}

Tryptophan fluorescence emission monitored with Perkin Elmer LS55 luminescence spectrometer using the same parameters as in previous Im9-lanthanide fluorescence measurements to study Im9 binding Ca^{2+} and Mg^{2+}. 1 µl aliquots of 5 mM $CaCl_2$ or $MgCl_2$ were added to 2 ml of 1 µM Im9 in 50 mM TEA buffer (pH 7.1) in an acid washed quartz cuvette, using a P2 Gilson pipette with the disposable tip.

The Effect of Al^{3+} on Im9

A Perkin Elmer LS55 luminescence spectrometer was used to investigate Im9 binding Al^{3+}. 2 ml of 1 µM Im9 in 50 mM TEA buffer (pH 7.1) was pipette into an acid washed quartz cuvette and scanned using the same parameters as in previous Im9-lanthanide fluorescence measurements. Aliquots of 1 µl of 5 mM $AlCl_3$ was added using a P2 Gilson pipette with the disposable tip.

Results and Discussions

Fluorescence Studies of the Effect of Lanthanide Ions on Im9

The presence of a unique conserved tryptophan residue (W74) in Im9, which is highly quenched in the native state, makes this protein easily amenable to analysis by fluorescence spectroscopy. A consistent enhancement of tryptophan emission and a red-shift to a range of 353-360 nm with increasing lanthanide concentration (La^{3+}, Eu^{3+}, Tb^{3+} and Lu^{3+}) were observed in all experiments. Figure 4 shows one example of these titrations. Im9 precipitation was revealed at early stages of titration, around ~ 30-40 µM of the metal concentration.

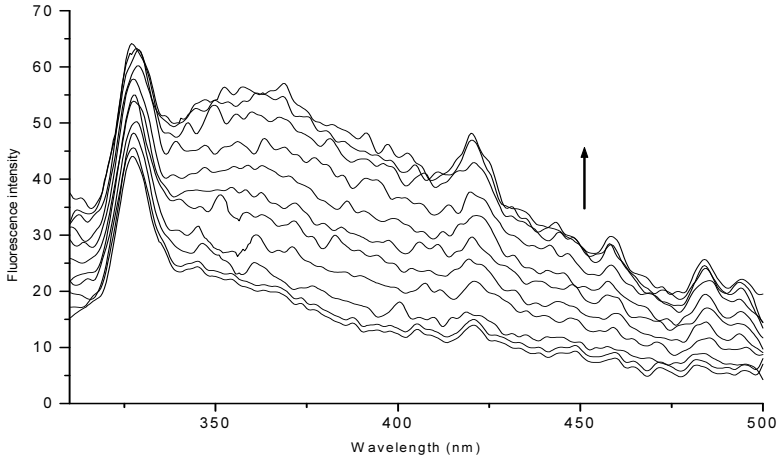

Fig. 4. Fluorescence measurement showing tryptophan emission change for 2ml of 1µM Im9 in 50 mM TEA buffer (pH 7.1) with aliquots of 5 mM $EuCl_3$.The arrow indicates the increasing amount of Eu^{3+} with successive traces corresponding to the addition of 1µl of Eu^{3+} solution up to 10µl, followed by the addition of 2µl of Eu^{3+} solutions.

A common feature of the spectra was the appearance of signals at 420, 458 and 485 nm in Fig. 4. Such signals increase in intensity as lanthanide concentrations increased. This probably is due to protein precipitation and light scattering. The band at 328 nm arises for tyrosine fluorescence. In proteins tyrosine fluorescence is often quenched or masked by tryptophan fluorescence[23]. However, in the folded Im9, the emission from tryptophan 74 is quenched[6], consequently the tyrosine fluorescence can be observed.

The enhancement of tryptophan fluorescence with the addition of lanthanide ions was not accompanied by a significant increase in Eu^{3+} fluorescence emission (Fig. 5), as occurred with these metal ions binding to other proteins, including colicin E9 DNase. Tb^{3+} and Eu^{3+} ions generally show luminescence emission at 545 and 615 nm, respectively, which can be enhanced when these ions are bound to a protein[24].

A plot of fluorescence intensity change as a function of lanthanide ions concentration (Fig. 6), revealed a marked increase in tryptophan fluorescence with increasing metal ion concentrations. This graph was similar to that described by other researchers for the denaturation of Im9 with urea[4,25-27].

