Optical Investigations of Neurohypophysial Excitability and Amyloid Fibril Formation

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Optical Investigations of Neurohypophysial
Excitability and Amyloid Fibril Formation

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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# Table of Contents

List of Tables ................................................................................................................................... iii

List of Figures ................................................................................................................................... iv

Abstract ........................................................................................................................................... vi

Chapter 1: Dynamic Modulation of Neurohypophysial Excitability .................................................. 1
  1.1 Introduction ............................................................................................................................... 1

Chapter 2: Background ...................................................................................................................... 4
  2.1 Electrical Signaling in Neurons ................................................................................................. 4
  2.2 The Neurohypophysis as a Model for Excitation Secretion Coupling .................................. 10
  2.3 Voltage-Sensitive Dyes (VSDs) ............................................................................................... 12

Chapter 3: Materials and Methods .................................................................................................. 17
  3.1 Tissue Preparation ................................................................................................................... 17
  3.2 Optical Recordings .................................................................................................................... 17
  3.3 Dye Staining and Pharmacological Interventions .................................................................... 20
  3.4 NEURON Simulations ............................................................................................................. 21

Chapter 4: Measuring the Action Spectra of VSDs in Intact Tissue ................................................ 25
  4.1 Introduction .............................................................................................................................. 25
  4.2 In Situ Measurements of the Action Spectra of Voltage-Sensitive Dyes ................................. 27
  4.3 Fourier Filtering of the Action Spectra .................................................................................... 28
  4.4 Summary and Discussion ......................................................................................................... 30

Chapter 5: Dynamic Modulation of Neurohypophysial Excitability via Potassium Accumulation ......................................................................................................................... 33
  5.1 Introduction .............................................................................................................................. 33
  5.2 Modulation of Neurohypophysial Excitability Depends on the Overall Level of Activity ...... 34
  5.3 Extracellular Potassium Accumulation and Neurohypophysial Excitability ......................... 36
  5.4 Lack of Evidence for the Role of GABA<sub>A</sub> Receptors .................................................... 40
  5.5 Role of Ca<sup>2+</sup> Influx in Amplitude Modulation of Population Action Potential .............. 41
  5.6 Temperature Effects on Excitability Changes .......................................................................... 43
  5.7 Comparing Numerical Predictions and Optical Recordings of AP Waveforms .................. 44
  5.8 Summary and Discussion ......................................................................................................... 47
Chapter 6: Amyloid Fibril Formation ..............................................................50
6.1 Introduction .........................................................................................50

Chapter 7: Background .............................................................................53
7.1 Amyloid Fibrils ..................................................................................53
7.2 Fourier Transform Infrared Spectroscopy (FTIR) ..............................54
7.3 Intrinsic Fluorescence Spectroscopy ..................................................61

Chapter 8: Materials and Methods ..........................................................63
8.1 Protein Sample Preparation .................................................................63
8.2 FTIR Spectra Measurements .................................................................64
8.3 Intrinsic Tryptophan Fluorescence Spectroscopy ..............................65

Chapter 9: Evolution of Secondary Structure of Lysozyme Amyloid Assembly .............67
9.1 Introduction ........................................................................................67
9.2 Changes in Secondary Structure Upon Amyloid Fibril Formation ..........68
9.3 Differences in Secondary Structure Between Oligomeric and Oligomer-Free Pathways .................................................................................................71
9.4 Time-Course of the Structural Evolution in the Oligomeric Pathway ....73
9.5 Summary and Discussion ...................................................................74

Chapter 10: Amyloid Growth under Near-physiological Conditions ...............76
10.1 Introduction .......................................................................................76
10.2 Temperature-Induced Denaturation of Lysozyme ..............................77
10.3 Secondary Structure of Amyloid Fibrils Grown in Denaturing and Native Conditions .................................................................................................78
10.4 Lack of Growth Along Monomeric Filament Pathway Under Near-physiological Conditions .................................................................79
10.5 Summary and Discussion ...................................................................81

References ...............................................................................................82
List of Tables

Table 3.1  NEURON Simulation Parameters.................................................................24
Table 7.1  Amide Bands............................................................................................57
List of Figures

Figure 2.1  Action Potential Propagation Along an Axon ...........................................7
Figure 2.2  State Variables and Time Constants vs. Membrane Potential for Na⁺ and K⁺ Channels ...........................................................................................................9
Figure 2.3  Electron Micrograph of an Axon Terminal and Pituicyte .........................11
Figure 2.4  Structure of di-4-ANEPPS ........................................................................13
Figure 2.5  Schematic of the Fluorescence Emission Spectrum of an Electrochromic Dye .......................................................................................................................15
Figure 3.1  Microscope and Recording Chamber ..........................................................18
Figure 3.2  Frankenhaeuser-Hodgkin (F-H) Space ........................................................22
Figure 4.1  In Situ Fluorescence Emission and Action Spectrum of di-4-ANEPPDHQ ......26
Figure 4.2  Fourier Analysis of the Action Spectrum for di-4-ANEPPDHQ .................28
Figure 4.3  Testing the Effects of Fourier Filtering on Fluorescence Spectra ...............29
Figure 4.4  Action spectra for Various VSDs .................................................................31
Figure 5.1  Optical Recordings of Neurohypophysial Excitability vs. Stimulation Frequency and Intensity ...............................................................................................34
Figure 5.2  Modulation of Excitability in Slices vs. the Intact Neurohypophysis ..........36
Figure 5.3  Effects of Varying External Potassium Concentration on AP Modulation ....37
Figure 5.4  Excitability Responses in Hypertonic Saline ..............................................38
Figure 5.5  Effects of Ouabain on Neurohypophysial Excitability ..............................40
Figure 5.6  Excitable Responses in the Presence of the GABA<sub>A</sub> Blocker Bicuculline ........41
Figure 5.7  Lack of Calcium Dependence for AP Depression ......................................42
Figure 5.8  Neurohypophysial Excitability Changes at Physiological Temperatures .......................... 44
Figure 5.9  Numerical Predictions of $K^+$ Accumulation Effects on AP Responses and Comparison with In-Situ Optical Recordings ........................................................................ 46
Figure 7.1  Cross-$\beta$ Structure of Amyloid Fibrils ........................................................................ 54
Figure 7.2  Molecular Vibration Modes .......................................................................................... 56
Figure 7.3  Schematic of Bruker Optik Vertex 70 FTIR Spectrometer ............................................. 58
Figure 7.4  FTIR Interferogram and Transmission Spectrum .............................................................. 59
Figure 7.5  ATR Crystal .................................................................................................................. 61
Figure 9.1  AFM Images of Monomeric Filaments and Oligomeric Protofibrils ......................... 68
Figure 9.2  Infrared Spectroscopy of Late Stage Fibrils shows characteristic $\beta$-sheet Peaks ................................................................................................................................. 70
Figure 9.3  Difference Spectra for Monomeric Filaments and Oligomeric Protofibrils ............ 72
Figure 9.4  Time-Course of Difference Spectrum for Lysozyme Solution Undergoing Oligomeric Fibril Growth ................................................................................................................. 73
Figure 10.1  Thermal Denaturation Curve for Lysozyme ................................................................. 77
Figure 10.2  Difference Spectra for Amyloid Aggregates Grown Under Denaturing or Near-Physiological Conditions ........................................................................................................... 78
Figure 10.3  Stability of Monomeric Filament Seeds at 37 °C in pH 7 Buffer ............................... 79
Figure 10.4  Failure of Monomeric Filaments to Seed Amyloid Growth Under Near-Physiological Conditions .......................................................................................................................... 80
Abstract

This dissertation describes the work done on two distinct projects. In the first part I sought to unravel the mechanisms that underlie the activity-dependent modulation of response in the excitation-secretion coupling of the neurohypophysis. In the second part, I optically monitored and analyzed the secondary structure changes accompanying amyloid fibril formation along multiple pathways, under both denaturing and near-physiological conditions.

Neuronal plasticity plays an important role in regulating various biological systems by modulating release of hormones or neurotransmitters. The changing response to the same stimulus, depending on the context and previous stimulation events, is also the basis of learning and all higher order brain functions. The mechanisms behind this modulation are widely varied, and are often poorly understood in specific tissues. In this work, we examined excitation-secretion coupling in the neurohypophysis, a tissue composed of densely packed axons that secretes the hormones arginine vasopressin and oxytocin. The release of hormones depends not only on the overall level of activity in the gland, but also upon the specifics of the temporal pattern of stimulation. By optically monitoring the electrical activity using voltage sensitive dyes, we were able to investigate this plasticity in the intact gland. Varying extracellular potassium concentration in the bath, increasing interstitial space via hypertonic saline, and retarding potassium reuptake with ouabain all showed that extracellular potassium accumulation drives the depression of excitability. This effect is hidden from glass micro-electrode recordings because of the inevitable damage sustained by the surrounding tissue. Furthermore, no calcium
mediated release mechanism played any significant role in the depression. Numerical simulations confirmed the findings and give more insight to the details of the mechanism.

Deposits of amyloid fibrils, long, unbranched polymeric protein aggregates, are the molecular hallmark for a variety of human diseases, including Alzheimer’s disease, Parkinson’s disease, and type II diabetes. While the amyloid fibrils all share a characteristic cross-β sheet structure, the proteins that make up the aggregates have no unifying theme in either native structure or function.

In this research, I characterized the structural reordering that accompanies this aggregation using Fourier transform infrared spectroscopy (FTIR). Hen egg white lysozyme forms fibrillar aggregates with two distinct morphologies, depending on the growth conditions. At acidic pH with low ionic concentrations, lysozyme forms the fibrils with standard amyloid morphology. These aggregates are long and stiff but with the cross sectional area of a single monomer. At higher salt concentrations, the aggregation follows another pathway, under which oligomers initially form and later assemble into protofibrils. The oligomeric protofibrils are thicker than the monomeric filaments, but are much more curvilinear. These fibrils are not universally recognized as amyloidogenic aggregates. Using FTIR, I showed that both this aggregates are indeed amyloid structures, but that they are structurally distinct.

