The opioid peptide dynorphin A – Biophysical studies of peptide–receptor and peptide–membrane interactions

Johannes Björnerås
The opioid peptide dynorphin A

Biophysical studies of peptide–receptor and peptide–membrane interactions

Johannes Björnerås
To my family.
List of Papers

The following papers, referred to in the text by their Roman numerals, are included in this thesis.

PAPER I: Direct detection of neuropeptide dynorphin A binding to the second extracellular loop of the κ-opioid receptor using a soluble protein scaffold.

PAPER II: Membrane interaction of disease-related dynorphin A variants.

PAPER III: Analysing the morphology of DHPC/DMPC complexes by diffusion NMR.
Johannes Björnerås, Mathias Nilsson and Lena Mäler, Submitted.

PAPER IV: Resolving complex mixtures: trilinear diffusion data.

PAPER V: The membrane interaction of dynorphin A depends on lipid head-group charge.
Johannes Björnerås, Astrid Gräslund and Lena Mäler, Manuscript.

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Contents

List of Papers vii

Abbreviations xi

1 The science of life 1
  1.1 What this thesis is about . . . . . . . . . . . . . . . . . . . . . 2
  1.2 What you will find in this thesis, and what you will not . . . . 3

2 Lipid environments 5
  2.1 Biological membranes . . . . . . . . . . . . . . . . . . . . . . 7
  2.2 Mimetics . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 10

3 Peptides and proteins 13

4 Methods 17
  4.1 Nuclear magnetic resonance spectroscopy . . . . . . . . . . . . 17
     4.1.1 General theory . . . . . . . . . . . . . . . . . . . . . . . 17
     4.1.2 Chemical shifts . . . . . . . . . . . . . . . . . . . . . . . 19
     4.1.3 Dynamics . . . . . . . . . . . . . . . . . . . . . . . . . . . 20
     4.1.4 Diffusion . . . . . . . . . . . . . . . . . . . . . . . . . . . 25
  4.2 Multi-way decomposition of experimental data . . . . . . . . . 28
     4.2.1 Two-way decomposition . . . . . . . . . . . . . . . . . . 29
     4.2.2 Three-way decomposition . . . . . . . . . . . . . . . . . . 29

5 The interaction between dynorphins and opioid receptors 31
  5.1 The opioid system . . . . . . . . . . . . . . . . . . . . . . . . 31
     5.1.1 Opioid receptors . . . . . . . . . . . . . . . . . . . . . . 32
     5.1.2 Dynorphin A—an opioid ligand . . . . . . . . . . . . . . 34
     5.1.3 Dynorphin A and the κ-opioid receptor . . . . . . . . . . 35

6 The interaction between dynorphins and lipids 39
  6.1 Peptides and membranes . . . . . . . . . . . . . . . . . . . . . 40
Abbreviations

AMP     anti-microbial peptide
CD      circular dichroism
CNS     central nervous system
CPP     cell-penetrating peptide
CSA     chemical shielding anisotropy
DHPC    1,2-dihexanoyl-
         sn-glycero-3-phosphocholine
DMPC    1,2-dimyristoyl-
         sn-glycero-3-phosphocholine
DMPG    1,2-dimyristoyl-
         sn-glycero-3-phospho-(1’-rac-glycerol)
DOR     δ-opioid receptor
DynA    dynorphin A
EL2     extracellular loop II
FCS     fluorescence correlation spectroscopy
GPCR    G-protein coupled receptor
GUV     giant unilamellar vesicle
IDP     intrinsically disordered protein
IUP     intrinsically unordered protein
KOR     κ-opioid receptor
LUV     large unilamellar vesicle
MOR     μ-opioid receptor
NMDA    N-methyl-D-aspartate
NMR     nuclear magnetic resonance
PC      phosphatidylcholine
PCA     principal component analysis
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>PDB</td>
<td>Protein Data Bank (database)</td>
</tr>
<tr>
<td>PDYN</td>
<td>proenkephalin-B</td>
</tr>
<tr>
<td>PG</td>
<td>phosphatidylglycerol</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>RDC</td>
<td>residual dipolar coupling</td>
</tr>
<tr>
<td>RF</td>
<td>radio-frequency</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SOD1</td>
<td>super-oxide dismutase 1</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
</tr>
<tr>
<td>tcf</td>
<td>time-correlation function</td>
</tr>
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</table>
1. The science of life

*I believe a leaf of grass is no less than the journey-work of the stars, And the pismire is equally perfect, and a grain of sand, and the egg of the wren.*

– Walt Whitman, *Leaves of Grass*

It is only with the wisdom of a philosopher or the courage of a fool that one dares to define human nature. And possessing nothing of the former and very little of the latter, it is with some reluctance that I, for the sake of this chapter, put forward two suggestions of things that makes us human: curiosity and an urge to control. First, the desire to find out, to understand, to know seems hard-wired into us. We start asking questions as kids and never really stop. Because even though the playful, wild, unquenchable lust for knowledge that permeates our younger years may eventually fade, some questions remain unsatisfyingly unanswered in almost everyone. Questions about the stars and what lies beyond them, about time, about the beginning and end of the world, and about ourselves—birth, life, death and the meaning of it all. Linked to this is our will to control our lives and the world we live in. We want to exert some power over our fragile existence, we like to predict, prepare and plan. And over the course of our brief human history we have created different systems of practices and thought to channel these two desires, systems out of which science is one.

*Life science* is the science of life or, to phrase it differently, the scientific study of living systems. As touched upon above, there are many astounding aspects of life and living organisms, and many unanswered—perhaps unanswerable—questions. Life is, and has probably always been, a source of amazement and wonder. In addition to such philosophical aspects, many areas within life science have a huge practical impact on our lives. Consider, for example, research on diseases such as malaria or tuberculosis, on neonatal intensive care, on small-scale water filtration, or on bone-anchored hearing devices. The work of life scientists can and will change individual lives completely. Because of these sometimes direct and large consequences for us humans (and for other living, sensible things), life science is symbiotically linked to the ethics and norms of society. For me it is this combination of the awe-inspiring fundamental problems and the strong interplay with the world surrounding the laboratory that makes life science so interesting and important. It is this combination that confers a not so small responsibility on life scientists, and gives meaning to the daily labour.
1.1 What this thesis is about

This thesis belongs formally to the area of biophysics, a sub-field of life science. As the name suggests, biophysical research has traditionally been based on physics methods, particularly different forms of spectroscopy, as well as on theoretical work and mathematical modelling, but that definition is too narrow to capture all the biophysical science of today. My area of the biophysics field concerns very detailed—molecular or even atomic—aspects of living systems, whereas other scientists in the field are interested in whole molecules and upwards, past networks of molecules to cellular organelles, complete cells and even groups of cells. A more important question, however, than to which category or life science sub-field this thesis belongs, is what it is about.

When I am asked ‘What do you work with?’ I usually reply that I do research on a molecule present in the brain, a signalling compound similar to endorphin, the substance responsible for effects such as the ‘runner’s high’. This is half true, a part of this thesis is about dynorphin A, a peptide (small protein) that modulates neural signalling; more specifically it is about 1) how dynorphin A interacts with a part of the κ-opioid receptor, a dynorphin binding partner in a brain signalling chain, and 2) how dynorphin A interacts with lipids in bilayers. The other half of the truth, and the other part of this thesis, concerns systems that try to mimic biological membranes. It involves the characterisation of such mimetic systems, as well as work on methods that aid the characterisation.

On a more general level, the results in this thesis are connected to two phenomena that are important, maybe even necessary, in living systems: compartments and signalling. Although the definition and classification of life is not clear-cut, some things are generally accepted as vital elements of life. A living organism 1) has some sort of information storage capability (such as DNA), 2) is able to reproduce and evolve, and 3) is able to convert and use energy. In addition to this all known living systems have some way of keeping things in confined spaces. The most obvious example is an overall barrier that creates an inside of the organism separated from the surroundings, and beneath this surface the organism may harbour a remarkably complex architecture of biological membranes. Many physiological responses depend on processes taking place at these boundaries, for example whether a specific particle (molecule, virus, etc) is able to pass through a cellular membrane. Dynorphin A interacting with lipids is an example of such a process; and the study of membrane mimetics is in extension a study of the biomembranes themselves.

A second feature that is characteristic of living organisms and, in some respect, connected with compartmentalisation, is a means of sending, receiving and processing signals. Signalling systems may facilitate rather simple pro-
cesses such as microbes sensing an energy source and moving towards it, or very complex events such as the massively parallel neural communication in a human brain. At its most general, a biological signalling system involves a ligand—the physical embodiment of the signal, and a receptor—something that ‘reads’ the signal through interaction with the ligand. Dynorphin A interacting with the κ-opioid receptor is a part of such a signalling system.

1.2 What you will find in this thesis, and what you will not

This thesis contains a number of published and unpublished articles. This material as a whole constitutes physically the second half of this thesis, but in terms of underlying work and intellectual effort it is the foundation. I share the responsibility and the credit for this work with my co-authors. In the first half of the thesis I try to provide a context for the articles; to give an idea of where the work presented in this thesis fits in the great fresco of life science.

First, in Chapter 2, lipids and lipid systems are introduced, with a bias towards their role in biological systems. The physico-chemical properties of lipids are briefly discussed, as well as how these properties underlie the thermodynamical behaviour of lipid ensembles. This chapter also includes a section on lipid systems as tools in biochemical and physical research.

Chapter 3 treats peptides, which is the class of biomolecules to which dynorphin A (DynA) belongs. Again the chapter begins with a general introduction to peptides, after which follows a section on their biological function. Two aspects of this latter subject, namely peptide–receptor and peptide–lipid interaction, are expanded into two chapters of their own, 5 and 6 respectively, but before this, in Chapter 4 is an introduction of the main methods used in this thesis work. Finally this first half of the thesis is concluded with Chapter 7 where the main findings of the thesis work are summarised, put in context and used as the starting point for a discussion of possible extensions of the projects as well as alternative routes ahead.

There is not room for a detailed description of any method used, or a comprehensive historical review of a particular research field; but for the interested reader there should be references with scopes complementary to this thesis. As even the number of references is limited, however, a lot of brilliant work and brilliant researchers have been left out altogether, for this I am sorry. With this said, my hope is that anyone with an interest in life science will find something in this thesis that is both understandable and interesting—no humble hope indeed.
2. Lipid environments

Lipids are small biological molecules that are in general soluble in nonpolar solvents but have a much smaller, or vanishing, solubility in polar solvents such as water. Lipids may be defined and classified inductively, e.g. through their solubility in various solvents, or based on how they are extracted from biological membranes. Deductive approaches have also been suggested, giving more stringent and systematic definitions and classifications. Whichever approach is used, most lipids share a few characteristics that will be discussed here. Most importantly, many lipids are amphipathic, i.e. they have both hydrophobic and hydrophilic properties, with the hydrophobicity often coming from fatty acid hydrocarbon chains. In a polar solvent such as water contact between the hydrophobic parts and the solvent molecules is energetically unfavourable, thus leading to the lipids spontaneously assembling into a rich variety of supramolecular structures. The architecture of these structures, their morphology, depends on both environmental parameters such as temperature, concentration and pH, and on lipid molecular properties, of which a few are listed below:

**Hydrophobic moiety**: This part of a lipid is typically made up of fatty acids, i.e. hydrocarbon chains attached to the headgroup via a carboxyl group. A particular lipid may have up to four such chains, not necessarily of the same type. The chains usually contain an even number of carbons, from two to thirty-six, where in biological membranes the lengths are often between fourteen and twenty-two. The carbons in the chain can be joined by single or double bonds. The length and degree of unsaturation of the chain in a particular lipid type strongly influence what type of assemblies an ensemble of such lipids will form under various conditions. The physical chemistry of the chain also influences other thermodynamic properties such as phase transition temperatures.

