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SHORT COMMUNICATION

Evaluating protein:protein complex formation using synchrotron radiation circular dichroism spectroscopy

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ABSTRACT

Circular dichroism (CD) spectroscopy beamlines at synchrotrons produce dramatically higher light flux than conventional CD instruments. This property of synchrotron radiation circular dichroism (SRCD) results in improved signal-to-noise ratios and allows data collection to lower wavelengths, characteristics that have led to the development of novel SRCD applications. Here we describe the use of SRCD to study protein complex formation, specifically evaluating the complex formed between carboxypeptidase A and its protein inhibitor latexin. Crystal structure analyses of this complex and the individual proteins reveal only minor changes in secondary structure of either protein upon complex formation (i.e., it involves only rigid body interactions). Conventional CD spectroscopy reports on changes in secondary structure and would therefore not be expected to be sensitive to such interactions. However, in this study we have shown that SRCD can identify differences in the vacuum ultraviolet CD spectra that are significant and attributable to complex formation.

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Key words: synchrotron radiation circular dichroism spectroscopy; latexin; carboxypeptidase A; protein–protein interactions; secondary structure; complex formation; new methods.

INTRODUCTION

Circular dichroism (CD) spectroscopy measures the differential absorption of right and left handed circularly polarised light by chiral samples. When applied to proteins, conventional CD is measured in the far UV range (~250–190 nm) and reports primarily on the transitions of backbone amide groups, which give rise to characteristic spectral shapes for different types of protein secondary structure elements. The technique has been widely used for many years to demonstrate that a protein is folded[1] and to empirically assign percentages of secondary structure by comparison with reference datasets from proteins with known three-dimensional structures.[2,3] Protein CD spectra in the vacuum ultraviolet (VUV) range (<190 nm) have been less well characterised due to limitations in conventional CD instrumentation (low light flux from xenon arc lamps at low wavelengths) and strong absorption of buffer components in this region.[4] However, the high intensity and broad wavelength range of synchrotron radiation[5] can be used to measure CD, resulting in greatly improved signal-to-noise ratios and allowing the

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measurement of spectra well into the VUV range. These advantages of synchrotron radiation circular dichroism (SRCD), as well as rapid scan times and fewer restrictions on buffer requirements have enabled the development of several novel applications for SRCD in structural biology in recent years.[6] By extending spectra into the VUV range, SRCD increases the information content of the spectrum and permits more precise and detailed secondary structure analyses. Furthermore, the increased information content in the low wavelength region may make it possible to detect tertiary and quaternary structural changes in proteins.[6] To determine if this is possible, in this study, we examined if SRCD could be used to analyse a rigid-body protein/protein interaction that does not involve a net change in secondary structure, but does produce new quaternary interactions between polypeptide chains upon complex formation. To do so we have compared the SRCD spectra of the protease enzyme carboxypeptidase A and its protein inhibitor latexin with the spectrum of a complex of these two proteins. This system is ideal for such studies since the crystal structures of the both the isolated proteins and the complex are available, and show no significant changes in secondary structure in the complex relative to the isolated proteins. We have then investigated a number of features of the intermolecular interactions as potential sources of the spectra differences detected in the VUV region, including aromatic residue exposure, aromatic–aromatic interactions, and changes in rigidity of the interface residues.

