Structural studies of an ecotin variant

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Introduction

Ecotin is a canonical serine proteinase inhibitor from the periplasm of *Escherichia coli* [1]. One of its characteristic features is that it is a potent inhibitor of a variety of serine proteinases that have very different substrate specificities. Ecotin is a homodimeric protein. The two subunits are held together mostly by their long C-terminal strands that are arranged in a two-stranded antiparallel β-sheet. One ecotin dimer can chelate two proteinase molecules, each of them bound to both subunits of ecotin simultaneously through two different sites; namely the specific primary and the nonspecific secondary binding sites. However, the secondary site is very small, and it was not evident whether it is a real recognition site or rather an accidental contact surface [2]. With site-directed mutagenesis of the gene, we removed the last 10 amino acid residues from the C-terminus of ecotin [3]. The resulting mutant was evaluated by biochemical assays such as analytical gel filtration and fluorescent techniques and found to be monomeric. However, upon saturation with trypsin, the monomeric mutant formed not a heterodimer but a heterotetramer complex with the proteinase. This complex was indistinguishable from the one that is formed between wild-type ecotin and trypsin. This finding led us to conclude that the secondary site of ecotin is a real recognition site. However, in a recent paper, Yang and colleagues [4] reported that in their experiments, the same truncated form of ecotin behaves as a dimer. To get direct evidence about the quaternary structure of the truncated form of ecotin it was crystallized and high-quality data have been collected at the Advanced Photon Source.

Methods and Materials

Crystals of a C-terminal truncated version of ecotin were grown by vapor diffusion at room temperature using the hanging-drop method. A crystallization buffer containing 2 M ammonium sulfate and 0.1 M MES (pH = 6.5) was mixed with an equal volume of protein solution. The resulting rod-shaped crystals belong to space group C2 with cell parameters of a = 91.48 Å, b = 46.67 Å, c = 105.70 Å, and β = 121.48°; the crystals contain two molecules per asymmetric unit. For data collection, crystals were dipped in artificial mother liquor containing 10% glycerol and flash frozen in liquid nitrogen. A 1.95 Å data set was collected at the Structural Biology Center (SBC) beamline 19-ID and processed using DENZO and SCALEPACK. The final data set contained 25,299 unique reflections (redundancy 3.8) with an Rmerge (I) of 4.0% and an overall completeness of 90.4%.

Results

The structure was solved by molecular replacement using AmoRe and a model consisting of residues 1–132 of Protein Data Bank entry 1ECY. The rotation search using all reflections between 12 and 4 Å yielded two peaks. After a translation search and rigid-body refinement of the first rotation solution candidate, the best solution had a correlation coefficient of 0.358 and an R-value of 50.6%, with the highest noise peak at 0.302 and 52.9%. This solution was fixed, and a subsequent translation search for the second monomer resulted in a clear solution with a correlation coefficient of 0.532 and an R-value of 43.9%. At this point, 5% of all reflections were sequestered for monitoring the free R-value. Alternative cycles of density fitting (using program O and refinement with program Refmac) applying tight, noncrystallographic symmetry (NCS) restraints between the two monomers gave an R-value of 27% for all reflections between 6 and 1.95 Å resolution. Further refinement with loose NCS restraints, placement of water molecules with program ARP, and the addition of four sulfate ions resulted in the current R-value of 24.0% (with a free R-value of 30.2%) for all reflections between 6 and 1.95 Å.

Discussion

The current model consists of residues 1–86 and 92–132 of each ecotin monomer, two pairs of sulfate ions, and 192 water molecules. Of all non-proline/non-glycine residues, 88% are in the "most-favored" region of the Ramachandran plot. Two segments (residues 76–80 and residues 85–92) of both monomers have poorly defined electron density. The structure shows that the two NCS-related monomers interact to form a homodimer. However, the dimerization mode is completely different from that observed for native ecotin. We are currently characterizing this interface in detail and evaluating its biological relevance.

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