To provide a reference point for the lanthanide induced effect, fluorescence studies of the denaturation of Im9 with addition of GdmCl, increasing temperature and decreasing pH were carried out.

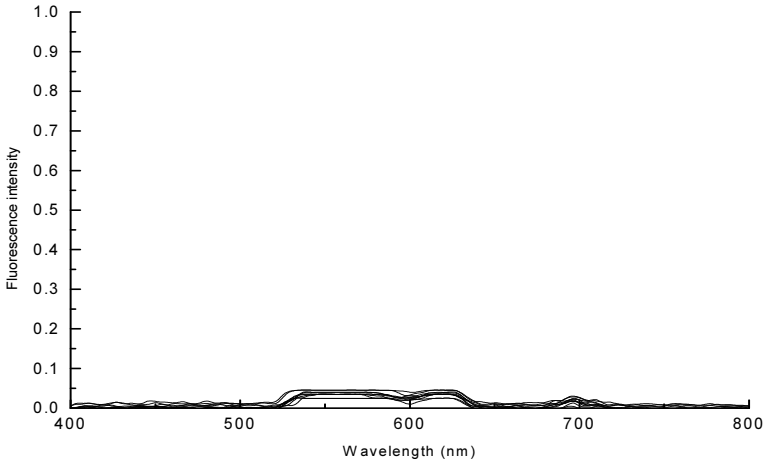

Fig. 5. Luminescence measurement showing a negligible Eu^{3+} emission signal due to Im9 binding.2ml of 1μM Im9 in 50mM TEA (pH 7.1) with aliquots of 5mM $EuCl_3$.

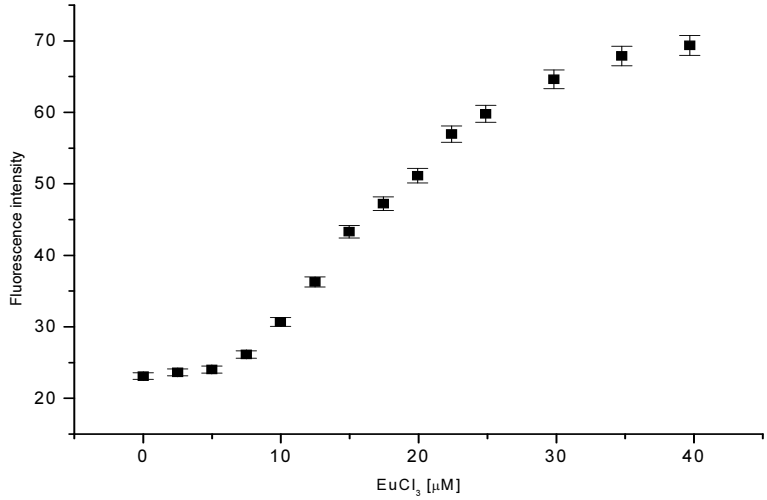

Fig. 6. Fluorescence intensities change of Im9 at 354nm as a function of $EuCl_3$ concentrations. Error bars refer to deviation of the spectrum from a smoothed line.

CD Study of the Effect of Lanthanide Ions on Im9

Investigation of the effect of Eu^{3+} on Im9 by circular dichroism (CD) spectroscopy was made to confirm that Im9 was indeed unfolding. CD is a powerful tool for determining secondary structure and for monitoring conformational changes in proteins[28]. Figure 7 showed the far-UV CD-spectrum (190-260 nm) of Im9 in 50 mM TEA buffer (pH 7.1) with addition aliquots of $EuCl_3$ stock solution. In the absence of Eu^{3+}, the spectrum showed a

strong double minimum at ~209 nm and 222 nm, and a stronger maximum at ~192 nm, which were characteristic peaks for α-helical protein. A consistent reduction in these peaks with increasing lanthanide concentration was observed. The plot of CD intensity change as a function of $EuCl_3$ concentrations (Fig. 8), revealed a similar graph that was obtained with fluorescence measurement for the same experiment.