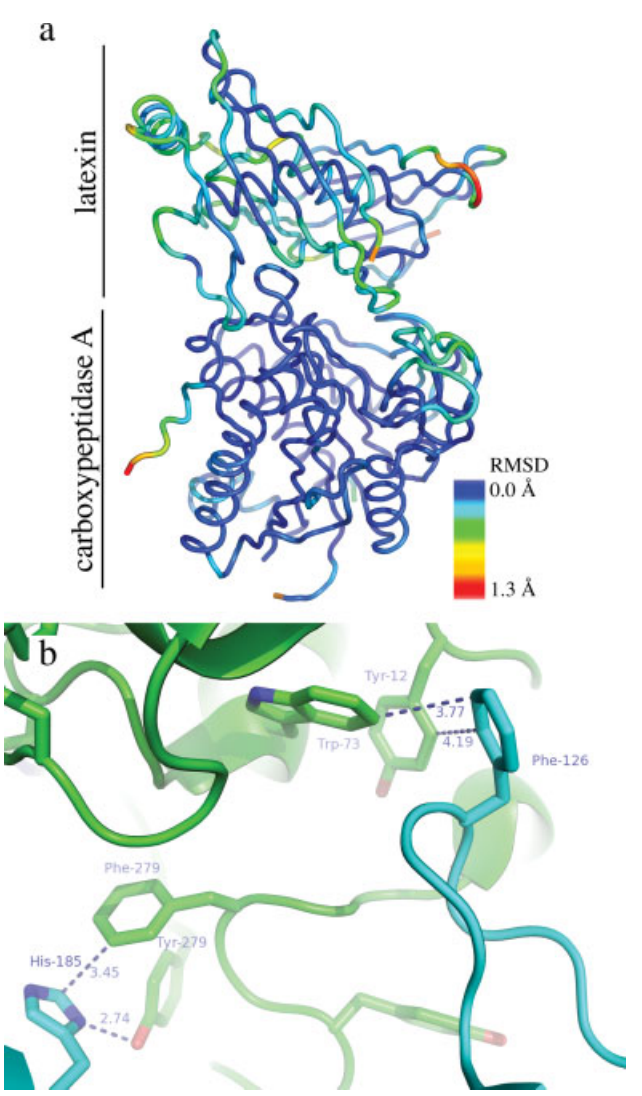

Figure 1

*(**a**) Crystal structure of the complex of human latexin and human carboxypeptidase A4 (2BO9) coloured according to rms deviations between the complex and uncomplexed murine latexin (1WNH) and human carboxypeptidase A4 (2BOA) proteins. To generate the figure the rmsd values were written to the B factor column of the pdb file of the CPA4:latexin complex crystal structure and a backbone trace generated and coloured in a spectrum from low (blue) to high (red) with the program Pymol.[8] (**b**) Interactions between aromatic residues at the interface between carboxypeptidase A and latexin (2BO9). The proteins are shown in a cartoon representation. Latexin is coloured blue and carboxypeptidase is coloured green. Aromatic side chains (Phe, Tyr, Trp, His) are shown in stick representation with noncarbon atoms coloured by element. Distances between the nearest side chain atoms of neighbouring aromatic residues are shown. The figures were prepared using the program Pymol.[8]*

EXPERIMENTAL

Analysis of protein structures

Crystal structures of the complex of human latexin and human carboxypeptidase A4 (2BO9) and uncomplexed murine latexin (1WNH) and human carboxypeptidase A4 (2BOA) proteins were superimposed using the ccp4 program lsqkab.[7] The root mean square deviations (rmsds) for latexin and carboxypeptidase A4 are 0.53 Å and 0.36 Å, respectively. To generate Figure 1(a) the rmsd values were written to the B factor column of the PDB file of the CPA4:latexin complex crystal structure and a backbone trace generated and coloured in a spectrum from low (blue) to high (red) rmsd values with the program Pymol.[8] Secondary structure compositions were calculated from the structures using the DSSP algorithm.[3] The surface area of the aromatic side chains buried upon complex formation was calculated using the CCP4 program "Surface."[7]

Protein samples

Cloning, recombinant expression in *E. coli* and purification of murine latexin are as described in articles by Aagaard *et al.*[9] and Cowieson *et al.*[10] Bovine carboxypeptidase A (Sigma-Aldrich C 0261) and the latexin:carboxypeptidase A complex were prepared as described in Mouradov *et al.*[11] The 1:1 complex was isolated using gel filtration. Samples were dialysed into 50 m*M* Na Phosphate pH 7.4, 150 m*M* NaF using Slide-A-lyzer mini dialysis units [MW 10 K cutoff] (Pierce Biotechnology). Prior to data collection all samples were briefly centri-

fuged at 10,000*g* to remove any particulate matter. Concentrations were then determined using the absorbances of the denatured proteins in 6*M* guanidine hydrochloride at 280 nm.[12] The concentration of carboxypeptidase A was 2.1 mg/mL, and latexin and the complex were both 10 mg/mL.

CD spectroscopy

SRCD spectra were measured on beamline CD12 at the Synchrotron Radiation Source, Daresbury, UK. Following each beam fill the instrument was calibrated for spectral magnitude and ratio using camphorsulphonic acid (CSA).[13] The CSA concentration was determined from its UV absorption peak at 285 nm where $\varepsilon_{285} = 34.6\ M^{-1}\ \mathrm{cm}^{-1}$.[14] The instrument parameters were as follows: step size 1 nm, slit width 0.1 nm, dwell time 1 s, and temperature 4°C. Spectra were measured over the wavelength range from 280 to 170 nm.

Conventional CD spectra were measured with a Jasco J-810 instrument over the wavelength range from 260 and 182 nm. Reported spectra have a 1 nm interval, and were measured with a 1-nm slit width, 1.2-s dwell time and temperature 20°C.