Biomembrane lipids can be divided into three main classes: 1) glycerophospholipids (phospholipids), 2) sphingophospholipids (sphingolipids) and 3) sterols and linear isoprenoids, where the first two both have acyl chains as described above. The members of the third class, e.g. cholesterol, are all derived from isoprene precursors, and will not be further discussed here. The interested reader is referred to Luckey.
**Backbone and head group:** Phospholipids and sphingolipids differ in the chemistry of the backbone, i.e. the moiety that joins the fatty acid chains and the head group. In phospholipids the backbone is a glycerol group, where two of the carbons are bound to the fatty acid chains, while the third is attached to a phosphate. To this molecule is then attached a head group moiety, where the available head group types vary between organisms. In the work described in this thesis only phosphatidylcholine (PC) and phosphatidylylglycerol (PG) lipids have been used, these are depicted in Figure 2.1. The ionic properties of the head groups are important for interactions with other lipids, as well as with proteins and peptides. Phospholipids may be anionic, carrying a negative net charge at neutral pH, or zwitterionic, with no net charge. PG is an example of the former, and PC of the latter. Sphingolipids have a sphingosine backbone instead of glycerol, but may otherwise have the same head group moieties as phospholipids.

**Overall molecular geometry:** The supramolecular behaviour of lipids, and their effect on e.g. membrane proteins, is also influenced by the overall shape of the individual lipid molecules. The effective size of the polar region relative the size of the hydrophobic region affects the intrinsic curvature of the surface of a lipid layer, and the total composition of different lipid types gives a net intrinsic curvature.

The discussion in the list above is biased towards bilayer-forming lipids, because these are one of the primary building blocks in biological membranes. But there are other classes of amphipathic molecules, or *amphiphiles*. One example is the detergents, a subgroup of lipids that in polar solvents form *micelles*, often spherical assemblies with a surface of polar headgroups protecting the hydrophobic core. For a thorough treatment of the physical chemistry of micelles see Wennerström, while a more practical approach to detergents in biochemistry is given by Garavito et al. Examples of lipid types together with examples of lipid self-assembly are shown in Figure 2.1.

It may be unfair to say that lipids have been neglected in life science research, but it did take some time before the scientific interest in lipids reached the same level as that enjoyed by DNA/RNA and proteins. A search in the database Web of Science for the topic ‘lipids’ from 1945 to 1990 gives about 50000 hits, while the same search for ‘DNA or RNA’ and ‘proteins’ gives roughly 150000 and 250000, respectively. One reason for this is that the structure–function relationship, in contrast to nucleic acids and proteins is not so easily found for single lipid molecules, but rather lies on the assembly/ensemble level, making it necessary to characterise an often complex phase behaviour. The interest in lipids has, however, grown and at the start of the
The term *lipidome* was added alongside the already established *genome* and *proteome*. For an excellent review of lipids, with a focus on physical chemistry, the reader is referred to Mouritsen’s book.[5] 

**Figure 2.1:** Schematic overview of lipids and lipid assemblies. a) Sodium stearate, the sodium salt of stearic acid. A common detergent in hand soap. b) DMPC molecule with hydrophilic region outlined, together with PG head group. c) DMPC, schematic depiction of head group and tail. d) Spherical detergent micelle. e) Lipid bilayer. f) Unilamellar vesicle.

### 2.1 Biological membranes

There is now strong support for the idea that lipids or lipid structures were present during the dawn of life,[12][13] although the details on how and when biological membranes entered the stage are still debated.[14] The fact is, that already in 1938 Oparin, one of the leading scientists in the formulation of early evolution in terms of physical chemistry, wrote in his seminal book *Origin of Life* about the ‘outstanding role’ of fat-like substances in biomolecular complexes.[15]

Moreover, in terms of function, one needs to look no further than to a single cell to see all the roles lipids play in a living organism. The most immediate function is the formation of lipid bilayers. In addition to an overall
envelope that creates an inside and an outside, the cellular interior is compartmentalised to various degrees of complexity, adding spatial constraint as a parameter for tuning the parameters and processes—chemical concentrations, gradients, enzymatic reaction rates, transport, storage, etc—that constitute the non-equilibrium state of life. Much work has gone into constructing theoretical frameworks and models of such processes; for example it was proven by Pólya in the 1920’s that for a random walk in one or two dimensions the probability of visiting every point is unity, while it is less than one in three dimensions. Adam and Delbrück used a more elaborate model to show that reducing the dimensionality from three to two may either decrease or increase the time it takes for a diffusing particle to encounter a fixed target (trap), depending on the ratio of diffusion coefficients and size of target compared to diffusion space. Additional work by the groups of McCloskey and Naqvi, together with more precise values for diffusion rates in biological systems, has seriously challenged the idea of dimensionality reduction as a straightforward way to increase reaction rates. Nevertheless, a straightforward geometrical calculation, gives a roughly 500-fold increase of a concentration of nanometer-sized objects when going from volume to surface in a sphere with a radius of 1 µm.

In the early 1970’s Jonathan Singer and Garth Nicolson published the fluid-mosaic model of a biological membrane, a model that over time became canonical, and will be taken here as the starting point for the development and refinement of biomembrane models. The interested reader is referred to a personal account by Singer of the early history of the model and the experimental and theoretical results on which it was built. The fluid-mosaic model incorporates a number of important features:

- As the name suggests, the biological membrane is a mosaic of phospholipid bilayer and lipid-associated proteins.
- The bilayer is very dynamic, indeed it is ‘...best thought of as a two-dimensional oriented viscous solution’.
- Based on the experimental evidence (at the time) it was impossible to say whether the bilayer was continuous or interrupted.
- Singer concluded that there is no long-range order, but probably short-range order, in the membrane.
- The proteins interacting with the membrane may be integral, meaning that they are immersed in the bilayer, or peripheral, meaning that they are not. (In the original model this distinction was made based on whether membrane disruption was needed to extract the proteins or not.)
Although this four decades old model has stood the test of time remarkably well, and the above list of features still encompasses many much more recent experimental results, the current biomembrane model has a few corrections and additional features. The two type-classification of membrane proteins can be more nuanced by separating membrane-inserting proteins, where a large portion but not the entire molecule is inserted into the bilayer, from proteins that are embedded in the membrane. For a detailed discussion of different degrees of membrane interaction, and possible protein classes, see Luckey. A more fundamental adjustment of Singer’s original model, where proteins were scattered sparsely in a uniform layer of phospholipids, is that in current models the membrane is much more protein-dense, and also that differing lipid types appear to be more segregated than previously believed, creating a more ordered and diverse environment. Membranes are, with Engelman’s words, ‘more mosaic than fluid’. A schematic picture of a biological membrane is shown in Figure 2.2.

**Figure 2.2:** Schematic overview of a biological membrane. a) Membrane-embedded proteins. b) Peptides interacting with the membrane surface and interfacial region. c) Lipid-interacting protein. Adapted from Engelman.

An important concept that has emerged is the lipid raft, meaning dynamic patches on the 10 to 100 nm length scale and enriched in, or exclusively containing, a limited number of lipid types. This concept of a segregated, and
(partly) controllable lipid micro-environment was introduced to explain ob-
servations of large heterogeneities in native membranes from e.g. epithelial
cells. Add this to the idea of a richer palette of lipid–protein interactions
than was originally understood, and the result is the model of today, where a
symbiotic interplay between lipids and proteins control not only protein as-
association, structure, stability and activity, but also such things as
membrane curvature and associated biological functions, transport pro-
cesses and signal transduction.

2.2 Mimetics

The complexity of almost any native biological membrane prohibits most atom-
level studies, and even molecular information on properties of, or processes
occurring in or at the membrane are often out of reach. The spatio-temporal
resolution of studies on native or near-native biological membranes is rapidly
increasing, however, thanks to development of both experimental and computa-
tional methods. A few examples from recent years of experimental methods
applicable to large biomembrane systems are atomic force microscopy, and femtosecond X-ray laser diffraction. Other techniques, such as magnetic
resonance force microscopy have shown promise, but are still under develop-
ment.

Another route is to create a system that shares the properties of interest
with the ‘real’ membrane environment that one wishes to study, but a system
sufficiently cut down in size and complexity for it to be manageable by the
methods that are of interest and available. Such an imitation of a real mem-
brane is often called a membrane mimetic. Just as for native biological mem-
branes, the mimetics exploit the self-assembly properties of lipid molecules,
and by tuning the aforementioned sample properties (lipid type, concentra-
tions, etc) quite a few reasonably well defined models are available. In the
work presented in this thesis the focus is on bicelles, a mimetic that is intro-
duced below alongside a few other systems, and discussed in more detail in
Chapter 6.

Micelles

An aqueous suspension of only detergent molecules will above a certain con-
centration form micelles, aggregates with a hydrophobic core and a polar re-
gion of detergent headgroups facing the water. Micelles have been used ex-
tensively to solubilise membrane-associated peptides and proteins to facili-
tate biophysical and biochemical characterisation; three examples out of many
are the study of an Arg-rich voltage-sensor domain from a K+ channel, and
the structure determination of the membrane protein VDAC-1 and OmpW by nuclear magnetic resonance spectroscopy (NMR). For surfactant/detergent systems in the specific context of NMR spectroscopy, the reader is referred to two excellent reviews by Stilbs and Furó.

Bicelles

In the ideal model, bicelles are disk-like objects, or larger sheets of lipid bilayers, where the edges (holes or rims) are stabilised by detergent molecules. A schematic picture of a small bicelle is shown in Figure 6.1 in Chapter 6. Similar to many native membrane surfaces, the bilayer part has a much smaller curvature than in micellar systems, something that has been established by e.g. studies of the curvature of helical peptides in micelles and bicelles. Furthermore, bicelles, in contrast to micelles, provide an opportunity of using many native types of lipids. Studies have also shown that some proteins are more stable and more active in bicelles than in micelles.

By varying the relative amounts of lipids and detergent, as well as parameters such as lipid type, ionic strength, temperature, pH and overall lipid concentration, the morphology of the bicelles changes, and there is no absolute consensus as to where the boundaries of different bicelle morphologies are in this parameter space. This question will be addressed more thoroughly in Chapter 6.

Vesicles

Vesicles (also called liposomes) are spherical objects made up of lipid bilayers. I will restrict the discussion here to unilamellar vesicles, which are shells of lipid bilayers, like solvent filled balloons as shown schematically in Figure 2.1. The accessible vesicle radii range from around 10 to 200 nm, and the size distribution of vesicles in a particular batch can be made reasonably narrow. A coarse division of unilamellar vesicle sizes into small (SUV), large (LUV) and giant (GUV) is sometimes made. The controllable size, and correspondingly controllable membrane curvature, together with the fact that many types of lipids may be used to form vesicles means that this membrane mimetic may be quite similar to a native membrane, in many aspects more so than any other mimetic. Vesicles are used in many biophysical fields, such as fluorescence spectroscopy, but their large molecular mass makes reorientation so slow that most solution-state NMR experiments are difficult or impossible to perform, as will be explained later.

In cells vesicles are utilised, among other things, as vehicles to transport various signalling molecules. The unravelling of this machinery started in the 1970’s, and awarded the 2013 Nobel prize in physiology and medicine.
to Rothman, Schekman and Südhof. Hence, it is not surprising that the field of research that concerns the use of vesicles for therapeutic interventions such as drug delivery is a very active one, with the market for injectable drug delivery technologies estimated to be worth more than 40 billion dollars by 2017.
In the summer of 1878 it was observed by George Francis that cattle died after drinking the water from Lake Alexandrina in Australia. He concluded that excessive bloom of the alga *Nodularia spumigena* ‘render[ed] the water un-wholesome’ and published his finding in Nature. Neither Francis nor anyone else at the time knew that the poisonous agents were peptides—it would take over a hundred years before such a toxic peptide was completely characterised after isolation from the alga.