While it is generally accepted that partial unfolding of the protein is a prerequisite for amyloid fibril formation, we found that native protein can be the substrate for amyloid growth when seeded with preformed oligomeric or protofibrillar aggregates. These seeded fibrils grown under near-physiological conditions are structurally indistinguishable from those grown from partially unfolded protein under denaturing conditions. This incorporation and restructuring of native monomers is characteristic of prion-like assembly.
Chapter 1

Dynamic Modulation of Neurohypophysial Excitability

1.1 Introduction

Understanding neuronal plasticity is an important aspect in unraveling the workings of the brain. The basis of learning and all higher order brain functions is that the same stimulus can lead to varying responses, depending on the context and previous stimulation events. This modulation can occur over time scales that range from hours down to milliseconds.

The mechanisms behind this modulation of excitable responses vary dramatically. In the case of long-term neuronal potentiation in the CA1 region of the hippocampus, the enhancement of synaptic efficacy is associated with morphological changes to the cells and the appearance of new dendritic spines (Engert and Bonhoeffer, 1999). In the same brain region, other groups have found that the calcium-binding protein S100B, produced in the supporting glial cells, modulates this long-term potentiation (Nishiyama et al., 2002). The origins of short-term facilitation and depression are also wide ranging. Modulation of excitability has been traced to the afferent release of neurotransmitters such as GABA (γ-Aminobutyric acid) (Engelman and MacDermott, 2004). Paracrine glutamate signaling from astrocytes has also been observed in the dorsal horn of rats (Bardoni et al., 2010). Additionally, changes in the ionic concentrations of the extracellular environment, particularly calcium and potassium (Lu et al., 2010; Jensen and Yaari, 1996), can have distinct effects on the excitability of neurons.
In this work, we examined excitation-secretion coupling in the neurohypophysis. The neurohypophysis is a neuroendocrine gland that secretes the hormones arginine vasopressin (AVP) and oxytocin (OT). The release of hormones from the neurohypophysis depends not only on the overall level of activity in the gland, but also upon the specifics of the temporal pattern of stimulation. We can optically monitor with high temporal resolution the effects that the patterns of stimulation have on excitable responses in the intact gland using voltage sensitive dyes (VSDs). The unique cellular structure of this tissue lends itself well to the use of optical measurements rather than only the local measurement of electrical responses using patch clamping. The neurohypophysis is mostly composed of a tightly packed collection of axons that enter through the narrow infundibular stalk. This density of excitable tissue and the convenient region at the infundibular stalk to initiate action potentials allow for a high signal to noise in the fluorescent measurements. That same density of axons also raises the question of whether or not the observed modulation in excitability arises from extrinsic sources such as afferent neurotransmitter release or glial feedback, or from the intrinsic geometry of the tissue. These effects would be difficult, if not impossible, to investigate using traditional electrophysiology because the glass electrodes alter the tissue environment surrounding the axon being patched onto.

In order to optimize the recording conditions for the voltage-sensitive dye measurements, we also developed a method to measure the action spectrum, or dye response spectrum, of a VSD in situ using a spectrophotometer coupled to a microscope via a fiber optic cable. The response of a dye to voltage changes can vary dramatically depending on the tissue and other factors. Because the shift in emission spectrum of an electrochomic dye during action potential
depolarization is typically quite small (<0.5 nm), it is crucial to determine the optimal recording parameters for a given dye and tissue combination.

Using this information about action spectrum of di-4-ANEPPDHQ, we chose appropriate conditions to monitor the excitability of the neurohypophysis while altering stimulation intensity and frequency, and performed various ionic and pharmacological interventions. The conclusions drawn from the optical recordings were then compared with numerical simulations of action potential propagation.
Chapter 2

Background

2.1 Electrical Signaling in Neurons

Electrical signals are transmitted along axons via action potentials. An action potential is a short (typically about 1 ms) depolarization of the membrane potential that propagates from the soma, or cell body, down to the axon terminus (Figure 2.1B). This signal can be passed to another neuron (synaptic transmission) or will trigger the release of neurotransmitters, hormones, or other signaling molecules to tissues throughout the body via the blood stream.

At rest, the electric potential across the membrane of an axon is typically in the range of -70 mV (inside against outside). This membrane potential is made possible by the ionic concentration differences across the axon membrane. In the case of two compartments with different salt concentrations separated by a membrane permeable to only one ion, that ion will diffuse down the concentration gradient. This imbalance of charge will give rise to an electric potential across the membrane, and equilibrium is reached when the chemical potential differences is balanced by the counteracting electrical potential difference across the membrane. The Nernst equation relates the ion concentrations in the two compartments, $c_a$ and $c_b$, to the resulting membrane potential, $\Delta V$ (Jackson, 2006). Here, $R$ is the universal gas constant ($R = 8.314 \text{ J K}^{-1} \text{ mol}^{-1}$), $T$ is temperature (K), $z$ is the ion charge (dimensionless), and $F$ is Faraday’s constant ($F = 9.648 \times 10^4 \text{ C mol}^{-1}$).
The environments about axon membranes contain more than a single salt, though, and the membrane is not equally permeable to all ions, so a more general equation is needed. The Goldman-Hodgkin-Katz voltage equation takes permittivity of multiple ions, $P_{\text{ion}}$, and the concentrations inside, $[\text{Ion}]_b$, and outside, $[\text{Ion}]_a$, the axon into account. Generally, this equation can accommodate any number of ion species, but the resting membrane potentials of axons are principally governed by only three types of ions: $\text{Na}^+$, $\text{K}^+$, and $\text{Cl}^-$.

$$
\Delta V = -\frac{RT}{zF} \ln \left( \frac{c_b}{c_a} \right)
$$

Outside the axon, there is a high concentration of sodium ions and low concentration of potassium ions (typically about 150 mM and 5 mM, respectively). Within the cell, these concentrations are reversed. The chloride concentration is on the order of 20 mM inside and 100 mM outside the axon. If the permittivity of any one ion is much larger than the others, this equation approaches the Nernst potential for that ion. In typical mammalian axons, $P_K >> P_{\text{Cl}}, P_{\text{Na}}$, so the resting potential is mostly set by the potassium Nernst potential (Jackson, 2006).

While the resting permittivity of the membrane is due to small leak currents and ion transporters, ion channels can selectively allow large conductances of ions across the cell membrane. There is a great diversity of channels, with over one hundred ion channel genes identified (Purves et al., 2008). These ion channels are formed from one or more transmembrane proteins. Membrane spanning domains of the proteins form a pore through which ions can...
diffuse into or out of the cell. The opening and closing of these pores can be controlled by membrane potential, ligand binding, mechanical distortion of the membrane, or even temperature fluctuations. Ligand-gated channels are crucial to cell to cell signaling and synaptic transmission, and stretch- and heat-activated channels are vital in the somatic sensory system, but voltage-gated channels drive action potential propagation.

Voltage-gated sodium and potassium channels are responsible for the membrane depolarization and repolarization during an action potential. An action potential is initiated when the local membrane potential is depolarized beyond a threshold potential, typically on the order of -50 mV. This causes the sodium channels in the immediate vicinity to open, allowing sodium ions to flow into the axon, following the concentration gradient. This current further depolarizes the membrane, and the depolarization spreads as the Na⁺ current passively flows down the axon. As the membrane becomes more depolarized, the sodium channels begin to inactivate while the potassium channel start to open. The flow of potassium from the high [K⁺] axon interior into the low [K⁺] interstitial space repolarizes the membrane as the action potential passes. The sodium channels remain inactive for a short (~10 ms) refractory period, preventing backward propagation of the action potential. Figures 2.1A and B shows a cartoon of action potential conduction (Adapted from Purves et al., 2008).

The gating kinetics of these channels were described and modeled by Hodgkin and Huxley in a series of papers in 1952. They shared the 1963 Nobel Prize in Physiology or Medicine with Sir John Eccles for this work. By controlling the membrane potential while monitoring the ionic currents, they were able to describe the channel activity as a function of voltage and time. When the membrane potential is given a depolarizing step from its resting state, the sodium initially flows into the axon through the open, activated channels. After a short
Figure 2.1 Action Potential Propagation Along an Axon

(A) A schematic of an axon shows the opening and closing/inactivation of sodium and potassium channels and ensuing ionic currents during action potential propagation. (B) The membrane potential at three points along the axon is plotted against time, highlighting the depolarization, repolarization, and hyperpolarization phases. (Figure adapted from Purves et al., 2008).
time (~ 1 ms) of being held at the depolarized potential, the sodium channel becomes non-conducting and the associated Na\(^+\) current ceases. This step is called inactivation, and follows a simple exponential decay. The opening of the channels, however, shows cooperative behavior, with the transition occurring sharply over a relatively narrow range of membrane potential values. The rising phase of this sigmoidal activation follows an exponential raised to the third power, implying that the opening of the channel may be dependent on three voltage sensors, or gates. The total conductance is written as:

\[ g_{Na} = \bar{g}_{Na} m^3 h \]

If all of the sodium channels are open and active, the conductance is \( \bar{g}_{Na} \). The gating parameter \( m \) gives the fraction of the voltage sensing units that are in the conformation needed for an open channel, while \((1-h)\) gives the fraction of the channels in the inactive state. Thus, \( m^3h \) gives the fraction of active, open channels. Both \( m \) and \( h \) are dependent on the membrane potential and relax from their initial values, \( m_i \) and \( h_i \), to their equilibrium values, \( m_\infty \) and \( h_\infty \), with time constants \( \tau_m \) and \( \tau_h \). The expressions for \( m \) and \( h \) are:

\[
m = (m_i - m_\infty)e^{-t/\tau_m} + m_\infty
\]

\[
h = (h_i - h_\infty)e^{-t/\tau_h} + h_\infty
\]

Similarly, Hodgkin and Huxley modeled potassium channels and found non-inactivating potassium channels in addition to the inactivating type. The inactivating potassium channels are modeled in the same manner as the sodium channels, but the expression for the conductance of
the non-inactivating channels lacks the factor of \( h \). The parameter \( n \) plays the role of \( m \) in potassium channels, with the conductance for a non-inactivating channel given below.

\[
g_{K,\text{non}} = \bar{g}_{K,\text{non}} n^4
\]

\[
n = (n_t - n_\infty) e^{-t/\tau_n} + n_\infty
\]

In modeling the neurohypophysis, four types of potassium channels are included: both inactivating and non-inactivating A-type (fast activating) and BK-type (slowing activating) channels. Plots of the equilibrium state variables and times constants versus membrane potential for sodium and A-type non-inactivating potassium neurohypophysial channels are shown in Figure 2.2.