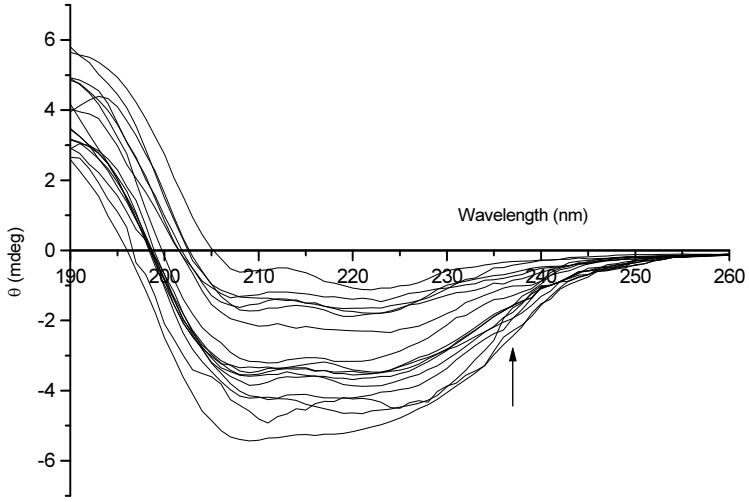

Fig. 7. CD measurement showing Im9 intensity changes for 0.2mg/ml of Im9 in 50 mM TEA buffer (pH 7.1) with increase aliquots of $EuCl_3$. The arrow indicates the increasing amount of Eu^{3+} concentrations.

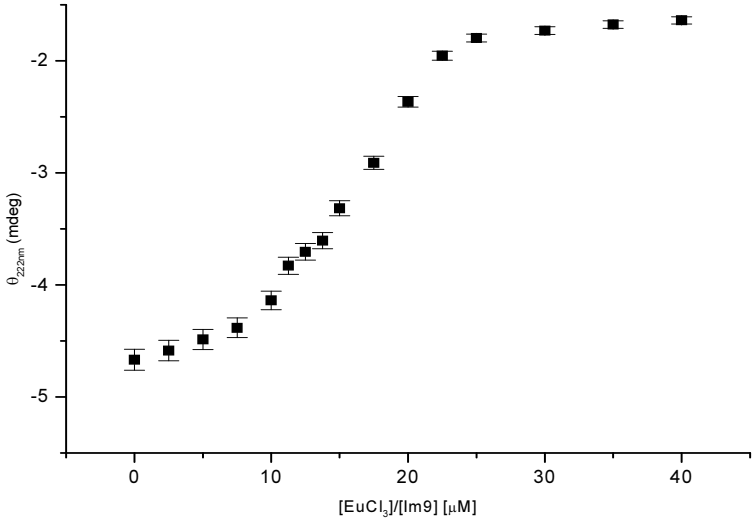

Fig. 8. CD intensities change of Im9 at 222nm as a function of $EuCl_3$ concentrations. Error bars refer to deviation of the spectrum from a smoothed line.

Lanthanide Ions Unfolded Im9

Investigation of lanthanide ion unfolded Im9 by luminescence and CD spectroscopy was made as follows:

Fluorescence Studies of Lanthanide Ions Unfolded Im9

A plot of fraction unfolded F_u as a function of lanthanide ion concentration (Fig. 9) revealed Im9 denaturation followed a two-state mechanism with increasing metal concentration. The values of F_u were calculated from each fluorescence measurements titrations, using Eequation (1).

$$F_u = (y_f - y) / (y_f - y_u) \qquad (1)$$

Where y is the observed fluorescence intensity, and y_f and y_u represent the values of y characteristic of the folded and unfolded states, respectively.

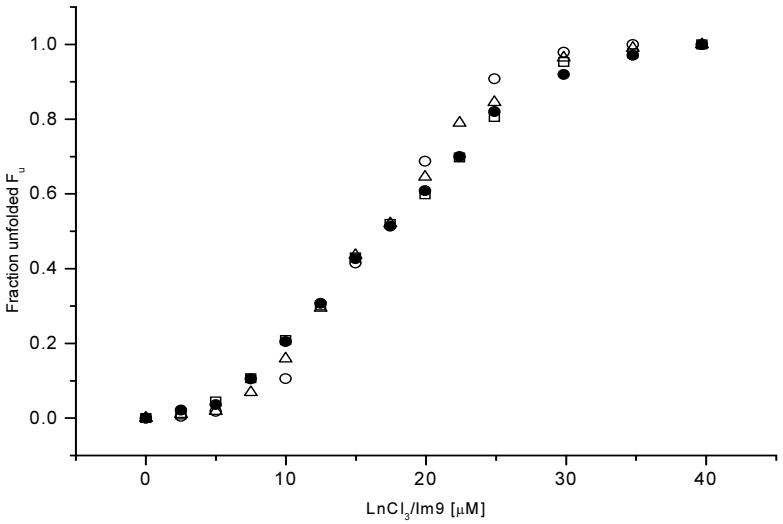

Fig. 9. Fraction unfolded (F_u) of Im9 at 354 nm as a function of lanthanide ion concentration. The values of F_u were calculated from equation [1]. The different lanthanide ions are La^{3+} (●), Eu^{3+} (o), Tb^{3+} (□) and Lu^{3+} (Δ).