Peptides, just like proteins, are a sub-group of *polypeptides*, i.e. biological polymers whose structural units are amino acids. One common and conceptually simple feature that may be used to distinguish peptides from proteins is size: peptides are shorter than proteins, usually not much longer than 50 amino acid units, residues, and often shorter than 30 residues. In terms of physico-chemical properties peptides and proteins are, to a large extent, overlapping, since the basic molecular architecture is identical. An example of peptide chemical structure is given in Figure 3.1.

Peptide properties are derived from the physical chemistry of the backbone and the amino acid side-chains. The torsion angles in the polypeptide chain, the pattern of hydrogen bonding between atoms in the backbone, the amount and distribution of hydrophobic and polar side-chains and the entropical cost of having more regularity in the polypeptide connect the conformation of the molecule to the free energy. If a single molecule is studied, the free energy of a particular conformational state determines the relative probability of the molecule to occupy that state. Any realistic biological system involves a large number of molecules, and at any given time there will be an ensemble of conformations, the distribution of which is determined by the energetic states of a single molecule, and by intermolecular interactions. On a local molecular scale the conformational state is called secondary structure, as exemplified by the helical segment of the peptide in Figure 3.1 d. Although tertiary structure is less applicable to peptides than to proteins, since the former are often too short to contain several structural elements with fixed intramolecular positions, peptides have been of great use as tools in fragment-based studies of the folding and tertiary structures of larger proteins.

The notion that the structure of a protein or peptide determines (much of) the biological function has been something of a paradigm, and has guided
much research in the field. And without doubt the structure–function relationship has proven extremely successful both as a guiding hypothesis in the prediction of the function of polypeptides with known structure or vice versa. There has also been great interest in coupling structure and function to sequence. In combination with the explosive increase in sequence information generated by the developments in genomics of the recent decades, there has been an incentive to determine protein 3D structures and to develop methods that accomplish such structure–function studies.

Figure 3.1: Different ways of representing peptides, here dynorphin A. a) Primary structure (one-letter nomenclature). b) Chemical structure (two residues shown). c) Stick model, chemical and spatial structure. d) Ribbon model, showing secondary structure.

The focus on structure has several causes. One is molecular stability, e.g. in the trivial sense that structured proteins are, in general, less prone to aggregate, less exposed to proteases, etc. Hence, these proteins have a longer life time, which facilitates experimental studies. Furthermore, observations are necessarily averaged over the time-scale of the experimental method, as well as over the molecular ensemble. Hence, an ensemble of molecules with a narrow distribution of conformational states is more easily characterised than one occupying a flat energy landscape. Also, methods based on diffraction, such as X-ray spectroscopy, require biomolecular crystals, something that strongly
favours order and stability.

In recent years, however, the structure–function paradigm has become more nuanced, with the emerging understanding that polypeptides that are intrinsically disordered (IDPs, sometimes IUP: intrinsically unordered protein) are both common and functionally important in organisms. Moreover, there are now more powerful methods available, that are capable of probing the dynamics of a biomolecular system, in addition to structural characterisation. Motional processes in a biomolecule are very complicated, they are often coupled, and the related observables span several orders of magnitudes. Consequently, studying and characterising dynamics require equally versatile methods, or the combination of several different methods. NMR spectroscopy provides a powerful and flexible approach in these studies, as will be discussed in Chapter [4]. Another example are computational methods, where accessible time-scales and system sizes have rapidly expanded in the last decades.

Despite their relative simplicity compared to proteins, peptides often have a rich and functionally important dynamical behaviour. For example, a biological membrane may create an environment in which certain peptides exist in an order–disorder dynamical equilibrium. An interesting observation here is that although the peptides are often more ordered in the membrane, it is not evident whether folding precedes or follows insertion. We will return to phenomena like these later, in the context of peptide–lipid interactions. Another example where the balance between order and disorder plays an important role is in aggregation processes, such as of the Alzheimer’s Aβ-peptide.

In biological systems peptides are found in a variety of contexts, just like proteins, and an extensive overview is beyond the scope of this thesis. Peptides may e.g. be present as units in supramolecular structures, such as spider silk or the amyloid fibrils that are the hallmarks of a number of devastating human diseases including the already mentioned Alzheimer’s disease. Another example is the role of peptides as toxins in e.g. the venoms of wasps and bees where the peptide melittin induces pores in membrane bilayers. In many organisms peptides are also important as a defense against microbes, something that has received much interest as a possible path towards developing novel antibiotics.

In biological systems peptides may also function as messengers. The phenomenon of signal reception and transmission is at the heart of any living entity, and the more complex the organism, the more intricate the structure of the information transfer network. For a unicellular organism it may be sufficient to have a nutrient sensor coupled to a motility device, but for higher organisms such as humans, collections of roughly 40 trillion cells, the situation is very different. Not only do individual cells throughout the organism need to respond to both intra- and extracellular chemical stimuli for them to function
in their local environment, information also has to flow on an organism-wide level. To meet this requirement evolution has created transport networks such as the cardiovascular and lymphatic systems, and the nervous system.

As implied above the carriers of information and the corresponding types of receivers and conductors may be of rather different character. The flow of information may be mediated by propagating electrical potentials, ions, chemical compounds, etc. This thesis will only discuss molecular ligand–receptor systems, and more specifically neuropeptide ligands and membrane protein receptors, in particular the DynA peptide and the κ-type receptor in the opioid system. This topic will be covered in chapter 5, while in chapter 6 the interaction between peptides and lipid environments will be treated, again with a strong focus on DynA.
Life science is a melting pot, where century-old traditions and knowledge from chemistry, physics, biology, medicine and mathematics meet and interact. This means that there is a plethora of experimental and theoretical methods, each one rooted to various extent in one or several of the sub-fields of life science. In this chapter only two methods and some applications of these methods will be discussed, the selection being based solely on the importance of the methods for this thesis work.

4.1 Nuclear magnetic resonance spectroscopy

The primary method I have used is nuclear magnetic resonance (NMR) spectroscopy, more specifically as applied to biological molecules (mainly lipids and polypeptides) in the liquid state, and this section is an overview of this. First an extremely condensed treatment of the general theory will be provided (primarily based on the standard textbooks by Levitt\cite{100} and Keeler\cite{101}) after which applications that have been exploited in the research treated in this thesis will be discussed.

4.1.1 General theory

All spectroscopic techniques probe a specific set of energy levels in the studied system by perturbing the system and detecting the response. The energetic regime investigated, and the equipment to perturb and detect the system, may vary substantially. In NMR, the energy levels of interest are defined by \textit{intrinsic angular momenta} (or \textit{spin}) of atomic nuclei, and the associated magnetic properties. Perturbations are created, and the nuclear response detected, through \textit{resonance} between the nuclei in the sample, exposed to a strong magnetic field, and the instrument electronics. A nuclear spin, $I$, is associated with a \textit{magnetic moment} $\mu_I$ which is parallel or antiparallel to the spin. The \textit{gyromagnetic} (or magnetogyric) ratio $\gamma$ is the constant of proportionality, according to:

$$
\mu_I = \gamma I
$$

(4.1)
The magnetic energy of a nucleus is a function of the angle between the magnetic moment and the applied field $B_0$, giving a set of energy levels. The energy difference between levels correspond to the resonance frequency, the Larmor frequency, $\omega_0$, of the nucleus:

$$\omega_0 = -\gamma B_0$$

(4.2)

In quantum mechanics energy is represented by the Hamiltonian operator $\hat{\mathcal{H}}$, while the description of the state of the system is contained in the wavefunction $\psi$. In the case of the interaction between a single nuclear spin and a static magnetic field of strength $B_0$ applied along the $z$-axis, the Hamiltonian is given by:

$$\hat{\mathcal{H}} = -\gamma B_0 \hat{I}_z$$

(4.3)

where $\hat{I}_z$ is the operator representation of $z$-angular momentum. The evolution of the system over time is given by Schrödinger’s equation:

$$\frac{d}{dt}\psi(t) = -i\hbar^{-1}\hat{\mathcal{H}}\psi(t)$$

(4.4)

The spin system generally contains multiple interacting spins, who may be affected by a number of factors in addition to the strong static field. Those factors may be external to the sample, i.e. caused by something in the environment, usually the spectrometer. One such perturbation that is a corner-stone of modern NMR, is the application of pulses of electromagnetic radiation at the Larmor frequency; such pulses are used to manipulate the overall state of the spin system. Other interactions are internal, such as the dipole-dipole coupling—the direct interaction of a particular nuclear magnetic moment and another magnetic moment (nuclear or electronic), or the J-coupling—the indirect interaction between two nuclei connected through chemical bond(s). In addition to the magnetic interactions certain nuclei are affected by electric fields, where the most important phenomenon is the interaction between the electric quadrupolar moment of the nucleus and the electric field gradient at the site of the nucleus. All such interactions may be represented by Hamiltonian operators, and the dynamics of the spin system can be calculated by solving the Schrödinger’s equation, given in Equation 4.4. The formalism of such spin systems and their time evolution under different types of interactions will not be described here. Instead a few NMR observables, and the information they provide, will be discussed.
4.1.2 Chemical shifts

NMR spectroscopy would be of limited use if every nucleus of a certain type had the exact same resonance frequency. Luckily, this is not the case. Each nucleus has a certain molecular surrounding, with a specific electron distribution, giving a local magnetic field that slightly alters the resonance frequency from the (theoretical) value of a nucleus only experiencing the applied static field. The frequency difference is called the chemical shift, and is generally a tensor. In solution, however, the rapid molecular reorientation averages the angular dependence to an isotropic chemical shift.

**Structural information from chemical shifts**  The chemical shift of the resonance frequency of a particular nuclear spin contains information on the local magnetic environment, which is primarily created by the electron distribution. Although in principle possible, the derivation of atomic coordinates directly from the chemical shift values is not feasible, due to limitations in the theoretical framework and computing capacity. Instead, a number of strategies have been developed to estimate protein structures from chemical shift information, each one using some combination of *a priori* knowledge of the molecular system, empirical knowledge, and molecular modelling. For example, atoms belonging to residues in ordered structural elements have different chemical shift values than corresponding atoms in disordered segments. Hence, a common strategy to derive structural information, or at least structural propensities, is to compare observed chemical shift values with database averages of values from different types of structural elements.

**The effects of ligand binding on the chemical shifts**  If a ligand binds to a receptor, some residues, both on the ligand and the receptor, necessarily have their chemical environment modified. Hence nuclei of either the receptor or ligand, or both, may be studied. Depending on the details of binding (energetics, kinetics, etc), these perturbations will have different effects; a case of ligand-induced structure would give large shift differences for the nuclei in the receptor backbone, while for weak binding even nuclei in the vicinity of the binding site may have changes close to the ‘chemical shift noise’ level, as seen in Paper I.

**Estimating solvent partitioning through chemical shifts**  In a situation where a molecule of interest may be in either of two chemical environments the chemical shift values may give information about the equilibrium distribution of molecules between the two environments. An example from the thesis
at hand is a system where a peptide is dissolved in a suspension of lipid complexes in buffer, as in Papers II and V. The time scale of the exchange between the environments strongly influences the properties of the observed spectrum. If the exchange kinetics is much faster than the difference between the resonance frequencies of a particular spin in the two environments, the result is a single narrow peak at the population weighted average of the two frequencies. If the exchange rate goes down, the peak gradually and is eventually split into two. This is the intermediate exchange regime. If the exchange is much slower, there will be two narrow peaks, one at each of the two frequencies.

4.1.3 Dynamics

Relaxation, i.e. the return from a non-equilibrium to an equilibrium state, is a rich and complex process in general, and this holds true also for the specific case of nuclei in NMR spectroscopy. For a complete treatment of relaxation in NMR, see e.g. Kowalewski et al. or Palmer.