![Figure 2.2 State Variables and Time Constants vs. Membrane Potential for Na\(^+\) and K\(^+\) Channels](image)

(A) The equilibrium values of the gating parameters for sodium channels (\( h_\infty \) and \( m_\infty \)) and non-inactivating A-type potassium channels (\( n_\infty \)). (B) The corresponding time constants for the gating parameters. These values were calculated by C. Brad Bennett (Bennett and Muschol, 2009).
2.2 The Neurohypophysis as a Model for Excitation Secretion Coupling

The neurohypophysis, or posterior pituitary, is a neuroendocrine gland that secretes the hormones arginine vasopressin (AVP) and oxytocin (OT). Arginine vasopressin and oxytocin are both nine residue neuropeptides that are synthesized in the magnocellular neurons of the paraventricular and supraoptical nuclei of the hypothalamus. They are packed into vesicles and transported down the axons of the magnocellular neurons to the neurohypophysis. These axons are densely packed in the tissue, along with supporting glial cells, called pituicytes, and blood vessels. The axons have a “beads on a string” morphology, with in-line secretory swellings distributed all along the axons. It is from these secretory swellings, or varicosities, that AVP and OT are released into the bloodstream.

Arginine vasopressin is crucial in maintaining cardiovascular homeostasis. As early as 1895, the vasopressor effect of extracts from the neurohypophysis was noted (Holmes, Landry, & Granton, 2003). This constriction of blood vessels is particularly important during hypovolemic shock, when AVP levels can reach 20-200 times the baseline concentration. Arginine vasopressin also produces strong antidiuretic effects that have been clinically exploited since the 1950s (Holmes, Landry, & Granton, 2003). To a lesser degree, AVP has been shown to play a number of roles in regulating social behavior in rodents. Knockout mice lacking the V1b receptor show a decrease in maternal aggression and social recognition. Injections of AVP, however, increase partner preference in male prairie voles (Caldwell & Young, 2006).

The early investigations into effects of oxytocin found that OT facilitates smooth muscle contractions in the uterus during labor (Lee, Macbeth, Pagani, & Young, 2009). Oxytocin release is highly elevated during suckling and plays an important role in lactation. Furthermore, the effects of oxytocin on social behavior are wide ranging. Oxytocin promotes partner
preference in female prairie voles, a behavior that is removed with the treatment of an OT receptor antagonist (Lee, Macbeth, Pagani, & Young, 2009). In human studies, oxytocin treatment was shown to increase generosity in a money splitting game (Zak, Stanton, & Ahmadi, 2007). When oxytocin was administered to autistic subjects, their comprehension of the emotions in content-neutral speech was improved (Hollander, et al., 2007). Oxytocin is also implicated in numerous other social and sexual bonding behaviors (Macdonald & Macdonald, 2010).

The axons from the hypothalamus, the pituicytes, and the blood vessels that comprise the neurohypophysis are extremely closely packed. Electron microscopy shows that the interstitial space can be as narrow as tens of nanometers (See Figure 2.3). This density of excitable tissue

![Figure 2.3 Electron Micrograph of an Axon Terminal and Pituicyte](image)

**Figure 2.3 Electron Micrograph of an Axon Terminal and Pituicyte**

An axon terminal (A) and pituicyte (P) from a mammalian neurohypophysis are shown, highlighting the tight packing of the cells. The scale bar is 224 nm. (Figure from Hatton, 2002.)
makes the posterior pituitary gland an attractive model for optical measurements of neuronal activity. Because the axons are so tightly packed, patch clamp electrophysiology, the standard method to investigate neuronal activity, fails to completely tell the tale of the electrical response to trains of stimuli. The glass microelectrodes used to probe the axons are able to yield detailed information on the behavior of individual neurons, yet the unavoidable destruction of the surrounding cells prevents the study of the interactions that occur within the intact tissue. By employing voltage sensitive dyes, we are able to accurately measure the electrical activity of the axons, including the effects that arise from interactions with neighboring axons.

It has been known for quite some time that dynamic changes in the excitable responses of neurohypophysial axons and their secretory swellings play a prominent role in modulating hormone release during patterned stimulation of the mammalian neurohypophysis (Gainer et al., 1986; Nordmann and Stuenkel, 1986; Dyball et al., 1988; Bourque, 1991; Jackson et al., 1991; Jackson and Zhang, 1995; Branchaw et al., 1998; Zhang et al., 2007). The question persists, however, whether such activity-dependent modulation of excitability in the intact neurohypophysis arises purely by regulation from within individual terminals or whether the complex architecture of neurohypophysial axons and their secretory swellings, as well as their integration into a tightly packed tissue, contribute to this activity-dependent modulation of release.

### 2.3 Voltage-Sensitive Dyes (VSDs)

Voltage-sensitive dyes (VSDs) are powerful optical indicators of electrical activity in tissues that are either inaccessible to electrode recordings (e.g. neuronal axons or dendrites) (Antic and Zecevic 1995; Nuriya et al. 2006; Muschol et al. 2003) or that require multi-site
Figure 2.4 Structure of di-4-ANEPPS
The structure of a common voltage sensitive dye, di-4-ANEPPS. From the left, it shows the hydrophilic propylsufonate head group, attached to the pyridinium acceptor, linked to the aminonaphthyl donor with an ethane linker, followed by two butyl chains. (Adapted from Loew, 2010, Figure. 2.2.)

recordings in order to unravel the complex spatio-temporal patterns of electrical activity (e.g. neuronal assemblies, neuroendocrine tissue, heart and muscle physiology) (Zecevic et al. 1989; Matiukas et al. 2006; Tai et al. 2004; Obaid et al. 1999). Since their discovery over forty years ago (Salzberg et al. 1972; Salzberg et al. 1973), fast response dyes have been shown to provide optical (absorption or fluorescence emission) responses linearly proportional to transmembrane voltage, and with response times sufficiently fast for faithful optical recordings of action-potentials from excitable tissues (Antic and Zecevic 1995; Salzberg et al. 1993; Zhang et al. 1998; Jin et al. 2002).
The most widely used VSDs are electrochromic dyes, in which the emission spectrum shifts as the membrane potential changes via a molecular Stark effect. While the structures of these dyes can vary, some trends do emerge. Typically, and common to the VSDs used in these investigations, one or more alkyl chains are attached to a donor group which is connected to an acceptor group with a linker. A hydrophilic head group is attached to the acceptor group. Figure 2.4 (Adapted from Loew, 2010) shows resonance structures for di-4-ANEPPS. The VSD used in most of our experiments, di-4-ANEPPDHQ is identical to di-4-ANEPPS except for the polar head group.

Upon absorption of a photon with an appropriate energy, the pyridinium moiety accepts the excited electron from the donor aminonaphthyl group. There are many energetically close sublevels in the excited state, and after a vibrational relaxation to a lower energy, but still electronically excited state, the molecule can emit a photon and return to its ground state. The lack of a single, well defined energy level for the excited state and for the ground state leads to broad excitation and emission spectra. This vibrational relaxation also causes the emission spectrum to be lower in energy (longer wavelengths) than the excitation spectrum.

The hydrophobic hydrocarbon chains on one end of the dye and the polar head group on the other end cause the VSD to be aligned perpendicularly to the surface when inserted into the axon membrane. While the membrane potential change during an action potential may only be about 100 mV, the lipid bilayer is only about five nanometers thick, so the change in electric field is on the order of $10^7$ V/m (Jackson 2006). This electric field along the length of the dye causes a molecular Stark shift that redistributes the energy states (Loew, 2010). Typically, this results in a blueshift in emission during action potential depolarization.
Figure 2.5 Schematic of the Fluorescence Emission Spectrum of an Electrochromic Dye

(A) Schematic of an emission spectrum of an electrochromic dye at rest (red) and during action potential depolarization (blue). The shift is greatly exaggerated to show detail. (B) Action potential of the dye. The spectrum at rest is subtracted from the spectrum during depolarization. The dashed vertical line indicates the wavelength of the maximum in resting spectrum, emphasizing the minimal voltage response at the dye’s fluorescence emission peak.

Usually, the emitted light is not recorded with a spectrometer, but a non-spectral intensity detector such as a photodiode array, photomultiplier tube (PMT), or camera with a charge-coupled device (CCD) detector (Antic and Zecevic 1995; Zhang et al. 1998). Because the fluorescent response is a shift in wavelength rather than a change in intensity, it is important not to simply record the intensity near the emission peak. The schematic in Figure 2.5 shows an electrochromic shift of an emission spectrum and its corresponding action spectrum, the
difference between the spectrum at rest and during depolarization. The emission spectra were modeled as Gaussian curves, and the magnitude of the shift is greatly exaggerated to more clearly show the two spectra.

