also lead to its accumulation. Both of these techniques have been used to trap the covalent glycosyl–enzyme intermediate of retaining β-glycoside hydrolases, such as the *Cellulomonas fimi* multi-functional cellulase/xylanase CEX, structures of which have been reported previously in *Nature Structural Biology*[6,7]. Historically, the intermediate for retaining α-glycosidases has proved difficult to trap. For example, the equivalents of the 2-fluoro-substituted sugars that were successful for β-retaining enzymes[6,10] are not inhibitors for α-glucosidases. The absence of three-dimensional information on the intermediate is reflected in a great deal of confusion and controversy in the published literature. The importance of the paper by Uitdehaag *et al.*[2] is that the covalent glycosyl–enzyme intermediate of an α-glycosidase/transglycosylase, a state which had previously eluded structural analysis, has now been trapped (Fig. 2).

Uitdehaag and colleagues[2] utilize the substrate 4-deoxymaltotriosyl α-fluoride in combination with the E257Q mutant of the catalytic acid/base residue. Removal of the terminal 4-hydroxyl substituent of the substrate prevents a second molecule from acting as an acceptor species that would otherwise have resulted in depletion of the intermediate by transglycosylation (Fig. 2). Mutation of the enzymatic acid/base prevents it's functioning as a base so that the breakdown of the intermediate, by hydrolysis, is also slowed. The fluoride leaving group on the substrate, however, requires little or no catalytic assistance from the enzymatic acid/base — hence the formation of the intermediate continues rapidly even on the mutated enzyme, leading to its accumulation. Taken together with previous analyses of β-glycosidases[6–8], the structures presented by Uitdehaag and colleagues[2] finally demonstrate that both retaining α- and β- glycosidases/transferases utilize a similar double-displacement mechanism, in which a covalent glycosyl-enzyme intermediate is formed and broken down through oxocarbenium-ion like transition-states. It is the stereochemistry that is different in each case: retaining β-glycosidase/transferases perform catalysis via an α-configured intermediate while retaining α-glycosidases/transferases react via a β-configured intermediate. The three dimensional realization of the distorted substrate and the covalent intermediate, provided by Uitdehaag *et al.*[2], will have profound implications for the design and synthesis of oligosaccharide inhibitors as therapeutic agents and mechanistic probes for enzymes utilizing α-linked substrates.

The α-amylase family is one of the largest of the many glycoside hydrolase families[11] and one of the most important due to the enormous application of these enzymes in industrial processes and the relevance of many of the enzymes therein to human disease. While a great deal of work remains to be done on these systems it is heartening that the covalent glycosyl–enzyme intermediate, proposed by Koshland almost 50 years ago, has finally been visualized on both α- and β-retaining enzymes.

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A new type of PDZ domain recognition

Hartmut Oschkinat

A new role for certain PDZ domains has been revealed — binding to non-terminal β-hairpin structures in other proteins. These interactions involve the same binding site as is used for binding to the canonical C-terminal peptide targets of PDZ domains.

Unpacking a new computer or stereo is fun, as long as the parts can be quickly assembled. The more specific the plugs and sockets are in color, shape or markings, the less frustration arises in connecting the units. The circuits that execute cellular operating programs similarly require precise assembly, and while molecular biologists have already unpacked a large number of components, they are busy trying to determine how all of the parts are 'wired' together. Adaptor domains (such as SH2, SH3, PH, PTB, WW, PDZ, SAM, EVH1, RA, Px, and so forth) interact selectively with their target sequences and function as molecular sockets, connecting signal transducing components to large protein complexes at focal points of biological activity, such as synapses, neuromuscular junctions, and tight junctions, or in apoptotic signaling pathways.

PDZ domains

One type of adaptor is the PDZ domain[1] (first found in the proteins PSD95[2], Dlg[3], and ZO-1[4]). These domains have remarkable selectivity towards their native targets, which are usually the extreme C-termini of proteins[5,6]. In many cases, PDZ domains bind specifically to the last five cytoplasmic C-terminal residues of membrane-associated receptors or channels[7,8]. A water molecule and the backbone amide groups of the PDZ domain, located in a loop formed by the GLGF motif (named for the residues it contains), are involved in hydrogen bonds

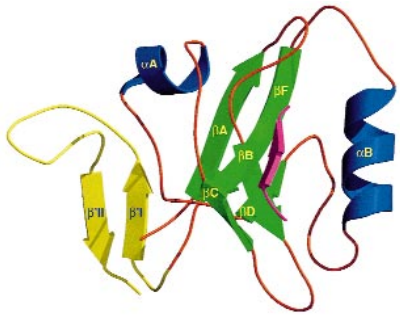

Fig. 1 Ribbon diagram of the nNOS PDZ domain–MelR peptide complex determined by NMR[13]. The MelR peptide is shown in magenta. The two C-terminal strands of the extension are in yellow. Kindly provided by ref. 13.

specifically to the terminal carboxyl group of the target peptide[9–11]. Peptide residues in the -1 to -4 positions (with respect to the C-terminal residue position) occupy pre-formed sites in the PDZ domain, and the peptide inserts as an additional β-strand into a groove (Fig. 1). A strong hydrogen bonding network involving both backbone and side chain atoms ensures considerable affinity and specificity.