Physically, relaxation in NMR is a consequence of small, local, time-dependent magnetic field fluctuations that, if they contain components at frequencies corresponding to energy differences between states in the spin system studied, induce transitions between these states. Since the transition probabilities are (slightly) larger in the direction towards low energy, after some time characterised by a relaxation time constant the perturbations cause the system to reach a steady-state population of the spin states affected by the perturbation. Following the discussion of e.g. Kowalewski and Mäler, such a perturbation may be represented by a stochastic function of time $Y(t)$, with an average value at time $t$:

$$\langle Y(t) \rangle = \int_{-\infty}^{\infty} yp(y, t) dy \quad (4.5)$$

Here $p(y, t)$ is the probability density, and if this density is independent of time, the stochastic process is stationary. An auto-correlation function may be defined as the average value of the product of $Y$ at two different times $t_1$ and $t_2$, and for a stationary process, this function will look as follows:

$$\langle Y(t_1)Y(t_2) \rangle \equiv G(t_2 - t_1) = G(\tau) \quad (4.6)$$

In the above equation $\tau$ is the separation between two time points, and $G(\tau)$ is the so called time-correlation function (tcf) of the stochastic process described by $Y(t)$. It describes the probability of two observations of $Y$ giving the same value, as a function of the time $\tau$ between the observations. Based on a few assumptions on the properties of the tcf, a reasonable form of $G$ is an exponentially decaying function:
\[ G(\tau) = G(0)e^{-\tau/\tau_c} \]  

(4.7)

where \( \tau_c \) is the correlation time of the perturbation. A Fourier transformation of \( G(\tau) \) gives a function that says how much effect the perturbation underlying \( G(\tau) \) has at any particular frequency. This distribution is the spectral density function, evaluated here for the function \( G \) as defined above:

\[ J(\omega) = 2 \int_0^{\infty} G(\tau)e^{-i\omega \tau} d\tau = G(0) \frac{2\tau_c}{(1 + \omega^2 \tau_c^2)} \]  

(4.8)

The perturbations, and associated spectral density functions, of interest here are time-dependent variations in the local magnetic field at specific molecular sites. Such perturbations are generated by various types of molecular motion, and hence the time constants and spectral densities contain information on these processes. Furthermore, relaxation involves a redistribution among the energy levels, and such redistribution is also caused by exchange processes. Hence exchange can be included in the theoretical framework of relaxation, and studied by similar experimental methods. An overview of molecular time scales and the associated NMR observables is shown in Figure 4.1.

In general, several relaxation mechanisms, some of them correlated, combine to give an effective relaxation from one particular state to another. This means that a particular relaxation rate is a sum of the spectral density functions of the relevant mechanisms, evaluated at the frequencies corresponding to the transitions involved in the relaxation. Here the discussion will primarily concern the relaxation of a \(^{15}\)N spin in the backbone of a protein in solution, even though some of the results are valid also for other cases. In a protein backbone the \(^{15}\)N nucleus has an attached \(^1\)H and relaxation is primarily caused by dipole–dipole interactions, and by chemical shielding anisotropy (CSA).

**Dipole–dipole interaction**

As the name suggests this is the direct interaction between two magnetic dipoles. In the case of NMR this means two nuclei, or the interaction between one atomic nucleus and one electron. The Hamiltonian representing the dipole–dipole interaction between two spins \( \hat{I} \) and \( \hat{S} \) may be found by translating the classical dipole–dipole interaction energy into the formalism of quantum mechanics:

\[ \hat{H}_{DD} = -\frac{\mu_0 \gamma_I \gamma_S \hbar}{4\pi r^3} \left( 3\hat{I} \cdot \frac{\mathbf{r}}{r^2} \cdot \hat{S} - \hat{I}\hat{S} \right) \equiv b_{IS} \left( 3\hat{I} \cdot \frac{\mathbf{r}}{r^2} \cdot \hat{S} - \hat{I}\hat{S} \right) \]  

(4.9)

In an IS spin system such as the \(^{15}\)N–\(^1\)H pair of nuclei previously introduced, there are four energy levels. Transitions between the levels are induced by
Figure 4.1: Time scales for NMR observables and physical processes in biomolecules, together with the corresponding frequency range. Adapted from Palmer.[108]
the dipole–dipole interaction described above with certain probabilities. For example $W_2$, the probability of a transition between the $\alpha_I\alpha_S$ and $\beta_I\beta_S$ energy states, is proportional to the spectral density at the sum of the two Larmor frequencies:

$$W_2 \propto b_{IS}^2 J(\omega_I + \omega_S)$$  (4.10)

**CSA interaction**  Earlier in this chapter, chemical shifts were introduced. The physical cause of chemical shifts are currents induced in the electron clouds surrounding the nuclear sites by the applied static field. These currents result in a small local magnetic field, a phenomenon that is called *chemical shielding*. Since the electron distribution is not uniform in the molecule, the induced currents have certain preferred orientations. Hence, also the induced magnetic field has a particular orientation in the molecular frame of reference, connected to the static field through a chemical shielding tensor $\sigma$: $B_0 - \sigma B_0$. In the case of rapid molecular reorientation, such as in a liquid, the chemical shift interaction is averaged to an isotropic value, as was stated earlier. This means that the orientational dependence of the chemical shielding is averaged out on the experimental time scale, and the effect on the resonance frequency is treated as if the shielding field was parallel to the external field. This does not mean, however, that the time-correlation function of the chemical shielding vanishes. Therefore, chemical shift anisotropy may contribute to relaxation and is indeed an important relaxation mechanism for nuclei in certain chemical environments.

**Protein and peptide backbone dynamics**  There are several ways of using NMR to study molecular dynamics. In recent years it has, for example, been great interest in NMR methods that probe motions on the microsecond to millisecond time scale, making it possible to study e.g. transition states in protein folding or aggregation. Here another methodology will be discussed, namely the study of peptide/protein backbone dynamics through measurements of the $R_1$ and $R_2$ relaxation rates, and the NOE factors, for $^{15}$N nuclei; see for example Kay et al. Mandel et al. and Mäler et al. As described previously, the rate constants are sums of spectral density functions describing relaxation caused by dipole-dipole and CSA interactions, according to the following expressions:
\[ R_1 = \frac{b_{NH}^2}{4} \left[ J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H + \omega_N) \right] + c_N^2 J(\omega_N) \] (4.11)

\[ R_2 = \frac{b_{NH}^2}{8} \left[ 4J(0) + J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H) + 6J(\omega_H + \omega_N) \right] \]
\[ + \frac{c_N^2}{6} \left[ 4J(0) + 3J(\omega_N) \right] \] (4.12)

\[ \text{NOE} = 1 + \frac{\gamma_H b_{NH}^2}{4\gamma_N R_1} \left[ 6J(\omega_H + \omega_N) - J(\omega_H - \omega_N) \right] \] (4.13)

where \( b_{NH} = \mu_0 \hbar \gamma_H \gamma_N / 4\pi r_{NH}^3 \) is the dipole–dipole interaction strength, \( \mu_0 \) is the permeability of free space, \( \hbar \) is Planck’s constant, \( r_{NH} \) is the length of the N–H bond vector, and \( \gamma_H \) and \( \gamma_N \) are the gyromagnetic ratios for \(^1\)H and \(^{15}\)N, respectively. \( c_N = \Delta \sigma \omega_N / \sqrt{3} \) is the CSA interaction strength, where \( \Delta \sigma \) is the CSA of the N spin. As can be seen in the equations, the \( R_2 \) relaxation rate contains a term proportional to \( J(0) \), meaning that this rate is affected by slow motions of the molecule, such as the overall tumbling. The effect of this is that the transverse relaxation rate in general increases with molecular size, up to a point where the signals cannot be detected. This is one of the reasons why solution-state NMR studies of membrane proteins are very challenging.

The observed relaxation rates may be analysed using Lipari-Szabo model-free formalism\(^1\) (equivalent to the two-step formalism by Halle and Wenerström\(^2\)). Here no particular motional model is assumed, but rather a separation of motional time scales. Using this assumption functional forms of the spectral density function can be constructed, depending on the details of molecular motion. For example, assuming isotropic rotational diffusion, one form of the spectral density function is the following:

\[ J(\omega) = \frac{S^2 \tau_m}{1 + \omega^2 \tau_m^2} + \frac{(1 - S^2) \tau}{1 + \omega^2 \tau^2} \] (4.14)

where \( S \) is a generalised order parameter, \( \tau_m \) is the overall molecular rotational correlation time and \( \tau = (1/\tau_m + 1/\tau_e)^{-1} \), where \( \tau_e \) is a correlation time describing intermolecular motion. Spectral density functions such as the above are fitted to the measured relaxation rate values and, if the assumptions are reasonable, the fitted parameters have physical relevance. For example the order parameter may correspond to the amplitude of N–H bond vector motions, and \( \tau_e \) to the correlation time of the local motion of this vector. Together these parameters give information on e.g. the flexibility of the protein backbone. Furthermore, any event that has an effect on protein backbone dynamics, such as a conformational change or interaction with another molecule, will cause
the fitted order parameter(s) and correlation time(s) to change. This type of
analysis was used in Papers I, II and V.

**Paramagnetic relaxation enhancement (PRE)** The magnetic moment of
an unpaired electron may enhance the relaxation of neighbouring nuclei through
the dipole–dipole mechanism. As previously described, the effect of this
interaction on the relaxation rates has a distance dependence of \(1/r^6\). The
dipole–dipole interaction energy is proportional to the product of the gyromagnetic
ratios of the two particles, and since the electron gyromagnetic ratio
is approximately 650 times larger than the nuclear equivalent, the relaxation
enhancement may be dramatic. Examples of applications of PRE include
studies of bilayer penetration of peptides or proteins by paramagnetic probes
on the lipids, or dissolved in the buffer. Such experiments were used
to estimate the positioning of DynA variants in bicelles in Paper II. Another
way to exploit this phenomenon is to attach a chemical moiety containing an
unpaired electron on a ligand. This results in a sensitive probe for ligand–
receptor interaction. This was used to study the interaction between DynA and
SOD1–EL2 in Paper II.

4.1.4 Diffusion

As discussed in the previous section, molecules undergo various types of mo-
tion. This section will focus on how translational diffusion is investigated
by NMR, a topic that has also been treated in several excellent review articles.

NMR measurements of molecular translational diffusion are based on the
application of magnetic fields whose strength vary as a function in position in
the sample. The most commonly used type is a linear variation along the z-
axis, often called a \(z\)-gradient. In the conceptually simplest experiment, dating
back to the early 1950’s and the work of Hahn and Carr and Purcell, the
pulse sequence is a spin echo, and spatial dependence is encoded and decoded
with two gradient pulses. The pulse sequence is depicted in Figure 4.2. In
the top panel, manipulation of the spins by RF-pulses is shown. Here a \(90^\circ\)
pulse moves the net magnetization vector to the \(xy\)-plane, where it produces a
decaying signal. After this follows a time period \(\tau\), after which a \(180^\circ\) pulse is
applied. The latter pulse has the effect of reversing the spin phase distribution
created by static field inhomogeneities, and after another interval \(\tau\) the signal
reemerges with an amplitude damped by a factor that depends on relaxation,
which we will not go into here. With the exception of small inhomogeneities,
molecules in different parts of the sample experience the same field strength,
and (magnetically equivalent) spins are completely refocussed, regardless of
where they are in the sample. Next we include the magnetic field gradients, shown below the RF-pulses.

\[ \begin{align*}
\text{Gradient} \quad \delta \quad \Delta \\
\text{a} \quad 180^\circ_y \quad 90^\circ_x \quad 1H \\
\text{b} \quad g \quad y \\
\text{c} \quad B_0 
\end{align*} \]

**Figure 4.2:** a) Basic pulse sequence for NMR measurements of translational diffusion. Schematic overview of the spin dynamics during the sequence, and the resulting spectra, in the b) absence and c) presence of diffusion. Adapted from Johnson.\(^{126}\)

In general a position-dependent field \( B_z \) is applied, and although the gradient \( g(r, t) \) of this field may have components in all spatial directions, the discussion here will be restricted to a \( z \)-gradient. We then have:

\[ g(z, t) = \frac{\partial B_z(t)}{\partial z} k \equiv g(t)k \quad (4.15) \]

The total magnetic field strength at time \( t \), as a function of position \( z \), will be a sum of the static field and the applied gradient field:

\[ B(z, t) = B_0 + g(t)z \quad (4.16) \]

The effect on the spins, in the absence of diffusion, is schematically shown in Figure 4.2b. Since the precession frequency is linearly dependent on magnetic field strength, an applied \( z \)-gradient pulse of duration \( \delta \) gives every spin a phase angle \( \phi \) dependent on position: \( \phi(z) = -\gamma B(z)\delta \). This means that two spins separated by a distance \( z \) acquire a phase difference \( \Delta \phi(z) = -\gamma \delta g z \) after
the gradient pulse (in the rotating frame). The situation after the first pulse in Figure 4.2 can be visualized as a ribbon of phases along the \( z \)-axis, as shown in the figure. The effect of the subsequent gradient pulse is to twist this ribbon into a helix. The 180° \( y \) pulse inverts the helix, while the final gradient pulse unwinds it, and at a time \( \tau \) after the 180° \( y \) pulse, the spin vectors are once again coherent, producing an echo. Again, if the spins do not move, the amplitude of the echo signal is dampened only as a consequence of relaxation, which is independent of the applied gradient strength. In such a case, the processed spectra would all have the same amplitude as a function of gradient strength, as shown in Figure 4.2 b. However, the molecules do undergo diffusion, and in a liquid this random motion through locations with different magnetic field strength cannot be neglected. The effect of diffusion is that the phase accumulated during the first gradient pulse is in general not negated by the second gradient pulse, giving an incomplete refocussing of phases, and a decreased signal amplitude. This is shown in Figure 4.2 c.