Widespread use of voltage-sensitive dyes has been hampered by several practical limitations, among them the small magnitude of the optical responses and the risk of phototoxic damage to the preparation. While detection of small fractional changes in optical properties improves with increased dye staining and illumination intensity, the risk of phototoxic damage is reduced by minimizing staining and light exposure. Due to these conflicting requirements on optical recordings, optimizing optical recording parameters for the specific dye/tissue combination used in a given experiment is important.
Chapter 3
Materials and Methods

3.1 Tissue Preparation

NIH Swiss female mice, typically 30- to 60-day-old, were euthanized and their pituitary glands were harvested following procedures approved by the Institutional Animal Care and Use Committee (IACUC protocol # 3174). The neurohypophysis along with a portion of the infundibular stalk and the surrounding pars intermedia were separated from the anterior pituitary. The intact neurohypophysis was mounted in a custom-designed optical recording chamber (Figure 1.3A) and perfused with oxygenated (95% O₂, 5% CO₂) physiological saline (154 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 10 mM glucose; pH 7.4). The infundibular stalk was placed between two platinum iridium (90/10%) field electrodes. The health of the neurohypophysis following dissection was assessed by measuring an intrinsic optical signal associated with excitation-secretion coupling in the unstained preparation (Salzberg et al. 1985). The preparation was allowed to rest for 20 min after dissection and typically for 10 min between each stimulus train.

3.2 Optical Recordings

All optical recordings were performed on an upright BX61WI microscope (Olympus America; Center Valley, PA) using a 20× 0.5 NA water-immersion objective (Figure 3.1).
Figure 3.1 Microscope and Recording Chamber

(A) The custom-designed optical recording chamber. (B) The chamber mounted on the stage of an Olympus BX61WI upright microscope with a top mounted Andor iXon high speed CCD camera.

Action potentials were initiated by brief (500 μs) bipolar shocks, supplied by an SIU-102 stimulus-isolation unit (Warner Instruments, LLC; Hamden, CT, USA). The stained preparations were epi-illuminated using high-power, low noise LEDs (Salzberg et al. 2005). For most of the recordings of transmembrane voltage, a 470 nm LED (MBLED; Thorlabs, Newton, NJ), driven at 100-200 mA with a stabilized constant-current LED controller (Mightex; Pleasanton, CA), was used as excitation light source. The illumination from the LED was bandpass limited with a 482/35 nm filter (Semrock; Rochester, NY), and fluorescence emission was selected with a 536/40 nm bandpass filter (Semrock). The filters were housed in a
fluorescein isothiocyanate (FITC) filter cube with a 500 nm edge dichroic beamsplitter (FITC-Di01-Clin-25x36; Semrock). To measure the emission spectra of the VSDs, the same LED and filter cube were used, but the emission filter was replaced with a with a 515 nm long-pass filter (OG 515 lp; Schott Advanced Optics; Mainz, Germany). The excitation light source for the recordings of intercellular calcium was provided by a 530 nm LED (M530L1; Thorlabs), driven at 100 - 300 mA. The illumination from the LED was bandpass limited with a 542/50 nm filter (Semrock), and fluorescence emission was selected with a 620/52 nm bandpass filter (Semrock) housed within a tetramethylrhodamine isothiocyanate (TRITC) filter cube with a 562 nm edge dichroic beamsplitter (FF562-Di03-25x36; Semrock).

To capture the brief and transient changes in fluorescence dye emission spectra induced by action-potential stimulation lasting only about 5 ms, we used an AvaSpec 2048-USB2 CCD-array spectrophotometer (Avantes USA; Broomfield, CO). The spectrophotometer was fitted with a 600 lines/mm grating and a 2048 pixel CCD with a detection range of 267 to 819 nm, resulting in a nominal resolution of 2.1 nm. On the CCD detector, however, the spectral data are sampled every 0.27 nm and with 16 bit resolution of intensity changes. A built-in order-sorting filter reduces contamination of the spectra from higher-order diffraction peaks. To preserve the small voltage-induced shifts in fluorescence emission, the default smoothing algorithm in the acquisition software had to be disabled. Light output from the camera port on the BX61WI microscope was focused with 10× 0.25 NA objective onto the 600 μm fiber-optic cable input to the spectrophotometer. To minimize dye bleaching and possible phototoxic damage to the preparation, LED illumination was limited to a total of 0.6 s prior to and during electrical stimulation. Synchronization of the LED illumination, the electrical stimulation of the
neurohypophysis, and the spectral recordings of dye emission was provided by an STG1004 stimulus generator (Multichannel Systems, Reutlingen, Germany).

For measurements of transmembrane voltage and intracellular calcium, images were acquired at up to 2,500 frames per second with a 128×128 pixel EMCCD camera (Andor iXon DV860BV; Belfast, Northern Ireland), cooled to -60 °C and using 16×1 binning. Action-potential modulation occurs spatially uniformly throughout the entire preparation (Muschol et al. 2003), so the camera was binned to allow for the higher acquisition rates of up to 2,500 frames per second, and fluorescence changes were integrated over the entire field of view (430 μm × 430 μm) to improve the signal to noise ratio. Synchronization of the electrical stimulation of the neurohypophysis with the LED control and camera recordings was provided by an STG1004 stimulus generator. The majority of measurements were performed at room temperature (24 °C). Under these conditions, action potentials are sufficiently broad that CCD recording at maximal speed (2.5 kHz) showed no under-sampling artifacts. In addition, the viability of the preparation is significantly extended. Control experiments at physiological temperature (37 °C) yielded qualitatively identical results but suggested quantitative shifts in the frequency- and stimulus number dependence of the observed effects.

3.3 Dye Staining and Pharmacological Interventions

To obtain optical recordings of the electrical activity, the preparation was stained with the fast-response potentiometric dye di-4-ANEPPDHQ (Obaid et al. 2004), obtained from Invitrogen (Carlsbad, CA). A 5 mM dye stock solution was prepared by dissolving the dye into a mixture of dimethyl sulfoxide and Pluoronic F-127 (90/10%). The dye stock solution was diluted in physiological saline to a concentration of 15 μM for staining, and the preparation was incubated
for 50 minutes. For measurements of intercellular calcium levels, the preparation was incubated with the membrane permeant calcium-sensitive dye mag-fluo-4, AM (Invitrogen) at 25 μM for 90 minutes. Again, a 5 mM dye stock solution was prepared with a mixture of dimethyl sulfoxide and Pluronic F-127 (90/10%). Pharmacological agents were generally added directly to the perfusion medium and administered tonically. Following solution changes, the preparation was allowed to rest for 15-25 min prior to any recordings.

The action spectra (i.e., the change in fluorescence dye emission induced by membrane depolarization) of di-4-ANEPPDHQ and four additional styryl-based voltage-sensitive dyes (di-8-ANEPPS, JPW 1114 (di-2-ANEPEQ), RH 414, and FM1-43) were determined. All dyes were obtained from Invitrogen. As in the case of di-4-ANEPPDHQ, stock solutions of di-8-ANEPPS, JPW 1114, and RH 414 were prepared by dissolving the dye into a (90/10%) mixture of dimethyl sulfoxide and Pluronic F-127 to a final dye concentration of 5–10 mM. FM1-43 was dissolved directly in distilled water instead. For staining of the preparation, 3–5 μL of dye stock was added to 1 mL of physiological saline for a final concentration of 20–40 μM. The preparations were typically stained for 45 min, with di-8-ANEPPS requiring 80–90 min incubation periods. Prior to fluorescence measurements, the dye solutions were washed out with physiological saline for 15 min.

3.4 NEURON Simulations

Numerical simulations of action potential propagation were performed using the multi-compartment modeling software NEURON, version 6.0.4 (Hines and Carnevale 1997; Carnevale and Hines 2006). The numerical model and physiological parameters used for our simulations of action potential propagation through individual neurohypophysial axons were developed in our
Figure 3.2 Frankenheuser-Hodgkin (F-H) Space
Schematic of the F-H space surrounding the axon in the NEURON numerical simulations. The potassium concentration in the axon interior and the extracellular space is fixed while the concentration in the F-H space is allowed to vary with activity.

lab, initially by C. Brad Bennett (Bennett and Muschol 2009), with voltage dependencies, gating kinetics, channel densities, and maximum conductances for these channels adopted from Jackson and Zhang (1995). These parameters are summarized in Table 3.1. Active and passive electrical properties of the model axons were matched to those of neurohypophysial axons and swellings. Besides voltage-gated Na\(^+\) channels, these axons contain both fast (A-type) and slow (BK-type) K\(^+\) channels (Bielefeldt et al. 1992; Thorn et al. 1991). All voltage-gated channels were distributed at uniform density along the surface of the axon. Standard Hodgkin-Huxley gating variables were used to model inactivating (m\(^3\)h) and non-inactivating (n\(^4\)) voltage-gated ion channels. The length of individual computational compartments along the axon cable was kept below 10 \(\mu\)m, compared to the electrical space constant of \(\lambda = 450\ \mu\)m or more for the axon. Typically, time steps of 25 \(\mu\)s were used to update all variables during simulations. AP propagation was initiated by current injection into the left-most computational compartment. NEURON provided direct output for the transmembrane potential and capacitive and ionic transmembrane currents. Potassium accumulation was introduced into this model by creating a
Frankenhaeuser-Hodgkin space: a thin (60 nm) layer of extracellular space enclosing the axon, in which potassium accumulates during action potential stimulation (Frankenhaeuser and Hodgkin 1956, Carnevale and Hines 2006). The thickness of the extracellular space was derived from an electron micrograph of a neurohypophysial axon (Figure 12 in Hatton 2002). Potassium clearance and diffusion were represented by exponential relaxation ($\tau = 800 \text{ ms}$) to the fixed external bath concentration (4.0 mM) outside this Frankenhaeuser-Hodgkin space (See Figure 1.4). The relaxation simulates the effects of both $K^+$ clearance via reuptake into various compartments (axons and glia) and radial diffusive transport following Fick’s law.
### Table 3.1 NEURON Simulation Parameters

