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Small heat-shock proteins (sHSPs) are a family of evolutionary conserved ATP-independent chaperones. These proteins share a common architecture defined by a signature α-crystallin domain (ACD) flanked by highly variable N and C-terminal extensions. The ACD, which has an immunoglobulin-like fold, plays an important role in sHSP assembly, mediating dimer formation of individual protomers, that then may assemble into larger oligomers. In vertebrate sHSPs the dimer interface is mediated by the symmetrical antiparallel pairing of two β-strands (β7), resulting in the formation of an extended β-sheet on one face of the ACD dimer. Recent crystallographic studies of isolated ACDs from a number of vertebrate sHSPs suggest a variability in the register of the β7/β7 interface, which may, in part, give rise to the polydispersity often seen with the full-length proteins. To further analyse the structure of ACD dimers we have employed a combination of X-ray crystallography and solution small-angle X-ray scattering (SAXS) to study the ACD-containing fragments of human HSPB1 and HSPB6. Unexpectedly, the obtained crystal structure of the HSPB1 fragment does not reveal the typical β7/β7 dimers, but rather hexamers formed by an asymmetric contact between the β4 and β7 strands from adjacent ACDs [1,2]. Nevertheless, in solution, both ACDs form stable dimers via the symmetric antiparallel interaction of β7 strands. Using SAXS, we show that it is possible to discriminate between different putative registers of the β7/β7 interface, and that under physiological conditions there is only a single register of the strands for both proteins [2]. Furthermore, we have solved the crystal structure of a fragment of HSPB6 including a portion of its N-terminal extension, the ACD and the C-terminal extension. The structure reveals a stable tetramer, as also confirmed by the SAXS data in solution. The novel tetramer formation is possible due to specific 'patching' of the β4/β8 side of the ACDs by a short hydrophobic motif found in the N-terminal extension.

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Keywords: small heat-shock protein, crystallography, small-angle X-ray scattering

MS51.P08

Acta Cryst. (2011) A**67,** C552

Using raman and fluorescence spectroscopies in protein crystallography

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Structural biology increasingly relies on the application of complementary methods to the same protein crystals that are used during X-ray crystallography experiments. We present here a comprehensive ensemble of improved spectroscopic setups aimed at analysing nano-volume samples, such as protein crystals or small amounts of concentrated solutions, by UV/vis absorption, fluorescence or Raman spectroscopy. *In crystallo* spectroscopy allows to verify the state of protein crystals in comparison to its solution state and, in combination with crystallographic data, to correlate structure and function. These techniques can be directly carried out on synchrotron beamlines, yielding immediate complementary information during X-ray data collection.

On the one hand, this yields information about, *e.g.*, the redox state of a metalloenzyme, the binding of a ligand, or the state of a photoactive protein. On the other hand, the onset and progression of X-ray radiation damage can be accurately monitored, as spectroscopic techniques are significantly more sensitive to subtle radiation damage than global crystallographic indicators such as changes of B-factors or loss of diffraction quality. Raman spectroscopy yields a wealth of information about chemical bonds at sub-atomic resolution and has opened considerable perspectives as a complementary tool to X-ray diffraction [1], and can be employed, for instance, to observe and quantify X-ray induced reduction of disulphide bonds in proteins [2].

In another application, we have studied the structural bases for the improved fluorescence properties of various green [3] and cyan [4] mutants of Green Fluorescent Protein. In particular, UV/vis absorption and fluorescence spectroscopies were used to monitor the X-ray induced bleaching of these fluorescent proteins, while crystallographic data recorded in parallel gives insights into the underlying structural changes.

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Keywords: raman spectroscopy, fluorescence spectroscopy, radiation damage

MS51.P09

Acta Cryst. (2011) A**67,** C552-C553

Structure of the second pair of fibronectin type III repeats of the integrin β4

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The integrin α6β4 is a component of the hemidesmosomes (HD), protein complexes that mediate the stable anchoring of basal epithelial cells to the basement membrane [1]. The cytoplasmic domain of the β4 subunit is unique among the integrin family and it is responsible for most of the intracellular interactions of α6β4, including the interaction with other hemidesmosomal components. The cytoplasmic region of β4 contains four fibronectin type III domains (FnIII1 to FnIII4) arranged in two pairs separated by a region named the connecting segment. Upstream of the FnIII1 domain there is a Calx- β domain [2], while a 90-residue long C-terminal tail extends downstream of the FnIII4 domain. It is proposed that prior to HD assembly the cytoplasmic domain of β4 adopts a closed conformation stabilized by an intramolecular interaction between the connecting segment and the tail; binding to plectin would unleash β4 and favour the association of β4 with other components of the HD [3].

We have combined x-ray crystallography, small angle x-ray scattering (SAXS), mutagenesis, and biochemical analysis to characterize the second pair of FnIII domains of β4. The crystal structure of the FnIII3 was phased by molecular replacement and it was refined against data to 1.6 Å resolution. The crystal structure of the FnIII4 was phased by single isomorphous replacement with anomalous scattering using a mercurial derivative, and the structure was refined against native data extending to 1.8 Å resolution. The structure of the FnIII3-FnIII4 region was analyzed in solution by using SAXS.