By varying the strength of the gradient pulses the signal amplitude is modulated, and it can be shown that the decay of the signal amplitude as a function of increased gradient strength ideally follows the Stejskal–Tanner equation:

\[
S(q) = S(0)e^{-Dq^2\Delta'} = S(0)e^{-D\gamma^2\delta^2g^2\Delta'}
\]  

(4.17)

where \( \Delta' \) is the time \( \Delta \) between gradient pulses as shown in Figure 4.2 minus a small correction due to diffusion during pulses. The attenuation factor \( D\gamma^2\delta^2g^2\Delta' \) in Equation 4.17 is the so-called Stejskal–Tanner factor. Data from a diffusion experiment are thus (ideally) bilinear with one spectrum for each level of gradient strength, and each of those spectra in general a superposition of spectra from components with different diffusion coefficients. As described above the diffusion modulates the spectral amplitudes and, as Equation 4.17 shows, these amplitudes theoretically have Gaussian dependence on gradient strength. Equivalently, the data are readily arranged as a 2D matrix with frequency along one dimension, and exponentially decaying amplitudes in the other dimension. In standard diffusion-ordered spectroscopy (DOSY) processing, an exponential function, such as in Equation 4.17 is then fitted to each peak or each spectral point in the data, and the diffusion constant \( D \) is extracted. Finally, the data are visualized in a 2D-plot with one spectral axis, and one with diffusion coefficient, with Gaussian peaks where the residuals from the fitting process give the peak widths. This gives a plot where signals from different molecules are spread out along a diffusion dimension, making the technique suitable for identifying components in a mixture.

Although the data are commonly represented as a two-dimensional plot, DOSY differs from ‘real’ 2D experiments where there are two temporal dimensions, each of which has a one-to-one mapping onto the frequency space.
through the Fourier transform. The diffusion dimension in DOSY is a statistical construct, with a function fitted to data. The problem is that this function may not be fully known, although the theoretical expression is available. One issue is that the decay often deviates from a simple exponential because of imperfections in the applied magnetic fields, etc (see for example Nilsson and Morris\textsuperscript{131}). A more fundamental problem is the fact that a peak in the frequency dimension may be a superposition of two signals, and if the peak height is fitted to a single exponential function, the apparent diffusion coefficient extracted will have a value in between the two correct values. Theoretically, the inverse Laplace transform could be used to map such superimposed decays onto the new dimension, in the same fashion as the Fourier transform, but the Laplace transform is mathematically different, with properties that make this procedure a numerically intractable problem for many realistic cases where the data contains noise. See Istratov et al.\textsuperscript{132} for a discussion of this.

4.2 Multi-way decomposition of experimental data

Multivariate analysis is a huge sub-field of mathematical and statistical analysis and can be described as the study of multiple variables and their interrelations. The simplest multivariate case would be two variables, here called two-way data, for example generated by a combined measurement of NMR frequency and diffusion coefficient for a nucleus, as shown in Figure 4.2. This is discussed below. Higher order data are generated by e.g. measurements on a certain observable on several occasions and on several samples, and the general multivariate situation gives multi-way data, arranged in multi-way arrays.

One area within this field is multi-way decomposition, in which the aim is to break down a data array into a number of components of low rank. The measured data are assumed to be a sum of components, each of which is a product of factors. The factors may, but need not, directly represent physical observables such as spectra. The last step is the actual decomposition, where the model is fitted to the data and the factors are estimated.

The underlying structure of the data may vary depending on the experimental origin, and hence also the choice of model to which the data is fit will be different. All applications of decomposition described in the articles of this thesis concern multilinear data, meaning that the factors are independent. The following discussion will be restricted to analysis of data with this particular property, and take as its starting point data generated by diffusion NMR. A review of multi-way methods to analyse 2D NMR data has been written by Pedersen\textsuperscript{133}. 28
4.2.1 Two-way decomposition

As discussed in the previous section and in Paper III, an example of bilinear two-way data is diffusion-edited NMR spectra. The data may be arranged in a matrix \( X \) \((I \times J)\) where each of the \( I \) rows is a spectrum of \( J \) points, recorded for a particular gradient strength level. If the sample contains \( N \) species, each one associated with a spectrum and a diffusion profile, the observed data \( X \) can be seen as a sum of \( N \) such \((I \times J)\) matrices, and written as:

\[
X = C^T S + E
\]  
(4.18)

Here \( C \) \((N \times I)\) contains the diffusion profiles and \( S \) \((J \times N)\) the spectra of the \( N \) components in the sample, and \( E \) contains measurement noise. A component here is anything with a distinct spectral profile and diffusion decay, and the aim of the analysis is to find the matrices \( C \) and \( S \) that best fit \( X \). It may be noted that this problem is a particular application of principal component analysis (PCA). Equation 4.18 can be written in an alternative element form:

\[
x_{ij} = \sum_{r=1}^{N} c_{ir} s_{jr} + e_{ij}, \quad i = 1, \ldots, I, \ j = 1, \ldots, J
\]  
(4.19)

4.2.2 Three-way decomposition

To extend the two-way model, we start from the element formulation of Equation 4.19, and trivially add a dimension:

\[
x_{ijk} = \sum_{r=1}^{R} a_{ir} b_{jr} c_{kr} + e_{ijk}, \quad i = 1, \ldots, I, \ j = 1, \ldots, J, \ k = 1, \ldots, K
\]  
(4.20)

This model is often called the PARAFAC model, from parallel factor analysis, as described by Harshman and others in the late 1960's. The model can also be written as a sum of outer vector products:

\[
X = \sum_{r=1}^{R} a_r \Delta b_r \Delta c_r + E
\]  
(4.21)

where \( X \) has the same meaning as before, but is now a three-way matrix, and the symbol \( \Delta \) is the outer vector (or tensor) product. The vectors \( a_r \) \((I \times 1)\), \( b_r \) \((J \times 1)\) and \( c_r \) \((K \times 1)\), describing the component factors, are often collected in matrices \( A \), \( B \) and \( C \). In contrast to the two-way case, the estimated factors of the type of three-way decomposition described here are unique (apart from trivial scaling and permutation), meaning that there are no two set of factors
with the same fit. This means that if the experiment is designed and performed in such a way that the data are indeed trilinear, the estimated factors will have direct physical relevance.

Since trilinearity is, in principle, the only condition on the data, three-way decomposition is extremely versatile. In the 1960’s the PARAFAC method was developed from factor analysis mainly by psychologists, in the particular area of psychometry, but already from the beginning the applicability of the method to data from such different fields as economy, electrical activity in the brain, and weather patterns was suggested\textsuperscript{136} Since then trilinear analysis has been used on data from a variety of experimental sources, and only a few examples will be given here. Apellof and Davidson used three-way decomposition to analyse fluorescence data from liquid chromatography experiments,\textsuperscript{138} while Lee and Ross applied trilinear decomposition to light spectroscopy data to study ligand binding to tyrosine.\textsuperscript{139} In the NMR field multi-way decomposition has been used to analyse e.g. non-uniformly sampled data,\textsuperscript{140} reaction kinetics,\textsuperscript{141} and toxin levels in the blood.\textsuperscript{142} In Paper IV of this thesis trilinear analysis is applied to diffusion NMR data on a mixture of three alcohols, and the power of the method in resolving heavily overlapping data is shown.
5. The interaction between dynorphins and opioid receptors

As touched upon earlier, one of the main areas of this thesis is how the peptide dynorphin A (DynA) interacts with a particular membrane receptor, an interaction that is a key part in the chain of events that constitute DynA signalling. On a general level DynA is a neuromodulator, where neuromodulation means the control, either through internal or external intervention, of activity in the nervous system. More specifically, DynA is produced and acts in the central nervous system (CNS), first and foremost as a part of the opioid system.

5.1 The opioid system

The opioid system is a collection of protein membrane receptors located in the CNS, and a number of ligands that interact with these receptors. Here the discussion will concern the vertebrate opioid system, which was formed early in vertebrate evolution. The ligands, the compounds that elicit their effects through the opioid system, are called opioids or opiates, and can be either endogenous, or exogenous, the latter produced in another organism or through chemical synthesis. The name of the system is derived from the opium poppy, from which the molecule morphine is isolated. The effects of this widely used drug—powerful pain relief but also abuse and addiction—illustrate some of the physiological processes mediated by the opioid system.

While a detailed model of the parts and mechanisms of the opioid system is only a few decades old, pure morphine was first isolated already in 1806 by Sertürner and a general knowledge of opiates and their effects on humans date back hundreds, or even thousands of years (see Brownstein or van Ree for interesting historical reviews). Much work in the opioid area has been motivated by the hope of replacing morphine as the prime analgesic with a drug that has fewer adverse side effects, a hope that has been rekindled by every breakthrough in the opioid research field. For example, the successful cloning of the opioid receptors in the early 1990’s prompted Reisine and co-workers to write that ‘The availability of the cloned receptors will facilitate the identification and development of more specific and selective compounds with
greater therapeutic potential and fewer undesirable side effects.\textsuperscript{150} Despite considerable efforts, however, an opioid ligand with high potency and no abuse potential remains to be designed, and the discussions regarding when, and how opioids should be therapeutically used is as active as ever.\textsuperscript{151}

A complete overview of the physiology of the opioid system is beyond the scope of this thesis, but in general this system is involved in the modulation of pain,\textsuperscript{152–153} as well as more complex behavioural processes,\textsuperscript{155} such as reward and addiction,\textsuperscript{156–158} and different types of mental disorders.\textsuperscript{159} Opioids have also been suggested to be a part of the immune response.\textsuperscript{160}

5.1.1 Opioid receptors

The existence of the opioid receptors (originally called opiate receptors) was demonstrated in the early 1970’s by the work of many researchers, but primarily the groups of Pert and Snyder,\textsuperscript{161–163} Goldstein,\textsuperscript{164} and Terenius.\textsuperscript{165–167} For a personal account, as well as an historical review of these years, see Snyder and Pasternak.\textsuperscript{168}