Axon and channel parameters used in NEURON simulations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$c_m$ Membrane capacitance</td>
<td>1 μF/cm$^2$</td>
</tr>
<tr>
<td>$r_a$ Cytoplasmic resistivity</td>
<td>100 Ω cm</td>
</tr>
<tr>
<td>$g_{Na}$ Maximum specific Na$^+$ channel conductance</td>
<td>39 mS/cm$^2$</td>
</tr>
<tr>
<td>$g_{K_{A,inact}}$ Inactivating A-type K$^+$ channel conductance</td>
<td>13 mS/cm$^2$</td>
</tr>
<tr>
<td>$g_{K_{A,non}}$ Non-inactivating A-type K$^+$ channel conductance</td>
<td>4.5 mS/cm$^2$</td>
</tr>
<tr>
<td>$g_{K_{B,K,inact}}$ Inactivating BK-type K$^+$ channel conductance</td>
<td>13 mS/cm$^2$</td>
</tr>
<tr>
<td>$g_{K_{B,K,non}}$ Non-inactivating BK-type K$^+$ channel conductance</td>
<td>13 mS/cm$^2$</td>
</tr>
<tr>
<td>$g_{leak}$ Maximum leakage channel conductance</td>
<td>0.055 mS/cm$^2$</td>
</tr>
<tr>
<td>$e_{leak}$ Leakage channel reversal potential</td>
<td>-66 mV</td>
</tr>
<tr>
<td>$e_{Na}$ Na$^+$ channel reversal potential</td>
<td>69.7 mV</td>
</tr>
<tr>
<td>$e_K$ Reversal potential for all K$^+$</td>
<td>Variable</td>
</tr>
<tr>
<td>$-[Na^+]_{in}$ Internal Na$^+$ concentration</td>
<td>10 mM</td>
</tr>
<tr>
<td>$-[Na^+]_{out}$ External Na$^+$ concentration</td>
<td>152 mM</td>
</tr>
<tr>
<td>$-[K^+]_{in}$ Internal K$^+$ concentration</td>
<td>130 mM</td>
</tr>
<tr>
<td>$-[K^+]_{out}$ External K$^+$ concentration</td>
<td>Variable</td>
</tr>
<tr>
<td>$T$ Temperature</td>
<td>24 °C</td>
</tr>
</tbody>
</table>

*Adapted from Bennett and Muschol 2009*
Chapter 4
Measuring the Action Spectra of VSDs in Intact Tissues

4.1 Introduction

Voltage-sensitive dyes (VSDs) have proven to be important tools for studying the electrical activity in excitable tissue non-invasively. There are, however, difficulties that limit the use of VSDs, such as the small magnitude of their optical responses and the risk of phototoxic damage to the preparation. Increased dye staining and illumination intensity improves the detection of the response, but increases the risk of phototoxicity. In order to balance these conflicting requirements, it is necessary to optimize the optical recording parameters for the specific dye/tissue combination used in a given experiment. Unfortunately, the staining and fluorescence characteristics of fluorescent VSDs tend to vary significantly from preparation to preparation. Fluorescence emission spectra of voltage-sensitive dyes, for example, can undergo significant shifts of their spectral emissions, depending on their immediate environment (Ross and Reichardt 1979). Also, as shown in Figure 2.5, the potentiometric changes in dye fluorescence are largest at wavelengths on either side of the peak in their resting fluorescence.

The measurement of the action spectrum of a VSD (i.e. the difference of the fluorescence emission spectrum during and prior to stimulation) is critical for optimizing optical recording parameters. However, the blue shifts of VSDs induced by typical membrane depolarizations
Figure 4.1 In Situ Fluorescence Emission and Action Spectrum of di-4-ANEPPDHQ

(Top) Superposition of the fluorescence emission spectrum of the electrochromic dye di-4-ANEPPDHQ measured in the stained mouse neurohypophysis, either at rest (blue) or during action-potential depolarization (red). (Inset): Enlarged version of the two spectra indicating the slight and asymmetric blue-shift induced by depolarization. (Bottom) The difference, or action spectrum, $\Delta F/F_{\text{max}} = (F_{\text{AP}} - F_0)/F_{\text{max}}$ for di-4-ANEPPDHQ obtained from the difference of 100 recordings (noisy gray trace) and after low-pass Fourier filtering (solid line). Here, $F_{\text{AP}}$ and $F_0$ refer to the fluorescence emission measured during action-potential stimulation and at rest, respectively. $F_{\text{max}}$ is the maximum in the resting fluorescence. Superimposed on the measured action spectra is the local derivative $dF_0/d\lambda$ of the resting fluorescence (hollow squares), rescaled to match the peak value in $\Delta F/F_{\text{max}}$. $dF_0/d\lambda$ values near the two spectrometer-induced kinks in the fluorescence spectra were excessively noisy and were removed from the figure (gap between 600 and 630 nm). The agreement between $\Delta F/F_{\text{max}}$ and $dF_0/d\lambda$ indicates that changes in transmembrane voltage uniformly shift fluorescence emission, consistent with the behavior expected for electrochromic dyes (Loew et al. 1979).
(100 mV or less) are small and difficult to detect (see Figure 4.1). Hence, resolution of these weak spectral shifts require long integration times and good control over transmembrane potential (Wuskell et al. 2006; Loew et al. 1981; Loew et al. 1979). Yet, optical measurement of voltage signals and, therefore, measurements of VSD action spectra are most useful in preparations which are not accessible to recordings with glass electrodes and thus lack control over transmembrane voltage. To address these problems, we developed an approach for in situ measurements of VSD action spectra by resolving the small spectral shifts induced during transient action-potential depolarization.

4.2 In Situ Measurements of the Action Spectra of Voltage-Sensitive Dyes

We measured the resting fluorescence emission spectra and action spectra of four electrochomic dyes: di-8-ANEPPS, di-4-ANEPPDHQ, di-2-ANEPEQ (JPW 1114), and RH 414. We have also included the voltage-sensitive dye FM1-43 for comparison and control. In contrast to the spectral shifts exhibited by the former, voltage changes predominately modulate the amplitude of FM1-43 fluorescence emission. Figure 4.1 displays the resting fluorescence-emission spectrum of di-4-ANEPPDHQ in the neurohypophysis averaged over 100 8-ms single-sweep recordings of a CCD-array spectrophotometer (AvaSpec 2048-USB2; Avantes USA; Broomfield, CO). Superimposed on the resting spectrum (solid line) is the emission spectrum recorded during action-potential–induced depolarization of the neurohypophysis, also averaged over 100 recordings. As indicated by the inserts in Figure 4.1, the emission spectrum of di-4-ANEPPDHQ is slightly blue-shifted during membrane depolarization. We estimated that the average wavelength shift induced during action-potential depolarization was slightly less than 0.4 nm.
4.3 Fourier Filtering of the Action Spectra

The action spectrum of the dye obtained from one single-sweep recording was too noisy to warrant analysis. To overcome this noise limitation, measurements of the resting and voltage-shifted emission spectra were repeated 100 times and the action spectrum was calculated from their averaged difference. The resulting action spectrum is shown as noisy gray trace in Figure 4.1. The overall shape of the action spectrum is clearly discernible after summation, yet the signal-to-noise (S/N) ratio is rather disappointing. Closer inspection of the difference spectra reveals that much of the noise seems to arise from broadband white noise, while most of the features of the difference spectrum are slowly varying with wavelength. This is confirmed by calculating the spectral power distribution of the action spectra. A typical power spectrum obtained via fast Fourier transform (FFT) of the raw difference spectra is shown in Figure 4.2.

![Fourier Analysis of the Action Spectrum for di-4-ANEPPDHQ](image)

**Figure 4.2 Fourier Analysis of the Action Spectrum for di-4-ANEPPDHQ**

Power spectrum for the action spectrum of di-4-ANEPPDHQ shown in Figure 4.1. Note the large amplitudes at low spatial frequencies, followed by white noise at higher frequencies. By zeroing out the high frequency noise components, the S/N ratio of the action spectra could be significantly improved.
The first 10–15 components of the power spectrum at low spatial frequencies rise by several orders of magnitude above the uniform background of white noise dominating high spatial frequencies. It is these low-frequency components of the Fourier spectrum that represent the portions of the action spectra slowly varying with wavelength. Hence, we low-pass filtered the summed action spectra by zeroing out all Fourier components below five times the root-mean-

![Figure 4.3 Testing the Effects of Fourier Filtering on Fluorescence Spectra](image)

(A) A single emission spectrum of di-4-ANEPPDHQ was recorded at very low illumination and short integration time. (B) The power spectrum of this artificially noisy emission spectrum shows strong low frequency components and a base level of high-frequency noise. (C) After low-pass filtering, the filtered emission spectrum (dashed line) was superimposed onto the sum of 100 emission spectra taken at much higher illumination intensity and with much longer integration times (solid line).
square amplitude of the white noise or beyond spatial frequencies of 0.1 nm$^{-1}$ or higher. Filtered action spectra were obtained via inverse FFT of the filtered Fourier spectra.

To ascertain whether low-pass filtering might alter the shape of the difference spectra, we recorded a single emission spectrum at a very low LED intensity (LED current set to 1 mA) and 2 ms integration time of the spectrophotometer (Figure 4.3A). This artificially noisy resting spectrum was Fourier-analyzed (Figure 4.3B) and transformed back into real space. The filtered spectrum was plotted together with the rescaled sum of 100 emission spectra for the same dye recorded at normal illumination (Figure 4.2C). Although the count rate of the summed resting spectra was over $10^4$ times that for the single, low-intensity measurement, low-pass filtering essentially reproduced the emission spectrum recorded at normal illumination intensities.