Figure 10 showed that $\Delta G_{N\text{-}U}$ varies linearly with lanthanide ion concentrations in the transition region. For estimating the conformational stability in the absence of the denaturant, ΔG^{H2O}, it was assumed that this linear dependence continued to zero concentration[29], and a least-squares analysis used to fit data to the Equation (2);[30]

$$F_u = \frac{\exp\{m([D] - D_{½}) / RT\}}{1 + \exp\{m([D] - D_{½}) / RT\}} \qquad (2)$$

Where R is the gas constant (1.987cal mol^{-1} K^{-1}) and T is the absolute temperature in Kelvin. m is a constant that is proportional to the increase in the degree of exposure of the protein on denaturation[31], and $[D]_{½}$, which measures the midpoint of the transition.

The values of, $D_{½}$, m and ΔG^{H2O} obtained with the four lanthanide ions (La^{3+}, Eu^{3+}, Tb^{3+} and Lu^{3+}) were almost similar, which indicated the same effect of different lanthanide ions (see Table1).

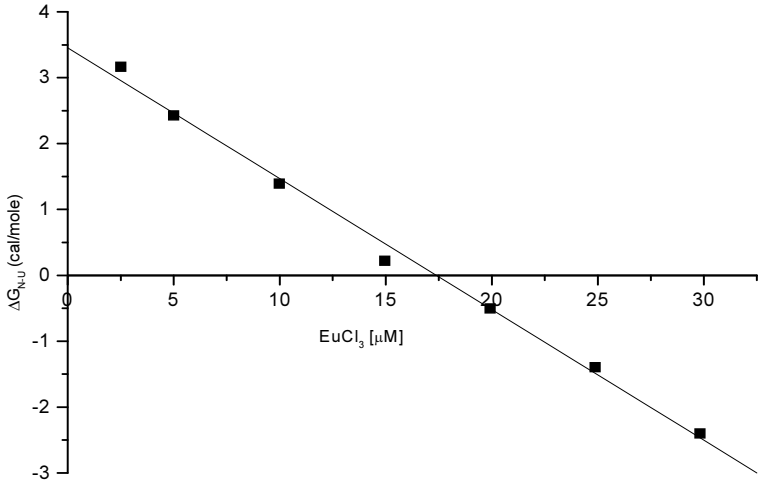

Fig. 10. Plot of ΔG_{N-U} against $EuCl_3$ concentration for Im9. ΔG_{N-U} was calculated from the data in Figure 9 using equation [3]. Line shows extrapolation to ΔG^{H2O} using least squares analysis, according to equation [2] with m= 0.18 kcal/mole/μM, ΔG^{H2O}= 3.45 kcal/mole, R=0.9973 and SD=0.163.

The values of, $D_{½}$, m and ΔG^{H2O} obtained with the four lanthanide ions (La^{3+}, Eu^{3+}, Tb^{3+} and Lu^{3+}) were almost similar, which indicated the same effect of different lanthanide ions (see Table1).

CD Study of Lanthanide Ion Unfolded Im9

F_u at 222 nm was calculated from the data in Fig. 9 using Equation (3)]

$$\Delta G_{N-U} = -RT \ln K \qquad (3)$$

F_u plotted as a function of lanthanide ion to protein concentration ratios. Figure 11 revealed that Im9 denaturation unfolded by a two-state mechanism with increasing $EuCl_3$ concentrations. Comparison between the fluorescence and CD results for Im9 equilibrium denaturation curves with increasing $EuCl_3$

concentration is presented in Fig. 11. The values of $D_{½}$ and m for Im9 denaturation by $EuCl_3$ were similar in both techniques, ($D_{½}$ ≈16.5 and m ≈0.19 kcal/mol).