The opioid receptors are divided into four subclasses: the κ-, μ- and δ-opioid receptors, (commonly abbreviated KOR, MOR and DOR) and the nociceptin receptor (sometimes orphanin FQ receptor). These four classes are all of the G-protein coupled receptor (GPCR) type. The GPCR membrane proteins are involved in chemical signal transduction and are currently at the center stage of life science; they constitute one of the most important classes of drug-targets,\textsuperscript{169} and in 2012 two of the pioneers in the field—Robert Lefkowitz and Brian Kobilka—were awarded the Nobel prize in chemistry for work on GPCRs. In the general agonistic situation, a ligand interacts with the receptor, increasing the probability of the receptor to interact with one or several guanine nucleotide binding proteins (G-proteins). At the other end of the spectrum are full antagonists, i.e. compounds that block receptor activation. The activity of GPCRs is much more nuanced than a simple on–off mechanism, however, and there is evidence for a rather complex GPCR conformational landscape.\textsuperscript{170,171} The G-proteins function as transducers, and possibly amplifiers, propagating the signal by modulating one or several effector systems.\textsuperscript{172,173}

As recently as 2005, there was only one GPCR crystal structure in the literature, that of bacteriorhodopsin,\textsuperscript{174} but the preceding decades had seen great progress in both the understanding of GPCRs, and in membrane protein production technologies. The result of this has been the determination of several new structures in the last few years,\textsuperscript{175,184} and at the time of this thesis, the three-dimensional structures of 25 GPCR proteins have been determined.\textsuperscript{188} However important these structures are to the understanding of biological systems, several questions regarding GPCR function remain to be answered and
several problems remain to be solved. One such aspect is the scarcity of NMR structures—to date, the CXCR1 receptor is the only GPCR who has had its structure determined with high resolution by (solid state) NMR. In contrast to X-ray crystallography, NMR spectroscopy may be used under near-native conditions and provides valuable information on dynamics on different time scales, in addition to the molecular structure. Another problem is the lack of structural information on peptide ligands bound to GPCRs where only one such structure exists, that of the CXCR4 chemokine receptor in complex with a cyclic peptide. In addition to being the endogenous receptor ligands, the complexity of peptides compared to small molecules allows for correspondingly complex modes of receptor interaction. For example, the sizes of most peptide ligands mean that they interact with larger regions of the GPCRs, involving e.g. the extracellular loops.

A compelling feature of the opioid receptors (as well as other GPCRs) is their combination of high similarity, with respect to both sequence (60 to 70%) and structure, and high specificity in their interaction with ligands (in particular peptides or peptide analogues). The opioid receptors all have a GPCR type A (rhodopsin-like) fold, with seven transmembrane helices linked together by intra- and extracellular loops, as is shown by the schematic drawing together with the κ-opioid receptor (KOR) X-ray structure in Figure 5.1.

Based on structural information from all four opioid receptors, as well as on an abundance of functional and biochemical studies, some conclusions regarding the similarities and differences within the opioid receptor family have been drawn. One of the main common features of the receptors is a binding pocket in the transmembrane region, a region that has a large inter-receptor sequence similarity while the termini, and extra- and intracellular loops of the receptors are quite different on a sequence level. Therefore, the loops have been hypothesised to provide the ligand selectivity properties of the opioid receptors. Surprisingly though, in the crystal structures also these latter regions are structurally rather similar, suggesting that the differences modulating ligand selectivity are very subtle. Another possibility is that the crystal structures show the proteins in similar conformational states (all four structures have an agonist bound in the transmembrane pocket), while there are several other available states for the native proteins. Following this latter line of reasoning, and taking the generally larger conformational freedom of the loop regions into account, it is reasonable to believe that the extra- and intracellular parts of the receptors may be more structurally diverse and more dynamic in the native environment than can be deduced from the crystal structures alone. Indeed, recent studies have shown that the extracellular regions of GPCRs may be conformationally regulated by ligand binding.

A strong argument in support of the selectivity-providing roles of the loops
are chimeric studies showing that the selectivity pattern of one receptor subtype may be transferred to another subtype by exchanging extracellular fragments. Later in this chapter we will return to the importance of the extracellular loops in the specific case of the \(\kappa\)-opioid receptor.

![Diagram of the \(\kappa\)-opioid receptor](image)

**Figure 5.1:** a) Schematic overview of the topology of the \(\kappa\)-opioid receptor (KOR). b) KOR crystal structure (PDB accession code 4DJH), with the extracellular loop II (EL2) highlighted in yellow. The DynA peptide, approximately drawn to scale, is included for comparison.

### 5.1.2 Dynorphin A—an opioid ligand

As mentioned in the opening of this section there are many types of opioid compounds of different origin, with different pharmacological profiles, evoking different physiological responses. No systematic overview will be provided here, instead the problem of selectivity between opioid receptors and ligands will be discussed with a particular focus on peptide ligands. These molecules, like the receptors, have several features in common; one example is the so-called enkephalin sequence: Tyr–Gly–Gly–Phe–Leu/Met, that constitutes the N-terminal sequence of all typical opioid peptides. This story of selectivity will be told from the viewpoint of the dynorphin A peptide, and start with a brief historical account.
In the 1970’s, a compound with distinct morphine-like properties was isolated from cow brain. The substance was later identified as an endogenous opioid peptide, named dynorphin after its high potency in an assay using the contractions of guinea pig ileum, and had its sequence determined (YG-GFLRIRPKLKDWNQ). Further research established that this peptide exerted its opioid effects mainly through interaction with the \( \kappa \)-subtype of the opioid receptors (KOR) and that there were two types of dynorphins, dynorphin A (DynA) and dynorphin B (DynB), emanating from the same precursor protein: proenkephalin-B (PDYN).

Slightly anecdotally, the early history of opioid peptides marks the culmination of a most interesting life science controversy, described from a sociological perspective in Collins’ and Pinch’s book The Golem. This controversy had started in the early 1960’s with results apparently showing that memories or capabilities could be acquired by an organism (planarian worm) through digestion of brain tissue from another, ‘trained’ organism. Other researchers questioned this, the debate raged on, and was eventually propelled into the area of opioid peptides when George Ungar, a pharmacologist at Baylor College of Medicine in Houston, published a study in Nature describing the isolation of ‘scotophobin’, a fifteen-residue peptide that transferred a specific behaviour (dark-avoidance) when taken from trained rats and injected in mice. Accompanying the article was a lengthy critical comment from one of the reviewers, a rather unusual thing in scientific publishing. Avram Goldstein at Stanford, one of the leading scientists in opioid research, tried to replicate Ungar’s remarkable findings but was unsuccessful, and wrote a critical comment on the experiments behind Ungar’s claims. The debate continued, but towards the mid-seventies a fragile consensual model had been established in which peptides could induce a general (such as alertness), rather than a specific (fear-of-the-dark), behaviour, and the neuropeptide research continued along this trajectory. Goldstein continued his work, and in 1981 he was the first to isolate DynA from natural sources and determine the amino acid sequence.

5.1.3 Dynorphin A and the \( \kappa \)-opioid receptor

In competition assays DynA has been shown to bind the \( \kappa \)-opioid receptor (KOR) with nanomolar affinity, with a five-fold or higher selectivity as compared to the \( \delta \) and \( \mu \) subtypes, depending on the specifics of the competing ligands. Conversely there are peptide ligands such as Leu-enkephalin, a pentapeptide comprised of the DynA N-terminus, that have very low affinity for KOR. So, what are the detailed features of this interaction, and where does the selectivity come from? To answer this question a large number
of research groups have used truncations, mutations, chemical modifications
and various forms of cyclisation of DynA (and other peptides) to map out
the physico-chemical and structural properties needed for potent, selective and
tunable opioid receptor agonist and antagonist effects (see Paper[11] and refer-
exences therein for a few examples). This vast body of research is not so easily
condensed into a few simple rules, but some aspects can be highlighted. First,
it is now generally accepted that the N-terminal residues of the opioid pep-
tides (the enkephalin sequence), in particular Tyr1 and Phe4, interact with
residues in the transmembrane binding pocket of the receptors to trigger re-
ceptor activation. This region of the ligand is often called the ‘message’, while
the C-terminal parts of peptide ligands are called ‘address’ regions; a concept
coined by Goldstein et al. in 1981[20]. Furthermore it has been shown that
the second extracellular loop of KOR (EL2) is needed for high affinity dynor-
phin binding. The position of EL2 in the KOR can be seen in Figure 5.1. To account for the importance of extracellular receptor regions in opioid
peptide binding, and for the mechanisms of ligand selectivity, a few different
concepts have been proposed:

**Binding/favourable interaction:** The message-address model depicts the
ligand as consisting of two, not necessarily completely distinct, parts. The N-terminal sequence (message) activates (or blocks) the receptor
upon interaction with residues in the transmembrane binding pocket. The remaining peptide region (address) needs to have a favourable inter-
action with extracellular parts of the receptor for the ligand to reach and
bind in the transmembrane site.

**Selection through exclusion:** In this model selectivity is proposed to be im-
paired through an exclusive mechanism, i.e. unfavourable energetics in
the interaction between ligands and extracellular receptor regions keep
‘unwanted’ ligands out[190].

**Conformational mechanisms:** In this hypothesis, the extracellular loops
constrain the positions of the transmembrane helices, and, as a conse-
quence, the positions of contact points in binding sites[197].

In the literature there is support for all the proposed mechanisms, and a
reasonable conclusion is that for any ligand–receptor system, all three models
will be valid, but to different degrees. In the case of DynA–KOR interaction,
the abundance of positively charged amino acid residues in DynA (the dynor-
phins are among the most basic naturally occurring peptides in the body) and
negatively charged residues in the EL2 of KOR, suggest that electrostatic in-
teractions are important, or even primarily responsible, for the interaction be-
tween the two. This hypothesis has been tested in several studies, with mixed
results. Schlechtingen and co-workers\textsuperscript{215} performed binding and activity studies with KOP and various analogues of DynA, showing that Arg residues, especially Arg6 and Arg7 were crucial for $\kappa$-selectivity. Ferguson, on the other hand, reported that replacing charged residues (but not all) in EL2 did not affect DynA affinity and function.\textsuperscript{215} Also modelling studies have downplayed the importance of Coulomb forces in comparison with hydrophobic interactions\textsuperscript{217} and experimental studies of the EL2 peptide in micelles lend some support to this.\textsuperscript{218}

As described in Paper I, we addressed the selectivity issue by using a construct protein to probe the interaction of the KOR second extracellular loop (EL2) with DynA. This engineered protein consisted of a soluble $\beta$-barrel scaffold onto which the KOR–EL2 was grafted. The scaffold was a variant of human superoxide dismutase 1 (SOD1) in which the native loops had been truncated,\textsuperscript{219} and the engineered protein was hence named SOD1–EL2. Our characterisation of the SOD–EL2 protein by various types of NMR experiments, as well as CD, showed a construct where the core part was structurally very similar to the ‘parent’ scaffold protein SOD1, a stable and mainly $\beta$-structured protein with short connecting loops. The EL2 segment taken from the KOR, on the other hand, was much more flexible. Moreover, we did not see any evidence of strong interaction between the EL2 and the scaffold, and concluded that the EL2 indeed behaves as a loop in SOD1–EL2.

In the investigation of the interaction between SOD1–EL2 and DynA several NMR methods were used. The effects of DynA on NMR chemical shifts of ($^{15}$N-labelled) SOD1–EL2 backbone amides were small and, for many residues, impossible to distinguish from noise. A few residues, mainly located in the EL2-region or in areas of the scaffold in close proximity to EL2, showed slightly larger shift differences, differences that also changed consistently upon titrated addition of DynA. Also the SOD1–EL2 backbone dynamics changed very little upon addition of DynA, and these differences in relaxation parameters were deemed too small to be used as conclusive evidence for ligand binding. A fitting of order parameters was done, suggesting an increase in backbone rigidity in the presence of ligand (unpublished results).