4.4 Summary and Discussion

We have determined the action spectra of several electrochromic voltage-sensitive dyes in an intact tissue during brief action-potential depolarizations. These action spectra were measured using a standard fiber-optically coupled CCD-array spectrophotometer. Because of the intrinsic noise and limited sensitivity of these commercial detectors and our requirement to resolve the spectral shifts induced by action-potential stimulation, fluorescence emission had to be summed over approximately 100 trials. Such summation, however, did suppress the noise level sufficiently to resolve the small difference spectra of resting versus stimulated fluorescence emission. Fourier filtering further improved the S/N ratios of the summed action spectra significantly. The action spectra of all styryl dyes, together with a marker indicating the peaks of their resting fluorescence, are displayed in Figure 4.4. Aside from significant amplitude difference variations in the net shift induced during action potential
Figure 4.4 Action spectra for Various VSDs
Action spectra, $\Delta F/F_{\text{max}}$, for five VSDs obtained from the mouse neurohypophysis during action potential depolarization. For each dye, the raw difference spectrum is shown in light gray, with the Fourier filtered spectrum superimposed as a solid line. All spectra are plotted to the same scale. The vertical lines indicate the location of the peak in the resting fluorescence emission. The action spectra of di-4-ANEPPDHQ, JPW 1114, RH 414, and FM1-43 were measured from the average of 100 spectra, while 200 spectra were averaged for di-8-ANEPPS.
stimulation, the action spectra of the four styryl dyes recorded from the neurohypophysis have similar shapes. More importantly, the action spectra reveal that the voltage-induced fluorescence changes \( \Delta F \) on opposite sides of the dye emission peaks (indicated by vertical reference lines in Figure 4.4) are rather asymmetric. This asymmetry is related to the asymmetric shape of the fluorescence emission spectra themselves. For a small and uniform wavelength shift \( \Delta \lambda \), the local change in fluorescence emission \( \Delta F \) should be directly proportional to the local slope \( \frac{dF_0}{d\lambda} \) of the resting fluorescence emission (Loew et al., 1979). Figure 4.1 shows the superposition of the measured action spectrum, \( \frac{\Delta F}{F_{\text{max}}} \), and the local derivative, \( \frac{dF_0}{d\lambda} \), of the resting emission of di-4-ANEPPDHQ. The two data sets are identical to within the noise, indicating that the asymmetry of the action spectra indeed arises from the asymmetric shape of the dye emission spectrum. Furthermore, the agreement between \( \Delta F \) and \( \frac{dF_0}{d\lambda} \) confirms that the voltage-induced wavelength shift, \( \Delta \lambda \), in fluorescence dye emission is independent of wavelength, consistent with the behavior of electrochromic dyes (Loew et al., 1979). Several of the other dyes in Figure 4.4 show apparent offsets between the peak in fluorescence resting emission and the zero crossing of the action spectra. These offsets are likely due to the significantly lower S/N ratios for the action spectra and the corresponding uncertainties in determining the crossover wavelength.

Overall, our measurement approach provides a convenient and cost-effective means for measuring the action spectra of voltage-sensitive dyes \textit{in situ}, in the preparation of interest, and with the same microscope setup used for fluorescence recordings of electrical activity. We presume that this approach could be extended to calibrate activity-induced changes to the emission spectrum against the absolute-voltage excursions during slow or fast changes in transmembrane voltage.
Chapter 5
Dynamic Modulation of Neurohypophysial Excitability via Potassium Accumulation

5.1 Introduction

Excitable responses in the intact neurohypophysis during near-physiological stimulation with trains of action potentials display a complex pattern of facilitation and depression. The observed amplitude modulation of the population action potentials depends on the frequency of stimulation as well as the total number of stimuli invading the intact neurohypophysis (Muschol et al. 2003). These changes are not due to altered spatial patterns of action potentials invasion but represents local modulation of the temporal pattern in excitable responses that occurs uniformly over the entire preparation (Muschol et al. 2003). Typical patterns of excitable responses for stimulation at different frequencies are shown in Figure 5.1A. Overall, the amplitude of local excitability responses tends to facilitate during low-frequency (≤ 5 Hz) stimulation while depression dominates at elevated frequencies or during sustained stimulation. Using high-speed optical recordings and potentiometric indicators dyes, we investigated which of the many potential mechanisms that can modulate excitable responses from neurohypophysial axons in the intact preparation actively contribute to it during elevated levels of near-synchronous activity. Such elevated levels of near-synchronous activity have been observed in vivo in lactating rats (Leng and Shibuki 1987) and during dehydration (Wakerley et al. 1978).
Figure 5.1 Optical Recordings of Neurohypophysial Excitability vs. Stimulation Frequency and Intensity

(A) Optical recording of action potential responses from the neurohypophysis during a train of 40 stimuli at 5 Hz, 15 Hz, and 25 Hz initiated by field stimulation using 5 μA current pulses. The traces were recorded from a single intact neurohypophysis stained with the voltage indicator dye di-4-ANEPPDHQ (10 μM; 50 min). The slow drift in the baseline of the optical traces is predominately due to contributions from glial depolarization to the population signal (Muschol et al. 2003). The right column shows the ratio of the optical voltage response elicited by the nth stimulus ($\Delta V_n$) compared to the first stimulus ($\Delta V_1$) during stimulation with bipolar current pulses of (B) 14 μA and (C) 5 μA, respectively. Amplitude values exclude the contributions from the glial baseline drift.

5.2 Modulation of Neurohypophysial Excitability Depends on the Overall Level of Activity

Beyond the previously reported dependence of excitable population responses on the frequency of stimulation and the number of stimuli, we noticed that neurohypophysial
excitability is a sensitive function of the overall level of neuronal activity. Figure 5.1A shows the optically recorded population action potentials elicited from the intact neurohypophysis in response to trains of 40 stimuli at 5, 15, and 25 Hz. Each stimulus results in a short optical transient reporting the changes in transmembrane voltage related to the action potentials invading neurohypophysial axons. At low stimulation frequencies, the amplitude of the optically recorded responses increases early within the train ($n_{stim} < 10$) while depression of excitability develops during sustained stimulation. As stimulation frequency is increased, depression begins to dominate facilitation from the outset. Figure 5.1B quantifies this amplitude modulation as ratio of the $n^{th}$ over the $1^{st}$ optical voltage signal, $\Delta V_n / \Delta V_1$, within a given stimulus train obtained with a stimulation intensity of 14 $\mu$A. Figure 5.1C shows the results of an identical experiment, but with the effective current of the stimulation-isolation unit reduced to 5 $\mu$A. Lowering the stimulation intensity by a factor of three decreased the amplitude of the initial optical response $\Delta V_1$ by about the same factor, indicating a near-linear relation between stimulation intensity and the number of activated axons. Reducing stimulation intensity consistently enhanced the facilitation of excitable responses, delayed the onset and reduced the amplitude of depression at all stimulation frequencies. Comparable effects were obtained when recording population responses from neurohypophysial slices of different thickness (150 $\mu$m vs. 300 $\mu$m) or from the intact preparation (Figure 5.2). Amplitude modulation recorded from thin slices resembled that observed for decreased stimulation intensity in the intact preparation. Hence, reducing overall neuronal activity (either via reducing stimulation intensity or via lesion of axons in slice preparations) also reduced the depression of excitable responses during bursts of activity.
Figure 5.2 Modulation of Excitability in Slices vs. the Intact Neurohypophysis
(A) Action potential responses during a train of 40 stimuli at 25 Hz optically recorded from either sagittal slices containing the infundibular stalk or from the intact neurohypophysis. AP trains were initiated by field stimulation using bipolar current pulses (14 μA). (B) Amplitude modulation of voltage responses within a 25 Hz stimulation train recorded from thin (150 μM) or thick (300 μM) slices as well as from the intact preparation.

5.3 Extracellular Potassium Accumulation and Neurohypophysial Excitability

Activity-dependent increases to interstitial K⁺ accumulation would provide a natural explanation for the enhanced depression of excitable responses with increases in overall neuronal activity. To test this hypothesis we used multiple interventions for manipulating the levels of K⁺ accumulation during neuronal activity and for investigating their corresponding effects on excitable responses. Figure 5.3 summarizes the changes in neurohypophysial excitability when altering potassium concentrations, [K⁺]ₜₐₐₙ, of the extracellular perfusion medium. Activity-dependent depression became progressively more pronounced as [K⁺]ₜₐₐₙ-levels were increased from 3.0 mM to 5.6 mM (control) and to 10.3 mM. Figure 5.3B quantifies the amplitude
Figure 5.3 Effects of Varying External Potassium Concentration on AP Modulation

(A) Excitable response from the neurohypophysis during a train of 40 stimuli at 15 Hz with a nominal stimulation intensity of 5 μA. The traces were obtained from a single preparation bathed in standard mouse Ringer's solution ([K$^+$]$_{out}$ = 5.6 mM, black), or at low ([K$^+$]$_{out}$ = 3.0 mM, blue) and high potassium concentrations ([K$^+$]$_{out}$ = 10.3 mM, red). Note the increase in depression and the reduced after-hyperpolarization as potassium concentration increases. (B) Modulation of voltage responses within a given stimulus train. (C) Superposition of the optically recorded waveforms for the three K$^+$ concentrations used in (A). Optical signal amplitudes are identical, indicating that there are no changes in the efficacy of field stimulation with [K$^+$]$_{out}$.

Modulation of population action potentials with increasing [K$^+$]$_{out}$. This trend clearly supports the role of potassium accumulation as cause of depression. One concern in interpreting these findings is whether [K$^+$]$_{out}$ might alter the threshold for action potential generation via field stimulation and, correspondingly, alter the number of axons depolarized during field stimulation. Reduced efficacy of field stimulation, if present, should result in corresponding reductions to the
optical response to the very first stimulus in the train. As shown in Figure 5.3C, no such reduction in the overall amplitude of the population response was observed.