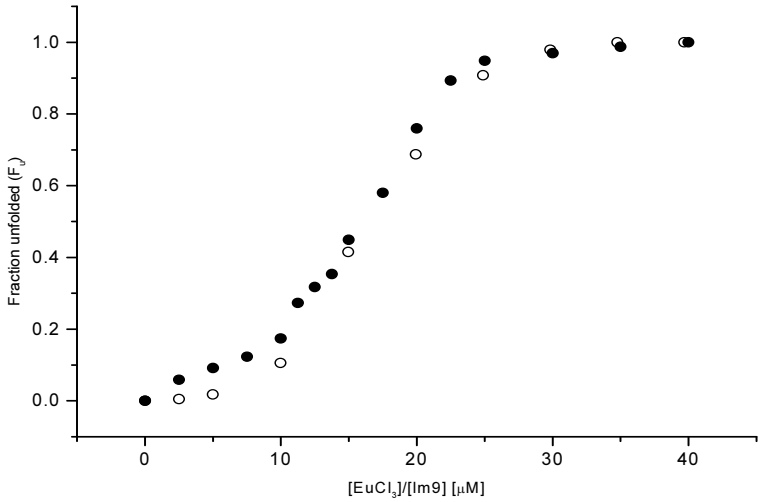

Fig. 11. Fraction unfolded (F_u) of Im9 as a function of $EuCl_3$ concentrations. The values of F_u were calculated from equation [1]. Filled circles are the CD data, and open circles are the fluorescence data.

Table 1. Thermodynamic parameters of Im9 denaturation with lanthanide ions.

Ln^{3+}	$D_{½}$ μM	m kcal/mole/μM	ΔG^{H2O} kcal/mole
La^{3+}	16.1 ±2.2	0.1 ±0.01	3.0 ±0.2
Eu^{3+}	Fluo. 16.9 ±1.2 CD 16.55 ±1.4	Fluo. 0.18 ±0.03 CD 0.19 ±0.01	Fluo. 3.5 ±0.12 ---------
Tb^{3+}	16.7 ±1.4	0.11 ±0.011	3.07 ±0.15
Lu^{3+}	16.6 ±0.5	0.12 ±0.01	3.0 ±0.15

Im9 Binding Ca^{2+} and Mg^{2+}

Following the tryptophan emission for Im9 with increasing $CaCl_2$ and $MgCl_2$ concentrations by fluorescence measurements showed slight or no change in tryptophan emission with both metals (Fig. 12 & 13).

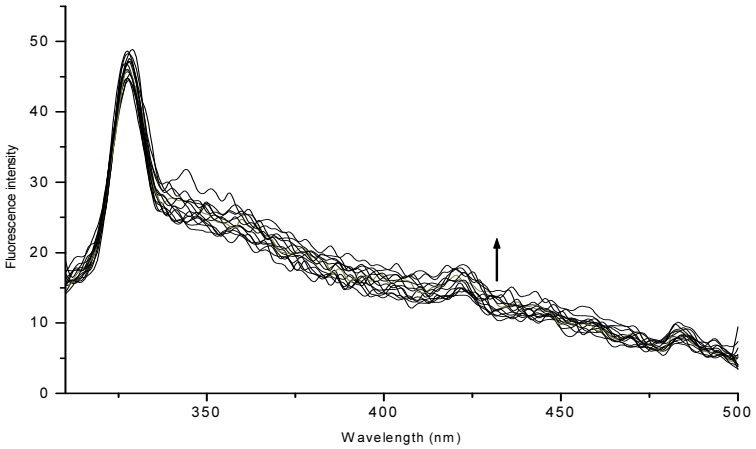

Fig. 12. Fluorescence measurements showing tryptophan emission changes for 2ml of 1µM Im9 in 50 mM TEA buffer (pH 7.1) with increasing aliquots of 5mM $CaCl_2$. The arrow indicates the increasing of $CaCl_2$ concentrations.