Chemical shift experiments were also performed in which unlabelled protein was titrated onto $^{15}$N labelled DynA. In this series of experiments SOD1–EL2, the EL2 peptide and the naked scaffold protein were used. Here the results more conclusively showed an interaction between EL2 and DynA. Both the EL2 peptide and SOD1–EL2 caused consistent changes in the chemical shifts of DynA, while no chemical shift changes were observed when the scaffold without the EL2 segment (SODnoloops) was added, showing that the EL2-loop is required for DynA interaction. Assuming a one-to-one type of binding, dissociation constants were estimated from the chemical shift data.
These constants were in the micromolar range, suggesting a remarkably weak interaction compared with the nanomolar affinities previously reported for DynA binding to the full-length receptor.\textsuperscript{220,221} This indicates that the extracellular interaction is very different from the transmembrane binding. From a biological point of view this makes a lot of sense, however, since a too strong binding of the ligand to the extracellular regions of the receptor could impair the binding of the DynA N-terminus to the KOR transmembrane pocket, or significantly slow down the release of DynA from the receptor.

Further interaction studies were performed with a paramagnetic probe attached C-terminally to DynA. As described in Section 4.1.3 these experiments give information on the distances between an unpaired electron in the probe and surrounding nuclei, through the effects of the electron on the relaxation of the nuclear spins. Using this technique DynA–EL2 interaction was confirmed, with the spin-labelled DynA strongly affecting the EL2 segment in SOD1–EL2 protein.
6. The interaction between dynorphins and lipids

In Chapter 2 it was argued that biological membranes are integral parts of living systems. The membranes will be encountered by other constituents of such systems, for example proteins and peptides, carbohydrates, ions, and water molecules. Consequently, a very large, very diverse and very vital area of life science deals with what happens in or at membranes. What passes through, what gets embedded, what stays at the interface, what disrupts the bilayer, etc. In addition to fundamental physico-chemical questions about e.g. lipid bilayer properties and about biological processes such as membrane protein translation, this research area also includes medical applications. An example is the delivery of drugs (or other therapeutic entities) into cells, or into specific cellular organelles.

In the same chapter the various types of membrane-interacting proteins—peripheral, membrane-inserted and embedded—were introduced. The importance of membrane proteins can hardly be overemphasised. This is shown both by results from genome analysis, where membrane proteins, e.g. receptors, transporters and channels, are predicted to constitute 20 to 30 % of all proteins, and by figures from industrial and academic drug discovery research, where approximately 50 % of all drug targets are membrane proteins. Traditionally, high resolution 3D structures of membrane proteins have been few due to the difficulties of crystallising membrane proteins for X-ray spectroscopy, and the inherent limitations in using solution-state NMR on particles with large molecular mass, as discussed in the Methods chapter. Although tremendous progress has been made in X-ray technology as well as in the NMR area (both solution- and solid-state), structure determination of membrane proteins is still by no means trivial. The fact that the largest database of membrane protein structures contains approximately 1300 entries, while there are over 85 000 structures of soluble proteins in the Protein Data Bank (PDB) says something about the discoveries that are still waiting to be made in this area. Interesting as this subject is, in this chapter the discussion will not concern large membrane proteins, but instead peptides. More specifically it will treat the interaction between peptides and the lipids of biological membranes, again with a particular focus on DynA.
6.1 Peptides and membranes

Although the question of biological insertion of hydrophobic peptides into the membrane during the translation process is important and fascinating, the focus here will be on peptides that encounter the membrane from a polar environment such as the cytosol, the synaptic cleft, or the aqueous buffer in a test tube. In many cases such peptides interact with a protein or another peptide in the membrane, a situation that was discussed in the previous chapter. Here we will look at interactions directly with the lipid environment. A few different factors will determine to what extent and in what way such interaction occurs. In many cases electrostatic interactions will be important, with charged residues of the peptide interacting with charged groups of the membrane lipids. Also the number and distribution of hydrophobic residues are important, in particular for determining if the peptide will penetrate into the hydrophobic core of the membrane or not. How well-structured the peptide is determines to what extent hydrogen bonds can be formed between backbone atoms and bonding partners on the lipid molecules.

The different amino acid residue types have more or less specific preferences for the various parts of the membrane environment. Very hydrophobic residues such as leucines and isoleucines are comfortable in the hydrophobic core of the bilayer, tryptophan side-chains are often located in the membrane interface region, while residues with charged side-chains prefer the aqueous environment outside of the membrane, or interact with phosphate moieties in the lipids. This characterisation is, however, slightly simplified. For example, arginine is charged at physiological pH, but the side-chain also has hydrophobic properties, conferred by the aliphatic chain. It has been shown that Arg residues may be part of transmembrane protein regions, and minimise the energy penalty by having the charged end of the side-chain ‘snorkeling’ to the more polar membrane interface region. Such complicated modes of membrane interaction may be found also for other residues.

There are various ways of estimating the energetic cost or gain in transferring the different types of amino acid residues from the solvent to the membrane (or the other way around), and by doing such estimates it is possible to construct scales of hydrophobicity. Three widely used scales are 1) the Kyte-Doolittle scale, \(^\text{231}\), 2) the Wimley-White scale, \(^\text{232}\) and 3) the ‘biological scale’ from von Heijne and co-workers. \(^\text{226}\) The first scale used a combination of water-vapor partitioning of side-chain analogues, and residue accessibilities derived from twelve soluble proteins, to get hydrophobicity values for all residues. Wimley and White looked at partitioning of short peptides into POPC vesicles to get free-energy values and compile a scale, while von Heijne et al. studied the energetics of Sec61 translocon-mediated insertion of different
peptides into a membrane. By using such scales it is possible to estimate how probable it is for a particular peptide to be inserted into the membrane, either by itself or by another mechanism.

The different modes of membrane interaction of peptides are rather diverse, both in terms of how their conformation changes when going from the polar to the hydrophobic environment, as well as if multimerisation is a part of the interaction mechanism. A common situation is when the interaction with the membrane induces structure in a peptide that is less ordered in solution; such peptides are often amphitropic, where the transition from a water-soluble to a membrane-bound form controls peptide activity. An example of such a peptide is gramicidin, an antimicrobial peptide that multimerises in the membrane to form β-channels through the bilayer.

A particular type of interaction is represented by amphipathic helices, where there is a tendency of one face of the helix to interact with the bilayer surface, keeping the helical axis perpendicular to the bilayer normal. As the surface concentration of peptides increases, this interaction may be followed by a more disruptive interaction with the bilayer, as is the case for many antimicrobial peptides (AMPs).

6.1.1 The use of bicelles in peptide–lipid research

As described in Chapter 2 there are a number of available lipid systems that provide simplified models of a real biological membrane, for experimental studies where a native membrane is too complex. Here I will discuss bicelles, one type of membrane mimetic, and how they may be used to study peptide–lipid interactions, particularly with NMR spectroscopy. A general overview of membrane mimetics in biomolecular NMR studies is beyond the scope of this thesis, but a good introduction is given by for example Warschawski et al.

Bicelles were introduced in the late 1980’s as a tool for structural characterisation of biomolecules, based on findings that certain mixtures of phospholipids and detergents formed a liquid crystalline phase that was oriented by the static magnetic field of an NMR spectrometer. This tendency of the bicelle to align in a magnetic field is, to a large extent, defined by the $q$-value, the ratio between the concentration of lipids, $c_{lipids}$, and detergent molecules, $c_{detergent}$ in the bicelles:

$$q = \frac{c_{lipids}}{c_{detergent}}$$

(6.1)

At certain ratios where $q \gtrsim 2$ the field-aligning phase is formed. These bicelles will in turn weakly align other biomolecules such as proteins, creating the opportunity of measuring residual dipolar couplings (RDCs) that may
When the q-value is below this approximate threshold, the tendency of the bicelles to align vanishes, and the correlations times of the molecules in the assemblies get sufficiently fast on an NMR timescale to give high resolution spectral information. This type of bicelles, sometimes called *isotropic bicelles*, have been widely used for studies of lipid-interacting proteins and peptides. A schematic depiction of a bicelle together with a peptide is shown in Figure 6.1. The detailed morphology of isotropic bicelles has been a topic of debate, and concerns issues such as to what extent lipids and detergent molecules are segregated, the phase behaviour of the lipids, the possible coexistence of multiple types of complexes in a sample, and the overall shape of bicelles.

Several groups have studied DHPC/DMPC bicelles and observed that certain nuclei in the two molecules have different chemical shifts in bicelle samples but identical chemical shift values when solubilised in non-polar solvents. This is a strong indication that there is segregation between the two molecules in the bicelles. Furthermore, attempts at determining the size and shape of bicelles under various conditions have been made through measurements of e.g. NMR relaxation and diffusion scattering methods and electron microscopy. For DHPC/DMPC mixtures at low
q-values and near room temperature, both Luchette et al.\cite{246} and Glover et al.\cite{247} proposed disk-like objects, clearly distinguished from mixed micelles, although the latter group noted a change in morphology below a total PC concentration of 130 mM. A recent report by Beaugrand et al.\cite{251} suggests that the DMPC and DHPC molecules are only segregated for $q \gtrsim 1$ and that concentrations lower than 250 mM may lead to larger aggregates. In Paper III of this thesis, we studied DHPC/DMPC, $q = 0.5$, bicelles with $^1$H and $^{31}$P NMR spectroscopy, together with the decomposition methodology discussed in Paper IV and showed that the lipids are largely segregated. Furthermore, we concluded that the only species present are bicelles and free DHPC, and that the morphology does not change qualitatively for concentrations down to approximately 75 mM. This work also demonstrated the applicability and robustness of two-way decomposition of diffusion NMR data as an approach in analysing data on lipid mixtures such as bicelles.

6.2 Membrane interactions of dynorphin A

In the decade after their discovery, dynorphins were found to cause a variety of physiological responses even when the opioid receptors were blocked by antagonists such as naloxone. These non-opiate effects, including paralysis\cite{252} changes in the motor system\cite{253} and neural cell death\cite{254}, have mainly been studied in cellular systems, mice or rats. In some cases the underlying signalling pathways have been shown to involve other receptors, such as the NMDA receptor\cite{252,255–259} but dynorphins also interact directly with membranes. This is likely to have biological functions, and will be discussed below. Moreover, it appears plausible that membrane and receptor interactions work in concert; a particular lipid-induced conformation could for example be preferred by the receptor. Such structure induction has been observed in model membrane systems.\cite{260–264} Moreover, the membrane may serve as a surface for peptide adsorption and accumulation prior to receptor binding, as has been suggested by Schwyzer and Sargent.\cite{264,265}

Reciprocal to being affected by lipids, DynA has been shown to cause effects on membranes. The peptide does for example translocate into cells\cite{266} and induce calcium influx into vesicles\cite{267} as well as leakage in liposomes.\cite{268} Furthermore, DynA has been suggested to induce formation of vesicles in ordered bilayers.\cite{269} It may be noted that some of these properties overlap with cell-penetrating peptides (CPP), an interesting class of peptides that has been much studied\cite{270,275} and also used as a tool to deliver cargo such as siRNA\cite{276} and proteins\cite{277} into cells.

In a fairly recent study specific mutations in the DynA precursor gene were linked to a neural disorder.\cite{278} Some of these DynA variants were studied bio-
physically by Madani et al. who showed that the membrane interacting properties differed substantially between variants.\textsuperscript{279} In Paper II we describe investigations by CD and NMR spectroscopy of the bicelle interactions of two of these variants. One peptide variant, R6W–DynA, was found to have clear lipid perturbing properties, with a strong association and comparatively deep N-terminal positioning in bicelles, while another, with an L5S mutation, interacted only weakly with the bicelles. The more aggressive variant, R6W–DynA, associated strongly to bicelles, and in certain regions of the peptide a moderate helicity was induced, but no fully formed secondary structure was detected. Such behaviour has been seen also in wild-type DynA.\textsuperscript{260} In contrast to this, strongly channel- or pore-forming peptides such as gramicidin A exhibit a much more evident structure induction in the bilayer.\textsuperscript{234,280,281}

In Paper II, as well as in previous studies\textsuperscript{260} it was shown that wild-type DynA associates strongly with bicelles, and to better understand this interaction we examined the detailed molecular dynamics of wild-type DynA in bicelles, as described in Paper V. Here three residues, G2, L5 and L12, were labelled with $^{15}$N nuclei, giving the opportunity to use a battery of NMR $^{15}$N-relaxation experiments, as described in Chapter I. Overall, the observed dynamics were quite different in the labelled sites, indicating that the DynA peptide has a complex motional behaviour in the bicelle medium. Analysis of the relaxation data within the model-free framework indicated that the bicelles restrict the dynamics of the peptide, slowing down local correlation times and giving rather large order parameters. The restriction was slightly stronger with anionic DMPG lipids present in the bicelles, and most evident for the L5 residue. Such charge-dependent dynamics has also been observed for the CPP transportan,\textsuperscript{283} although this peptide is more structured in bicelles. Overall, the results suggest that DynA has a residue-specific interaction with lipids, despite the fact that only limited peptide structure is induced by the lipids.
7. Conclusions and outlook

The last chapters contained summaries of the main findings of this thesis work, in the respective area. Here I will draw a few conclusions, and put these conclusions in context.