To further investigate the effects of extracellular potassium, we used hypertonic mouse Ringer's solution to expand the interstitial spaces and, thereby, reduce the levels of activity-dependent $[K^+]_{out}$ build-up. Adding sucrose (100 mM) to the standard physiological saline produces a 30% hypertonic solution. The membrane-impermeable sucrose increases the osmotic pressure which should cause the axons and pituicytes to shrink. Comparing images of the intact neurohypophysis in standard and hypertonic solutions, we determined that the overall dimensions of the preparation were only marginally reduced. Nevertheless, there was a modest

![Figure 5.4](image)

**Figure 5.4 Excitability Responses in Hypertonic Saline**

(A) Effects of hypertonic solution (30%) on the population responses from the neurohypophysis during a train of 40 stimuli at 25 Hz with a nominal stimulation intensity of 5 $\mu$A. Hypertonic solutions contained 100 mM sucrose added to the normal mouse Ringer's. (B) Amplitude modulation of the optical responses with a stimulus train.
(5-15%) but reproducible decrease in the optical signal to the first stimulus. We presume that this reduction was due, in part, to less intimate contact of the stimulation electrode with the tissue. To compensate for the slight amplitude changes in the population action potential, we adjusted the stimulation voltage so that the initial optical response amplitude in the hypertonic solutions matched those under control conditions. We also noticed that the AP undershoots became more pronounced in hypertonic solutions - consistent with the effect of expanded extracellular spaces on glial depolarization (Konnerth et al. 1987). Therefore, we presume that hypertonic conditions induce at least some cellular shrinkage and a corresponding increase in interstitial spaces. As shown in Figure 5.4, this expectation is consistent with the relieved depression in neurohypophysial excitability within a given stimulus train. The response of the preparation to hypertonic saline was most noticeable at high stimulation frequencies characterized by rapid $K^+$ -accumulation, though a modest reduction in depression was observable at low frequencies, as well.

Finally, we applied the $Na^+/K^+$ ATPase inhibitor ouabain (50 μM) in order to retard $K^+$ re-uptake into axons and pituicytes during repetitive stimulation. As shown in Figure 5.5, ouabain does indeed enhance depression of excitability. Ouabain effects were most pronounced at low stimulation intensities and frequencies, i.e. conditions normally exhibiting little depression. Under these conditions, $Na^+/K^+$ ATPase activity normally is able to clear the accumulating potassium. The reduced activity of the $Na^+/K^+$ ATPase in the presence of ouabain inhibits $K^+$ clearance sufficiently to induce depression even at these lower stimulation frequencies. The effects of ouabain also reduced the hyperpolarizing undershoots, as was already noticed when increasing extracellular $[K^+]_{out}$ levels. In the presence of ouabain we
Figure 5.5 Effects of Ouabain on Neurohypophysial Excitability
(A) Effects of the Na⁺/K⁺ ATPase blocker ouabain (50 μM) on excitable responses from the neurohypophysis during a train of 40 stimuli (5 μA) at 5 Hz. (B) Amplitude modulation of the optically recorded responses in (A).

observed more prominent depolarizations of pituicytes, as indicated by the enhanced baseline drift of the optical voltage signals.

5.4 Lack of Evidence for the Role of GABA<sub>A</sub> Receptors

GABA-ergic innervations of the neurohypophysis are well documented (Oertel et al. 1982, Vincent et al. 1982), and the role of ionotropic GABA<sub>A</sub> receptors during impulse propagation at individual terminals has been investigated. Hence, we wondered whether GABA<sub>A</sub> mediated inhibition contributed to the prominent depression of excitable responses in the intact neurohypophysis. Since field stimulation of the infundibular stalk automatically activates afferent fibers, including GABA-ergic fibers, we used bicuculline (100 μM) to block
**Figure 5.6  Excitable Responses in the Presence of the GABA\textsubscript{A} Blocker Bicuculline**

(A) Effects of the GABA\textsubscript{A} receptor blocker bicuculline (100 \(\mu\)M) on excitable responses from the neurohypophysis during a train of 40 stimuli (5 \(\mu\)A) at 25 Hz. (B) Amplitude modulation of the optically recorded responses in (A).

GABA\textsubscript{A}-R. As shown in Figure 5.6, application of bicuculline, if anything, slightly increased depression. This modest increase could potentially result from the release of GABA-ergic inhibition which, in turn, enhances local K\textsuperscript{+} ion accumulation. The main result is that GABA\textsubscript{A}-R mediated inhibition does not make a significant contribution to the short-term depression of excitable responses in the intact neurohypophysis, at least under conditions of elevated net activity prevailing here.

**5.5 Role of Ca\textsuperscript{2+} Influx in Amplitude Modulation of Population Action Potential**

More generally, we wanted to address whether Ca\textsuperscript{2+} influx played a role in the observed depression of excitable responses. Figure 5.7 compares the results of multiple interventions to
lower Ca\textsuperscript{2+} influx and their corresponding effects on AP modulation. As previously reported, Cd\textsuperscript{2+} did indeed suppress both facilitation and depression of population responses (Muschol et al. 2003). Yet, decreases of extracellular [Ca\textsuperscript{2+}] from 2.2 to 0.1 mM, prolonged exposure to the membrane permeant fast calcium chelator BAPTA/AM (50 μM) or application of Ni\textsuperscript{2+} (1 mM) all resulted in depression of excitable responses that was not statistically significantly different from their controls.

Similarly, simultaneous exposure to the specific Ca\textsuperscript{2+} channel blockers ω-conotoxin GVIA (10 μM) and MVIIC (5 μM) or the L-type channel blocker nicardipine (5 μM) had only minor effects on excitability loss, when compared to their controls. These

![Figure 5.7](image-url)

**Figure 5.7 Lack of Calcium Dependence for AP Depression**

(A) Excitable responses from the neurohypophysis during a train of 40 stimuli (5 μA) at 25 Hz in normal saline vs. 0.1 mM Ca\textsuperscript{2+} (B) Comparison of the maximum facilitation and depression of the AP responses within a train of 40 stimuli upon lowering external Ca\textsuperscript{2+} to 0.1 mM; using the Ca\textsuperscript{2+} buffer BAPTA/AM (50 μM); application of the specific Ca\textsuperscript{2+} channel blockers ω-conotoxin GVIA (10 μM), MVIIC (5 μM) or nicardipine (5 μM); or application of the Ca\textsuperscript{2+} channel blockers Ni\textsuperscript{2+} (1 mM) or Cd\textsuperscript{2+} (0.5 mM). The hatched bars are controls the shaded bars indicate the measured change in facilitation or depression during the various interventions.
results suggest that Cd\(^{2+}\) mediates its effect on neurohypophysial excitability by a mechanism other than block of voltage-gated \(\text{Ca}^{2+}\) channels. While we do not exclude \(\text{Ca}^{2+}\) contributions to facilitation, depression of neurohypophysial excitability during tissue-wide, elevated activity displays no discernable \(\text{Ca}^{2+}\) dependence. The lack of \(\text{Ca}^{2+}\) sensitivity implies that neither \(\text{Ca}^{2+}\)-mediated endogenous mechanisms within individual axons nor \(\text{Ca}^{2+}\)-dependent release from afferent, regulatory inputs make a significant contribution to the depression of excitable responses. The lack of \(\text{Ca}^{2+}\) sensitivity, however, is consistent with activity-dependent \(\text{K}^{+}\) accumulation as the prevailing mechanism underlying the depression of neurohypophysial excitability.

### 5.6 Temperature Effects on Excitability Changes

The above experiments were all performed near room temperature (24 °C). This temperature was chosen because the action potentials are slightly broader, thereby avoiding under-sampling of the fast optical transients at the maximal camera frame rate of 2,500 frames/sec. To determine whether the above observations persisted at 37 °C, we repeated two sets of experiments: the intrinsic modulation of neurohypophysial excitability (Figure 5.8A) and the effects of the \(\text{Na}^{+}/\text{K}^{+}\) ATPase inhibitor ouabain (Figure 5.8B). Overall, the modulation of excitable responses was qualitatively identical to the observations at 24 °C. However, the transition from facilitation to depression shifted to higher stimulation frequencies and/or higher stimulation numbers. Consistent with these shifts, the effects of ouabain at 37 °C shifted to higher frequencies, as well.
Figure 5.8 Neurohypophysial Excitability Changes at Physiological Temperatures

(A) Excitable responses from the neurohypophysis at 37 °C during a train of 100 stimuli at 25 Hz and 75 Hz during field stimulation with 14 μA current pulses. The short-term undulation in the apparent peak amplitudes of these optical recordings results from the under-sampling of the shorter optical transients at these temperature, even at the highest camera frame rate (2.5 kHz) for our system. This under-sampling artifact increases the noise in the amplitude modulation, but does not alter the overall modulation pattern. (B) Amplitude modulation of excitability for the data in (A), as well as for recordings at f_{stim} = 100 Hz. The increased temperature induces an overall shift in the transition from facilitation to depression of population responses to higher frequencies and large number of stimuli. (C) Effects of the Na^{+}/K^{+} ATPase blocker ouabain (50 μM) on the amplitude modulation of optically recorded excitable responses from the neurohypophysis at 37 °C during a train of 100 stimuli (14 μA) at 25 Hz.

5.7 Comparing Numerical Predictions and Optical Recordings of AP Waveforms

Optical recordings of neuronal responses with fast potentiometric dyes provide the unique ability to monitor electrical activity throughout an intact tissue. Preserving tissue
morphology is particularly important for characterizing the effects of K⁺ accumulation, as is apparent from the significant effects of slicing on population responses (see Figure 5.2). However, there are residual questions of whether the observed modulation of population amplitudes and their waveforms might arise from changes in the efficacy of field stimulation, changes in temporal summation of the APs, or changes in the spatial pattern and efficacy of tissue invasion by the AP. As indicated above (see Figure 5.3C), we can evaluate whether interventions alter the efficacy of field stimulation by comparing the optical responses during the first stimulus of a train. Similarly, summing optical signals exclusively near the site of stimulation indicates how optical waveforms are affected by, or reflect changes in, temporal dispersion of population responses.