For both SRCD and cCD spectra three scans of both the sample and the baseline (containing the buffer alone) were averaged and subtracted and smoothed with a third order Savitsky–Golay algorithm[15] using CDTool processing software.[16] For comparability, SRCD and cCD samples were measured using the same protein concentrations and the same cell pathlengths. Carboxypeptidase A and the complex were measured in a 21.8 μm and latexin in a 15 μm quartz cell (Hellma). Spectra are expressed in delta epsilon units, calculated using mean residue weights of 114.7 Da, 112.5 Da, and 113.5 Da, respectively, for latexin, carboxypeptidase, and the complex. The calculated mixed spectrum of a 1:1 complex was produced by the following equation to take into account the differences in numbers of peptide bonds in the two proteins: $[(\Delta\varepsilon_L \times N_L) + (\Delta\varepsilon_C \times N_C)]/(N_L + N_C)$, where the subscripts L and C indicate latexin and carboxypeptidase, respectively, and N is the number of peptide bonds (number of amino acids minus one) in each of the proteins.

RESULTS

Comparison of crystal structures

Carboxypeptidase A and its protein inhibitor latexin form a tight complex with a 1:1 stoichiometry. The structures of the two proteins individually and in complex have recently been solved by X-ray crystallography.[9, 17, 18] Superposition of the structures of the two individual proteins onto the complex reveals that the interaction is largely rigid [the rmsd of backbone atoms is less than 0.5 Å, Fig. 1(a)]. The rmsds for latexin and carboxypeptidase A4 are 0.53 Å and 0.36 Å, respectively. The interaction of the two proteins results in only a small proportion (~0.5% calculated from analysis of the structures using DSSP[19]) of the residues changing from a disordered to an ordered secondary structure. The small changes that are observed do not cluster around the protein interface and therefore are more likely to be a result of the differences in crystallisation conditions and packing than the protein:protein interaction.

The same is true for the crystallographic temperature factors, which are indicators of the mobility of regions of the molecules. While temperature factors in the latexin/carboxypeptidase A complex (2BO9) are on average lower than temperature factors in the two individual protein structures (2BOA and 1WNH), they are not relatively lower around the interface than in the rest of the complex (data not shown). Thus it seems unlikely that there are major changes in mobility of backbone atoms upon complex formation. Additionally, changes in the exposure and interactions of aromatic amino acids upon complex formation were investigated. Several aromatic amino acid side chains become buried upon complex formation (from carboxypeptidase A: Tyr 12, Trp 73, Tyr 198, Tyr 248, Tyr 277, and Phe 279; from latexin: Tyr 8, Phe 126, Trp 141, Trp 157, and His 185). Also, several form close aromatic–aromatic interactions with residues from the other protein. Specifically, the terminal hydroxyl of carboxypeptidase A Tyr 198 forms a hydrogen bond (2.7 Å) with the ND1 nitrogen of latexin His 185. In addition, there are hydrophobic contacts between carboxypeptidase A Trp 73 and latexin Phe 126 (3.8 Å), carboxypeptidase A Tyr 248 and latexin Trp 157 (5.0 Å), and carboxypeptidase A Phe 279 and latexin His 185 (3.4 Å) [Fig. 1(b)].

Conventional CD spectroscopy

The spectra of latexin, carboxypeptidase A, and the latexin:carboxypeptidase A complex were measured using a conventional CD instrument and the calculated complex spectrum was produced as noted above [Fig. 2(a)]. Although all of the spectra are somewhat noisy (a circumstance that could have been improved by increasing the concentration or the number of scans) they were obtained in a comparable manner to that used for the SRCD spectra reported below, so that a fair comparison could be made of the cCD and SRCD results.

The overall shape of the calculated and measured cCD spectra of the complex are very similar. Limited light flux (as measured by the HT voltage readings) at low wavelength means that valid data could not be measured below 182 nm, and that the error bars are very large in the wavelength region below 205 nm. Although the observed cCD spectrum is slightly less intense than the calculated cCD spectrum, because of the large error bars