In addition to this canonical mechanism of target recognition, it has been proposed that certain PDZ domains form heterodimers[12]. The PDZ domain of neuronal nitric oxide synthase (nNOS) interacts with both the PDZ domain of syntrophin and the second PDZ domain of PSD95[12], proteins which are found in the same cellular location. A ~30-residue C-terminal extension (colored yellow in Fig. 1) of the minimal nNOS PDZ domain is critical for these interactions. Whether both types of PDZ interactions — canonical peptide binding and PDZ–PDZ interactions mediated by the C-terminal extension — can occur at the same time has, until now, been unclear.

The structural basis of the mechanism by which two PDZ domains can interact has now been investigated by two groups, Tochio *et al.*[13], whose work is presented on page 417 of this issue of *Nature Structural Biology*, and Hillier *et al.*[14], whose results were just published in *Science*. Tochio *et al.* determined the NMR structure of the nNOS PDZ domain in complex with a target peptide derived from the melatonin receptor (MelR)[13], and Hillier *et al.* solved the X-ray crystal structure of a complex between the nNOS PDZ domain and the syntrophin PDZ domain[14].

Structures

The independent crystal structure and NMR results are highly complementary and reveal insights into a molecular mechanism that is likely to be used for regulating signaling pathways by alternative or simultaneous binding of proteins to the same PDZ domain. Both groups elucidated the structural requirements for the formation of PDZ heterodimers by solving structures of the nNOS PDZ with its C-terminal extension. Both of the new structures[13,14] reveal a highly conserved canonical PDZ fold comprising residues 1–100, including an intact binding site (to which C-terminal peptides bind[8,9,11]), and both show that the structure of the C-terminal extension (residues beyond 100) is a double-stranded β-sheet, with a loop connecting the two strands (Fig. 1).

Hillier *et al.*[14] also determined the structure of the nNOS PDZ domain in complex with the syntrophin PDZ domain. This structure demonstrates that the additional sheet and the loop in the extension of the nNOS PDZ domain form a 'β-finger' that inserts into the canonical peptide binding site of the syntrophin PDZ domain (Fig. 2). In this situation, the nNOS sequence H-L-E^{-3}-T^{-2}-T^{-1}-F^{0}- is analogous to the C-terminal peptide G-V-K^{-4}-E^{-3}-S^{-2}-L^{-1}-V^{0}-COOH (the superscripts indicate the occupied peptide binding positions in the complex). The binding of the non-terminal β-finger peptide by the syntrophin PDZ domain follows very similar selectivity-determining principles as are found in the case of canonical C-terminal peptide target binding.

The loop structure seen in the β-finger of the nNOS C-terminal extension may be required for high-affinity binding to the syntrophin PDZ domain, and possibly also to others. This is supported by the work of Gee *et al.*[15] who searched for syntrophin-PDZ-binding peptides. They found sequences of the type X-R/K-E-T-C-L/M-A-G-X-X-C (bold residues need to be conserved), in which a disulfide bond between the two cysteines was required for tight binding. The corresponding linear (non disulfide-linked) peptides did not show high affinity, suggesting that a loop conformation is important. In the extended nNOS PDZ domain, the loop structure, although exposed, is present even in solution[13], despite some hinge motion.

The presence of the loop in the β-finger probably allows the GLGF region of the peptide-binding site in the syntrophin PDZ domain to remain unchanged upon binding[14]. The structure of the complex between the two PDZ domains shows an important water molecule positioned in the GLGF loop region of the PDZ domain that has been predicted to be a conserved feature of peptide binding[9]. This water molecule should, in principle, be displaced if the bound peptide were to continue as an extended strand further into the binding site, and the looping of the β-hairpin likely prevents this displacement. Together, these results indicate that the conformation of the β-finger contributes favorably to the binding energy. Moreover, these findings suggest that PDZ domains