7.1 What are the most important findings of this thesis work, and how can they be built upon?

Below is a list of findings, followed by a discussion on each item in the list.

1. An opioid receptor extracellular loop may be studied in a soluble protein environment.

2. DynA binds weakly to the KOR–EL2 loop.

3. The molecular dynamics of the membrane interaction of DynA is delicately balanced, and is affected by single-site mutations, as well as small changes in lipid environment.

4. Multi-way decomposition methods may be applied to NMR diffusion data on bicelle samples, and provide information that is complementary to DOSY analysis.

5. Mixtures of DHPC/DMPC, q = 0.5, behave like classical isotropic bicelles at concentrations from 300 to 50 mM.

The first item on the list is a conclusion from Paper I. The concept of moving a segment of a protein to a new protein has been attempted with some success, but constructs with membrane protein segments in soluble proteins have been more difficult to design. Moreover, the idea of transplanting a site of weak binding rather than the main binding pocket is to my knowledge much less investigated. Our successful attempt suggests a more general opportunity of studying the weak interactions involved in ‘filtering’ of ligands in systems similar to DynA–KOR, such as the other opioid receptors, or other GPCRs in general. In such systems the much stronger interaction of the ligand
with the primary binding pocket of the receptor may obscure the more delicate dynamics and kinetics of the extracellular interactions.

The results on the interaction between DynA and SOD1–EL2 have a few interesting aspects. One is the small magnitude of the interaction; judging from the amount of positive and negative charge in DynA and EL2, respectively, one would be forgiven for expecting a stronger interaction. But such a feeble binding actually makes biological sense, a stronger binding could possibly have prevented the opioid core (N-terminus) of the DynA peptide from reaching the transmembrane interaction site.

Studies on a scrambled DynA peptide (unpublished), suggest that this also interacts weakly with the loop, hence apparently downplaying sequence specificity. An obvious continuation of this research is the design and production of constructs similar to SOD1–EL2 but with other (opioid) receptor loops could, and interaction studies of these with an expanded library of ligands, starting with other opioid peptides. A more complete experimental system based on the SOD1–EL2 methodology may be one way to map out some of the selectivity differences between the receptors in this family.

The third point highlights the fascinating ambiguity of DynA lipid interaction. In many ways the peptide is evidently membrane disrupting, but it does not form stable structures in the membrane. These properties are likely to have biological functions for e.g. receptor binding or peptide trafficking. It was recently shown that β-endorphins and other peptide hormones are stored in particular types of vesicles in the cell, tightly packed as amyloids. Such results add to a more detailed understanding of the properties, mechanisms and functions of similar peptides. Moreover, an increased knowledge on the role of electrostatics and hydrophobicity for e.g. transient pore formation is important for applications such as novel antibiotics inspired by antimicrobial peptides.

Possible routes forward with the research on the DynA–lipid interactions includes mutation studies with systematic residue variations. Another interesting issue to address is the possibility of cooperative behaviour such as accumulation of peptides on or in the membrane, and formation of intramolecular complexes. So far we have no evidence of such multimerisation, but it may occur under certain circumstances, contributing to membrane destabilisation and disruption.

Overall, the combined results from the studies of DynA interacting with KOR and membranes may be used to interpret the link between the DynA variants described in Paper II and SCA, a disease that they cause. Such an interpretation, it has to be remarked, needs to be made with some caution. The disease is caused by the death of certain neural cells in the cerbellum, and this cell death must in some way be coupled, at least partly, to DynA. The two DynA variants that we investigated had single-site mutations on residue five.
and six, respectively. For the latter variant, R6W, a charged amino acid is replaced with a tryptophan. This means substituting a very polar residue for a type that interacts strongly with lipid bilayers. Not surprisingly this variant had clear membrane-perturbing properties, and both these properties *per se* and their influence on DynA-mediated opioid receptor activity may contribute to cell malfunction. For the L5S variant, the relatively low membrane activity most likely excludes the possibility of induction of cell death through bilayer disruption. On the other hand, membrane-induced priming of the peptide for receptor interaction may also be abolished. Perhaps more importantly, the substituted residue belongs to the N-terminal enkephalin pentapeptide, common to all typical opioid peptides, and highly important for peptide-mediated receptor activation. Hence, a substitution in this sequence very likely diminishes the ability of the peptide to activate the receptor, and in extension decreases neural signalling.

Item 4) and 5) on the list concern bicelles, and methods to study them. Multi-way decomposition of NMR data is a powerful tool to analyse mixtures, something that is thoroughly discussed in Paper IV. In Paper III one type of multi-way analysis was employed to study fast-tumbling DHPC/DMPC bicelles, and one of the things we could show was that this method provides valuable information on samples where there are no resolved signals from the two species. As for the morphology of the samples, we detected only bicelles of one type, together with free DHPC, but no other complexes such as micelles. Both these findings are valuable when considering bicelles against other membrane mimetics for a particular experimental design.

An interesting continuation of these studies would be to increase the dimensionality of the data by additional measurements, and use three-way (or higher) analysis methods. Another possibility is fluorescence correlation spectroscopy (FCS) to get single-particle information on bicelle morphology.

7.2 Critical review of methodology, results and conclusions

Like in much of biophysical research, the approaches used here to study biological systems are very reductionistic. In the case of KOR-DynA interaction, only a fragment (less than 10% of the total membrane protein sequence) was studied, in an environment that lacks all membrane components. Likewise, in the membrane interaction studies, membrane mimetics involving a maximum of three different lipid types were used, and instead of the continuous and highly irregular surface of a biological membrane there was a suspension of small lipid complexes. How valid are these approaches?
Starting with the DynA–KOR-EL2 interaction studies, a criticism of our approach may be that a fragment taken out of a protein context is not representative of the native system and, hence, any ligand–interaction observed in the model system has no relevance for the interaction in the native system. In response to this there are previous studies showing that isolated membrane protein fragments keep many of the biophysical properties of the full-size system. A valid question is, however, the importance of a disulfide bridge in the native receptor, anchoring EL2 to a transmembrane helix. There are other such disulfide bridges, and even though these are, in general, critical for antagonist inactivation of the receptor, \[289\] the importance for the EL2–DynA interaction is difficult to assess since the loss of function may be a consequence of other effects caused by the removal of the cysteines.

Moving to the membrane interaction of DynA, the choice of bicelles as membrane mimetic system can be discussed. The arguments in favour of using bicelles were formulated in Chapter \[6\] and may be summarised in two sentences. First, for high-resolution solution state NMR experiments, systems much larger than bicelles are, in practice, impossible to use. Second, the primary alternative in the bicelle size range is a micellar system, but although micelles are often used to solubilise membrane-interacting proteins and peptides, in contrast to bicelles they do not contain a lipid bilayer environment, and they have a very curved surface. Also the types of lipids used in our studies may be questioned. The use of phosphatidylcholine (PC) lipids is motivated by the abundance of PC lipids in mammalian neural cell membranes. For example PC is the major component of rat neuronal plasma membranes, constituting almost 30% of total lipid amount.\[290\] PG is, on the other hand, not a major component in neural cells, but its overall negative charge is a surrogate for other charged lipids such as PS and PI which are present to various degrees in biological membranes. It is of course possible that the dynorphin peptides in the native cellular environment could interact mainly with membrane regions enriched in lipids with another headgroup than PC, but I am not aware of any such findings.
I dikten Sonnet to Science beklagar sig Edgar Allan Poe över hur vetenskapen, den ”gam vars vingar är den trista verkligheten”, hindrar det poetiska manöverutrymmet. Hur saker ändras när de väl träffats av vetenskapsens granskande blick. ”Det är ju du som [...] skingrat flodens trolska skymningsvakter, och älvors dans som genom kvällen drog” utbrister Edgar, ”och det är du som drivit bort i vinden, min sommars drömmar under tamarinden.”


Vissa ser det magiska och fantastiska också i det vetenskapliga, andra tycker, som Poe, att något går förlorat. Oavsett vilken åsikt man har är det svårt att förneka att de vetenskapliga modellerna av komplicerade mänskliga fenomen som minnen, personlighet, medvetande, känslor, och så vidare blir allt bättre. Sådana komplexa saker hänger ihop med våra hjärnor, och vad som händer i dem. Och här är signalering ett oerhört viktigt begrepp. Cellerna i hjärnan tar emot information i form av elektriska signaler från resten av nervsystemet, och denna information måste sedan processas, vilket innebär att hjärncellerna, neuronerna, måste kommunicera med varandra. Kommunikationen är till stor del kemisk, det är molekyler som skickas som signaler mellan cellerna. Sådana molekyler kallas signalsubstanser, eller neurotransmittorer, och en signal-
koppling mellan celler är en synaps. I varje millimeterstor kub i hjärnan finns det ungefär 80 000 neuroner och 4.5 miljoner synapser.

Den signalsubstans som står i centrum i denna avhandling är dynorfin A (DynA). DynA är en peptid uppbyggd av 17 aminosyrarester, vilket gör den ungefär hälften så stor som sin kanske mer kända släktling endorfin. Ändelsen -orfin i dessa båda signalsubstansers namn är ingen slump, både dynorfin och endorfin har smärtlindrande effekter som liknar morfin (endorfin betyder just “kroppens eget morfin”) och båda ämnena tillhör en större grupp molekyler som kallas opioida peptider. I Figure 3.1 visas olika avbildningar av en peptid. Produktion av DynA i det centrala nervsystemet hänger ihop med belönings- och beroendesystem, och forskare tror också att det är inblandat i vissa former av psykisk sjukdom, även om kopplingen inte är helt klar. Stora mängder av DynA kan verka bedövande och till och med paralyserande.


Ytterligare en intressant fråga är hur DynA växelverkar direkt med de lipider, fettläkande ämnen, som är en av de viktigaste byggenstenarna i biologiska membran. Hur forskare tänker sig att biologiska membran ser ut visas i Figur 2.2. Många peptider påverkar lipider, vissa kraftigt, som till exempel peptiderna i giftet hos bin och getingar, som gör hål i membranet. Andra peptider har mer

Sammantaget bidrar resultaten i denna avhandling till en bättre förståelse av hur DynA interagerar med receptorn KOR. Dessutom kan de metoder vi utvecklat användas för att studera liknande system där en peptid, eller annan molekyl, binder till en receptor. Även om vi inte arbetat med sådana tillämpningar, är peptid–receptor-system mycket viktiga inom läkemedelsutveckling, eftersom receptorer liknande KOR är betydelsefulla för en mängd biologiska processer, och därmed mycket viktiga mål för olika läkemedel.

Även resultaten av bicellstudierna är viktiga både för den grundläggande förståelsen av dessa membranliknande, eller membranmimetiska, system, och rent praktiskt för dem som använder biceller som ett verktyg i sin forskning.
I would like to warmly thank a number of people, whose generous contributions of time, skill and encouragement have brought this thesis into existence. This section has not room for everyone who deserves to be acknowledged, and I can only hope that you who are not named accept this general expression of gratitude. I could not have done it without you.

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