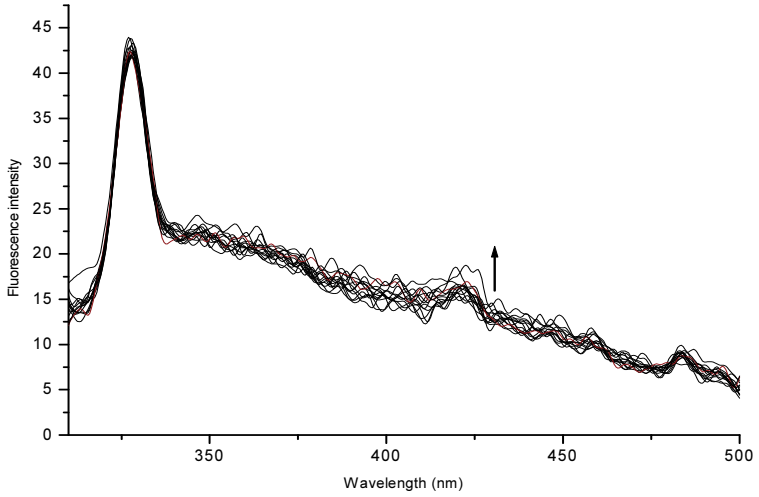

Fig. 13. Fluorescence measurements showing tryptophan emission changes for 2ml of 1µM Im9 in 50 mM TEA buffer (pH 7.1) with increasing aliquots of 5mM $MgCl_2$. The arrow indicates the increasing of $MgCl_2$ concentrations.

Those results could be a consequence of Ca^{2+} or Mg^{2+} ion binding being abolished or the binding was not being accompanied by a conformational changes.

The Effect of Al^{3+} on Im9

Figure 14 showed the Im9 fluorescence changes in the emission spectra with increasing aliquots of Al^{3+}. A consistent enhancement of tryptophan emission and a red-shift to 354 nm with increasing $AlCl_3$ concentration were observed. Im9 precipitation was detected at ~75-85 μM of metal concentrations.

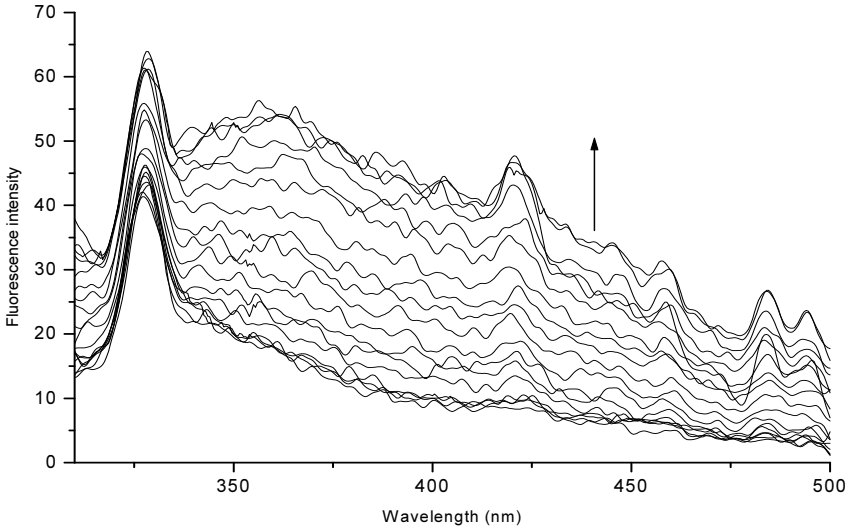

Fig. 14. Fluorescence measurements showing tryptophan emission changes for 2ml of 1μM Im9 in 50 mM TEA buffer (pH 7.1) with aliquots of 5 mM $AlCl_3$. The arrow indicates the increasing amount of Al^{3+} with the successive traces corresponding to the addition of 1μl of Al^{3+} solution up to 10μl, followed by the addition of 2 μl and then 5 μl of Al^{3+} solutions.

By plotting the fraction unfolded as a function of $[Al^{3+}]$ for the fluorescence measurements (Fig. 15), revealed that Im9 unfolding arises with the increasing of aluminium concentrations. The midpoint $D_{½}$ of the unfolding Im9, where 50% unfolded protein was 30.5 ±1.8 μM and m = 0.1 ±0.04 kcal/mole.

For estimating ΔG^{H2O} of Im9 in the absence of the denaturant, $\Delta G_{N\text{-}U}$ values were plotted as a function of $AlCl_3$ concentrations. Figure 16 showed the same value of ΔG^{H2O} which was obtained from the unfolding with $LnCl_3$.