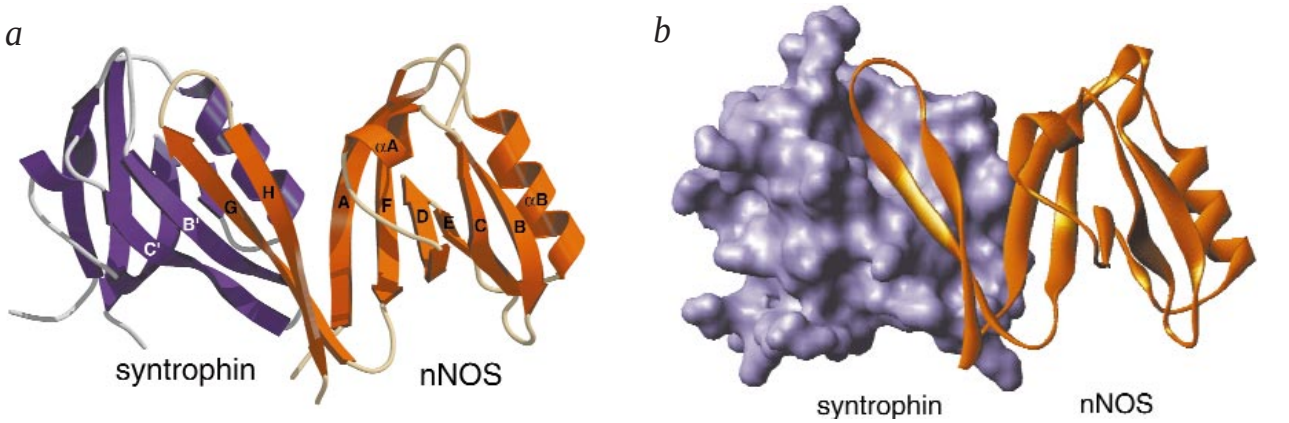

Fig. 2 ***a***,***b***, Two views of the nNOS PDZ domain–syntrophin PDZ domain complex, showing the nNOS β-finger bound in the canonical peptide-binding site of the syntrophin PDZ domain[14]. Kindly provided by W. Lim.

may bind in a general fashion to non-terminal sequences, possibly those with β-finger-type structures, regardless of whether or not they are found downstream of other PDZ domains. Indeed, the PDZ domain of the actinin-associated LIM protein (ALP), which is found in skeletal muscle[16], associates with a non-terminal sequence in a certain spectrin-like repeat of α-actinin-2. However, it still needs to be shown that the corresponding binding region shows a β-finger-type structure.

Two binding sites

The β-finger of the extended nNOS PDZ domain is situated opposite the canonical peptide-binding site on the other side of the nNOS molecule (Fig. 1), suggesting that the two separate binding sites probably cannot interact with each other without major structural rearrangement. This idea is supported by chemical shift comparisons of the complexes of MelR peptide bound to the short canonical domain and to the extended domain, which show that the structure of the peptide binding site itself (and the mode of binding) is independent of the presence of the extension[13].

Since the two interaction sites of the nNOS PDZ structure are on opposite ends of the domain, the two modes of activity can potentially be independent of each other — that is, binding of a C-terminal peptide and binding of another PDZ domain could, in principle, occur simultaneously. Thus, the extended nNOS PDZ domain is probably a bifunctional adaptor that can provide a link between various signaling components or between signaling proteins and the cytoskeleton. As an example, Tochio *et al.*[13] present a schematic diagram of a signaling complex proximal to the N-methyl-D-aspartic acid (NMDA) receptor involving PSD95 and CAPON[17], a novel cytosolic protein that binds to nNOS. This model emphasizes the separate binding sites and requires that CAPON bind to the canonical peptide-binding site of nNOS, leaving the nNOS extension available for interaction with PSD95. This model is supported by the finding that a known nNOS target peptide and a C-terminal peptide containing the last 12 residues of CAPON likely use the same canonical peptide binding site[13]. However, this idea is not in complete agreement with other studies in which binding of CAPON and PSD95 to nNOS was found to be competitive[17], a situation that was thought to be a possible mechanism for regulation of these signaling pathways. In those investigations, a 125-residue long C-terminal fragment of CAPON was used whereas Tochio *et al.*[13] used a short peptide. Thus, it remains a possibility that some of the non-terminal residues of the 125-residue long CAPON construct interact with the β-extension of the nNOS PDZ domain and prevent its interaction with PSD95.

Conclusion

The impact of these structures, which indicate a new mode of PDZ binding to non-terminal target sequences, is strengthened by recent evidence for the *in vivo* relevance of the interaction between the syntrophin PDZ domain and the nNOS extension. Both proteins occur in skeletal muscle cells, where syntrophin is attached to the dystrophin/utrophin network[18] and nNOS is found near the sarcolemma[19]. Kameya *et al.*[20] deleted the PDZ domain coding region from the syntrophin gene in a line of mice. In these mice, they find that nNOS is localized to the cytosol rather than the sarcolemma of the skeletal muscle cells. This altered localization strongly supports the idea that the β-sheet extension of nNOS[12] mediates a naturally occurring interaction. Thus, the nNOS–syntrophin case is the first demonstration (both *in vitro* and *in vivo*) of the use of a non-terminal peptide for PDZ domain–protein interactions. Most likely, many more examples of PDZ recognition of internal peptide sequences will be identified in the near future in the wide variety of signaling cascades known to be mediated by these versatile adaptors.

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