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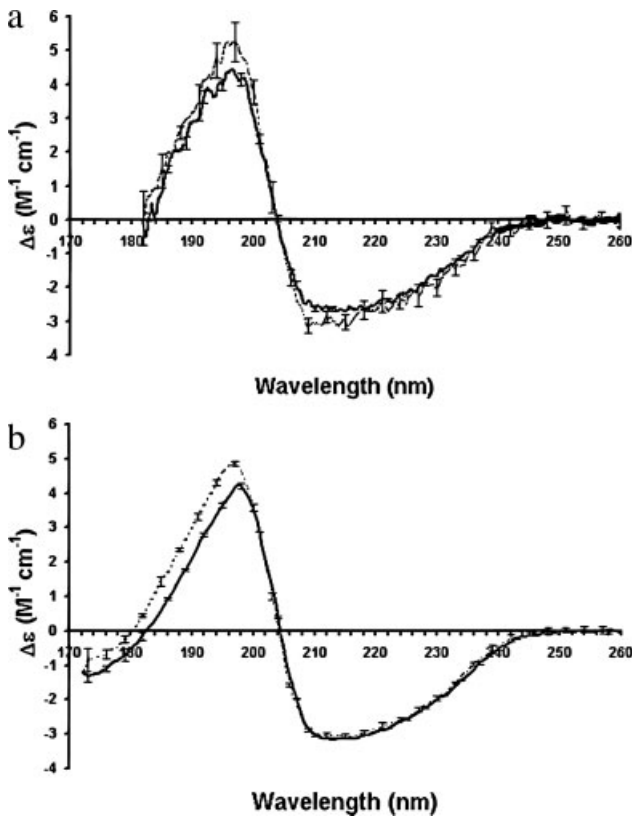

Figure 2

*(**a**) Conventional circular dichroism and (**b**) SRCD spectra of the complex between murine latexin and bovine carboxypeptidase A. The observed spectrum of the complex is indicated by the solid line, and the calculated spectrum derived from the spectra of the two proteins measured in isolation is indicated by a broken line. Error bars plotted on the spectra correspond to one standard deviation of the measurements.*

in the measurements, these differences are not statistically significant. Hence, from this data it would not be possible to state that complex formation was observed.

Synchrotron circular dichroism spectroscopy

The SRCD spectra of carboxypeptidase A, latexin, and the complex formed between these two proteins [Fig. 2(b)] were measured and the calculated complex spectrum was produced as above. The calculated and measured complex spectra in the wavelength range above 200 nm were nearly identical (and the small error bars support this conclusion). This suggests, as seen in the crystal structures, there is essentially no change in the secondary structure upon complex formation. However, significant changes are seen in the low wavelength data (<200 nm) that can be attributable to complex formation. There is a slight red shift of the peak around 197 nm, a significant decrease in the magnitude of this peak, and either a narrowing of this peak or the disappearance of a shoulder at lower wavelengths. Thus the SRCD detects some intermolecular interactions that do not involve secondary structural changes.

DISCUSSION

The nature of the complex formed between carboxypeptidase A and its tissue inhibitor latexin is well defined by X-ray crystal structures of these proteins on their own and in complex.[9, 17] The crystal structures suggest that interaction between the two proteins is a rigid body event without the creation of new secondary structure elements or ordering of disordered residues[18] in the ordered environment of the crystal. In this study, we demonstrate this is also the case in solution by comparison of the SRCD spectrum of the complex in the wavelength range from 200 to 260 nm with the summed spectra of the two proteins measured separately. However, a significant difference is observed between the measured and calculated SRCD spectra at wavelengths below 200 nm. Thus, in the case of latexin and carboxypeptidase A, SRCD simultaneously demonstrates the presence of a protein–protein interaction, by spectral changes at low wavelength, as well as providing information about the rigid-body nature of the interaction, indicated by the overlay at higher wavelengths.

The SRCD data obtained under comparable sample conditions to that used to obtain the cCD spectra have significantly better signal-to-noise levels, enabling detection of spectral differences with higher certainty. In this system, no secondary structure changes occur upon complex formation. While it is not possible to confidently identify any significant spectral changes associated with complex formation from the cCD data, comparison of the calculated and observed SRCD spectra of the complex of latexin and carboxypeptidase A provides evidence that a complex has been formed and strongly suggests the VUV region of the CD spectrum is affected by factors other than secondary structure. Possible sources of these differences could be: burying of a number of aromatic residues, especially tyrosines,[20] that are surface-exposed in the individual proteins, new aromatic–aromatic interactions formed in the complex, decreased mobility of backbone atoms in the complex leading to lower dynamic sampling, or tertiary/quaternary interactions that involve either interactions of adjacent secondary structural elements in a way that modulates their spectral properties or involves charge transfer interactions between elements that become spatially adjacent in the complex.

Examination of the crystal complex suggests that all of the above, except the changes in mobility, are potential contributors to the differences seen. Changes in the surface accessibility of Tyr[20] and Phe (Evans, Slingsby, and Wallace, in preparation) residues and interactions can

cause significant alterations in the transition ~190 nm. Although we cannot definitively state which (or which combination) of these mechanisms is contributing, this study is the first demonstration that SRCD can provide a useful means of detecting complex formation, even in the absence of secondary structural changes.

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