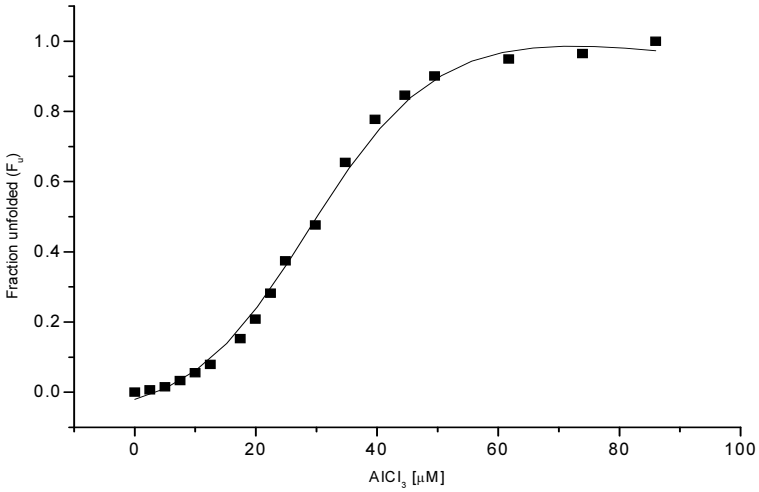

Fig. 15. Fraction unfolded (F_u) of Im9 as afunction of $AlCl_3$ concentrations. The values of F_u were calculated from equation [1]. Solid line shows curve fit according to equation [2.8] with $D_{½}$ = 30.5 ± 1.8 μM and m = 0.1 ± 0.04 kcal/mole/μM

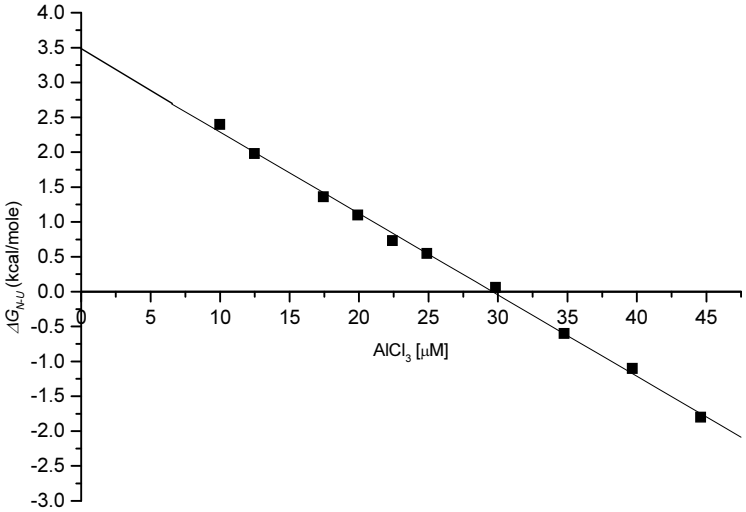

Fig. 16. Plot of ΔG_{N-U} against $AlCl_3$ concentrations for Im9. ΔG_{N-U} was calculated from the data in Figure 15 using equation [3]. Line shows extrapolation to ΔG^{H2O} using least squares analysis, according to equation [2] with m= 0.11 kcal/mole/μM, ΔG^{H2O}= 3.45 kcal/mole, R=0.9986 and SD=0.0745.

Although protein denaturation induced by lanthanide ions has not previously been reported, protein denaturation induced by other inorganic salts has been reported by Tanford[31,32]. Im9 unfolding was confirmed by a consistent increase in the measurable intensities of signals diagnostic of unfolded Im9 with the increasing lanthanide ion concentrations, accompanying with negligible changes in Eu^{3+} or Tb^{3+} fluorescence emissions. In addition, intensity changes plotting as a function of lanthanide ion concentrations (Fig. 6) were found to be similar to the equilibrium denaturation graph of Im9 with GdmCl or urea made by myself and other researchers[26,27]. Comparing the Im9-unfolding caused by lanthanide ion addition with the Im9-unfolding by other denaturation methods showed that both unfolding methods gave a similar ΔG^{H2O} value ~3.5 kcal/mol. The value of ΔG^{H2O} obtained from different denaturants for the same protein is generally similar[33]. The m values of GdmCl and lanthanide ion denaturation of Im9 were found to be different. While the higher m value was found with GdmCl, which reflect an increase in the surface area exposed in the denatured state, a lower m value was found with Im9 denatured by a small lanthanide ions concentration, relative to GdmCl concentration.

In order to ensure specificity of the unfolding effect of lanthanide ions on Im9, investigations of binding Ca^{2+} and Mg^{2+} with the Im9 were carried out. Eu^{3+} binding with other protein (*e.g.* lysozyme) was also investigated as a control (data not shown). It has been found that unfolding effect can only be achieved by lanthanide ion interaction with Im9. This was confirmed by the negligible tryptophan emission change on addition of Ca^{2+} and Mg^{2+} on Im9, as well as, the addition of Eu^{3+} ion to lysozyme.

