Chapter 5

Conclusions and outlook

In this thesis, the three-domain protein pleckstrin is studied by NMR and biochemical methods. The results presented in chapters 2-4 provide novel insights into pleckstrin's role in the platelet signalling network. Furthermore, a new method to obtain medium- to high-resolution structures by NMR is developed. This method can be generally applied to multi-domain systems (chapter 4).

The high-resolution structure of pleckstrin's C-terminal PH domain (C-PH) and the identification of the high-affinity ligand PtdIns(3,4)P_2 for this domain suggest a previously unknown direct mechanism for the regulation of pleckstrin. The profile of the lipid second messenger PtdIns(3,4)P_2 is quite well described in platelets, but not in other cell types. Its delayed appearance and sustained elevation which is coupled to pleckstrin phosphorylation may control pleckstrin's localisation and activity in the later stages of platelet activation. The spatio-temporal distribution of PtdIns(3,4)P_2 and pleckstrin in activated platelets will be a very interesting subject for future studies in order to investigate whether there is indeed a direct correlation. Also, pleckstrin's localisation could be monitored in transfection experiments where issues concerning the mutual dependence of phosphorylation and PtdIns(3,4)P_2 signalling can be more easily addressed.

From a structural point of view, the high selectivity of C-PH for PtdIns(3,4)P_2 is quite surprising because it could not have been predicted from the ligand-free structure. This implies that the ligand specificity of an uncharacterised PH domain will still need to be determined experimentally. The conserved motif on the β1 and β2 strands of PH domains seems to be a requirement for high affinity binding, but other determinants in the specificity determining regions decide which ligand fits into the pocket. Binding site mapping by NMR titration experiments identified two regions in C-PH that could serve

to select for PtdIns(3,4)P_2: the β1−β2 loop and the β7 strand. The details of ligand recognition will be elucidated in a structure determination of the C-PH PtdIns(3,4)P_2 complex which has already been initiated. Since no complex structure of a PH domain with PtdIns(3,4)P_2 has been solved to date, there would be considerable interest for such a high-resolution structure.

The NMR characterisation and subsequent spin-label surface scanning of the DEP_C-PH favours a model of this double-domain construct where the two domains are independent of each other and the linker is flexible in its central residues. The phosphoinositide binding properties of DEP_C-PH seem to be exactly the same as for C-PH alone, which supports the conclusion from the NMR experiments. The study of this protein was complicated by its inherent tendency to partially aggregate in solution. These aggregation tendencies are also observed for the full-length molecule and are one important reason why it is so difficult to work with full-length pleckstrin.

The preliminary structure of N-PH_DEP shows that the two domains form an extensive domain-domain interface and adopt a “closed” conformation. This is the first structural evidence that there is an intramolecular interaction between pleckstrin’s domains. The closed conformation of N-PH_DEP is consistent with the view that N-PH is inactivated in the unphosphorylated state of pleckstrin. The DEP domain may block the access to the functionally relevant areas of N-PH. Hence, an auto-inhibition model for unphosphorylated pleckstrin is proposed. Further refinement of the N-PH_DEP structure will provide a more detailed understanding of the molecular mechanisms involved. Moreover, efficiently PKC-phosphorylated NMR samples of pleckstrin are now available, so that the structural effects of phosphorylation can be directly studied. Eventually, the auto-inhibition model will have to be tested in structural studies of full-length pleckstrin.

The application of the site-directed spin-labelling approach to the pleckstrin protein which contains seven native cysteins is a break-through for obtaining medium-resolution structures of multi-domain proteins by NMR. The strategy to eliminate accessible cysteins one by one while monitoring correct folding by NMR is generally applicable. Already from the preliminary structure determination of N-PH-DEP it is apparent that this strategy will be of considerable utility for tackling similar structural problems.

A very large number of single domain structures from X-ray crystallography and NMR are now available, but only a much smaller number of structures of multi-domain proteins and protein complexes. In many cases, several or all domain components of a system are known, yet their interaction and mutual regulation await investigation. The combined spin-label/RDC approach presented in this chapter is ideally suited for this type of application. Any two-domain protein or protein complex for which the individual domain structures are known can be analysed. Once the native cysteins have been eliminated, the structure determination is relatively straight-forward and very fast compared to conventional NOE-based NMR structure calculations. Thus, this method should become of substantial utility for a large variety of applications and in particular for proteins that are “uncrystallisable”, like pleckstrin. The use of spin-labels combined with RDCs for the determination of the domain arrangement of full-length pleckstrin is already underway.