The effectiveness of salts in precipitating serum globulins has been investigated by Hofmeister, over 100 years ago. Protein unfolding occurs when the balance of forces between the protein interaction either with itself or with its environment is disrupted. The disruption of this balance of forces may be as simple as a perturbation of the normal water structure around the protein.

The Hofmeister series has been ordered according to the effect of ions on water structure[34], which is;

$$NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} > Al^{3+} > \text{guanidinum hydrochloride}$$

The ions in this series before Na^+ are known as water-structure makers (exhibiting weaker interactions with water than water with itself) and they stabilise proteins. The ions after Na^+ are known as water-structure breakers (exhibiting stronger interactions with water molecules than water with itself) and they destabilise proteins. Na^+ has little effect on water structure or in protein stability[35].

In order to incorporate the Ln^{3+} ions within the Hofmeister series, the effect of Al^{3+} on Im9 was investigated. This showed that the Im9 unfolding arises with increasing aluminium concentration. The equilibrium thermodynamic parameters for Im9 unfolding by addition of Al^{3+} ions were midpoint $D_{½}$ (50% unfolded protein) = 30.5 ±1.8 μM, m = 0.1 ±0.04 kcal/mole and ΔG^{H2O} = 3.45 ±0.06 kcal/mole. In contrast, the corresponding thermodynamic parameters of Im9 unfolding by lanthanide ion were $D_{½}$ = 16.5 ±1.3 μM, m = 0.12 ±0.02 kcal/mole and ΔG^{H2O} = 3.4 ±0.1 kcal/mole. The difference between the $D_{½}$ value and the similarity in ΔG^{H2O} value strongly suggested that Ln^{3+} ions should be placed within the Hofmeister series between Al^{3+} and guanidinium hydrochloride.

Conclusion

Fluorescence and CD measurements showed that Im9 unfolding arises with the increasing lanthanide ion concentrations. In summary; two conclusions can nevertheless be drawn from experiments. Firstly, Im9-unfolding with lanthanide ions is likely to result from an interaction between lanthanide ion and negative patch of Im9 surface, thereby decreasing the stability of the folded state. Following denaturation, protein aggregation occurred, with increasing lanthanide ion concentrations. An alternative binding model suggested that Im9 could exist in a competitive equilibrium between the folded state with its interior hydrophobic core and the unfolded state with a high density of water surrounding both polar and non-polar groups. In this scheme the presence of metal ions, which will be present as metal-aqueous complexes of the type $[M(H_2O)_n]^{m+}$ ion could lead to a shift in the equilibrium to form unfolding state.

Finally, results suggested that Ln^{3+} ions should be placed within the Hofmeister series between Al^{3+} and guanidinium hydrochloride.

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انهيار طي انثناءات بروتين المناعة بواسطة أيونات اللانثانيد

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المستخلص. هدف هذه الدراسة هو استعمال بروتين المناعة (Im9) لإنشاء بروتوكول معين لربط أيونات اللانثانيد به، وذلك لتوظيف أيونات اللانثانيد كتحقيق مطيافي لدراسة تركيب البروتين، ودراسة التفاعلات داخل الجزئي. الكولسين هو بلازما البروتين المشفر ضد البكتريا، وهو يخلق بواسطة بكتريا معينه لقتل بكتريا أخرى. وهذا البروتين يحتوي على ثلاثة أجزاء: حقل انتقالي بطرف نتروجيني، وحقل معرف فيه المستقبل الممركز، وحقل سام للخلايا بطرف كربوني. وتركز هذه الدراسة على ربط أيونات اللانثانيد ببروتين المناعة (Im9) المرادف لبروتين (E9), والذي يعادل عملية القتل لبروتين الكولسين (E9) في الخلية المنتجة له. ولقد تم فحص تأثير ربط معظم أيونات اللانثانيد مع هذا البروتين، وباستعمال مطياف التألق ثم مراقبة التغير للانبثاق الاستشعاعي الصادر من الحمض الأميني التربتوفان والموجود في البروتين، وكذلك الطاقه الرنينية المتألقة المنقولة من البروتين إلى أيون الاربيوم (Er^{3+}) والايتربيوم (Tb^{3+})، واللذان يعتبران أقوى انبثاقاً استشعاعيا من بين جميع أيونات اللانثانيد. وقد أظهرت النتائج تحلل البروتين (Im9) عند إضافة أيونات اللانثانيد.