Again, we saw no significant changes to temporal dispersion of the signals in any of the above experiments. We have previously shown that the observed modulation is also not due to changes in the spatial pattern of AP invasion (Muschol et al. 2003). The question remained whether K⁺ accumulation alone could account not just for the prominent modulation of population action potential amplitude, but might also underlie the observed differences in optical waveforms within a given stimulus train, including the modest broadening of action potentials and the loss of AP undershoots. The subsequent numerical simulations, therefore, were intended to determine which of the many features in our optical recordings of population responses were consistent with K⁺-accumulation mediated changes to single AP amplitudes and waveform. Our numerical simulations of individual action potential responses to trains of stimuli were based on a Hodgin-Huxley style numerical model that included realistic axon morphologies, ion channel properties (Na⁺ as well as A- and BK-type K⁺ channels with experimentally determined activation/inactivation parameters) and channel densities.
Figure 5.9 Numerical Predictions of K+ Accumulation Effects on AP Responses and Comparison with In-Situ Optical Recordings

(A) Numerical simulation of the effects of extracellular K+ accumulation on local action potential responses during a train of propagating action potentials. The dashed reference line indicates the resting potential of the axons. (B) Superposition of the optically recorded waveforms (black) and the results of numerical simulations of AP waveforms (blue) near the beginning of a stimulus train (2nd stimulus) vs. the end of a stimulus train (40th stimulus).

(Jackson and Zhang 1995, Bennett and Muschol 2009). A thin boundary layer was added to account for the effects of K+ accumulation during electrical activity. Simulations excluding this boundary layer resulted in action potential responses that were essentially unchanged throughout the entire stimulus train. Inclusion of K+ accumulation resulted in three major changes to AP amplitudes and waveform: action potential amplitudes became progressively more depressed (Figure 5.9A), AP undershoots systematically decreased in amplitude, and the AP waveform concurrently broadened during repeated stimulation (Figure 5.9B). Figure 5.9B provides a more detailed comparison of the numerically predicted AP waveforms vs. the waveform of population
action potentials optically recorded near the beginning and towards the end of a stimulus train. All three major features of the optically recorded population responses (depression of AP amplitudes, slight broadening of AP waveforms, and loss of after-hyperpolarization) are closely mirrored by the simulated waveforms of individual action potentials, when including K\(^+\) accumulation into the model.

The numerical simulations also provide insights into the mechanisms driving the observed waveform changes. The progressive loss of a hyperpolarizing phase results from two factors accompanying extracellular K\(^+\) accumulation: a decrease in the chemical potential difference driving K\(^+\) ions across the membrane and, to a lesser extent, a decrease in the conductance of all voltage-gated K\(^+\) channels. The net loss in transmembrane K\(^+\) current extended the time required to repolarize the membrane, leading to the observed broadening of AP waveforms. Finally, the decreasing rate of membrane repolarization causes the membrane potential difference to decrease by the time the next stimulus arrives (see reference line in Figure 5.9A). The drift in membrane potential results in progressive inactivation of voltage-activated Na\(^+\) channels and subsequent depression of AP amplitudes. This effect is particularly prominent since the Na\(^+\) channel inactivation curve is steeply voltage-dependent near the membrane resting potential (Jackson and Zhang 1995). Decreasing this steep voltage-dependence of the Na\(^+\) channel inactivation parameter h indeed relieved the observed depression of AP amplitudes in our simulations.

5.8 Summary and Discussion

The above optical measurements confirm that potassium accumulation is the dominant mechanism for inhibiting excitability of neurohypophysial axons and secretory swellings during
physiologically relevant levels of elevated and synchronized activity (Poulain and Wakerley, 1982; Leng and Shibuki, 1987). The close agreement of the numerically predicted AP modulation via $K^+$ accumulation near individual fibers to the optically recorded population AP waveforms (see Figure 5.9) indicates that the latter reflect predominately changes of individual AP responses and not changes to the overall number of actively fibers and/or effects of temporal dispersion to population signals. Furthermore, the numerical simulations confirm that $K^+$ accumulation not only reduces the overall amplitude of individual action potentials but also leads to a progressive loss of AP after-hyperpolarization and to a modest broadening of AP waveforms during trains of stimuli. Both effects are readily apparent in the optical recordings (Figure 5.1A) but, in the absence of independent confirmation, had not been identified as direct consequences of potassium accumulation. Finally, the combination of optical recordings and numerical simulations suggest that the effects of potassium accumulation on action potential responses are surprisingly complex and malleable, varying with the overall level of activity as well as with the temporal pattern of stimulation (see Figures 5.1B and 5.1C). The lack of discernable effects by the GABA$_A$ receptor blocker bicuculline (see Figure 5.6) suggests that GABA-ergic modulation, in contrast, plays a comparatively minor role. The absence of calcium-dependence (see Figure 5.7) of activity-dependent depression further confirms that the observed depression does not arise from exogenous or endogenous release of inhibitory neurotransmitters. The electrophysiologically documented GABA$_A$-R mediated inhibition of neurohypophysial excitability (Jackson and Zhang, 1995; Zhang and Jackson, 1995) versus the apparent absence of GABA-ergic modulation in optical recordings of electrical responses in this work is intriguing. It is conceivable that these differences reflect genus-specific differences between mice and rats. We would like to suggest that this apparent contradiction, instead, arises from the difference in
the “operating conditions” of the neurohypophysis probed by local electrophysiological recordings versus field stimulation during optical recordings. Single unit recordings are more likely to detect regulation of basal or slow irregular patterns of activity and release, while optical recordings more faithfully detect modulation during fast continuous or phasic burst activity in the intact preparation (Poulain and Wakerley, 1982). Additionally, effects that arise due to the compact nature of the tissue will inevitably be reduced or eliminated when the environment surrounding the axons is disrupted by a glass electrode. Hence, optical recordings supplement our understanding of the various mechanisms that modulate neurohypophysial excitability. Specifically, our measurements suggest that regulation of neurohypophysial excitability and release is not just dependent on the temporal patterns of activity, but shifts with the overall level of neuronal activity itself. This would imply that proper understanding of neurohypophysial excitability requires measurements of neuronal behavior over a range of different activity levels.
Chapter 6

Amyloid Fibril Formation

6.1 Introduction

Amyloid fibril deposits are the molecular hallmark for a variety of human diseases, including Alzheimer’s disease, Parkinson’s disease, type II diabetes, and hemodialysis-associated amyloidosis (Kelly 1996; Chiti and Dobson 2006; Miyata et al. 1993). Amyloid fibril formation can be biologically functional such as in extracellular adhesion of bacteria, transmission of inheritable traits in yeast (Halfmann et al., 2012), or tight packing of peptide hormones into electron-dense secretory granules (Fowler et al., 2005). Unraveling the mechanisms driving amyloid fibril formation and determining the features that render this process either pathogenic or functional represents a significant scientific challenge.

Amyloid fibrils are non-branching polymeric aggregates of proteins that can reach many microns in length and, typically, are stained by the dyes Congo Red or Thioflavin T. Besides these morphological and tinctorial features, amyloid fibrils share a characteristic cross-β sheet structure in which at least portions of the individual polypeptide backbones of the proteins are aligned perpendicular to the main fibril axis and are linked to each other via intermolecular hydrogen bonds (Jahn et al., 2010). The structural and morphological similarities of amyloid fibrils are in stark contrast to the wide variety of functionally and structurally distinct proteins that can form amyloid fibrils (Chiti and Dobson, 2006; Koo et al., 1999). This has lead to the supposition that amyloid fibril self-assembly is yet another example of a generic protein phase
separation process (Dobson, 1999). There is, however, an important difference between amyloid fibril formation and “standard” protein phase separation phenomena such as crystallization and liquid-liquid demixing. While the latter mostly preserve the native protein structure, the natively folded or intrinsically disordered protein needs to undergo significant restructuring in order to assume the cross-β sheet structure of the amyloid fibril state (Uversky et al., 2006; Uversky and Fink, 2004). How these conformational constraints on aggregation affect the propensity of a given protein to form amyloid fibrils, when and how such restructuring occurs, and whether restructuring precedes or is induced by the formation of specific aggregation intermediates remains an open question.

One of the goals of this research was to characterize the structural reordering that accompanies this aggregation using Fourier transform infrared spectroscopy (FTIR). FTIR spectroscopy is quite sensitive to the secondary structure of proteins, particularly the β-sheet motif associated with amyloid fibril formation. Using this technique, we have investigated the structure of hen egg white lysozyme (HEWL) as it forms amyloid fibrils under various growth conditions. In acidic conditions at elevated temperature, lysozyme forms fibrils along two distinct pathways. In low salt conditions, monomeric filaments grow after a pronounced lag period. These aggregates are long and stiff but with the cross sectional area of a single monomer. At higher salt concentrations, the aggregation follows an oligomeric pathway. Upon incubation, monomers come together to form oligomers which then assemble into protofibrils. The oligomeric protofibrils are thicker than the monomeric filaments, but are much more curvilinear. At physiological pH, only the protofibril pathway seems to be accessible.

It has traditionally been held that partial denaturation of protein is a prerequisite for amyloid fibril growth. However, by seeding a solution of natively folded monomeric lysozyme
with preformed oligomeric and protofibrilar aggregates, we have grown amyloid fibrils under near-physiological conditions (pH 7; 37 °C). We confirmed that HEWL is indeed natively folded at 37 °C by monitoring the intrinsic fluorescence of the tryptophan residues as a function of temperature. Additionally, FTIR spectroscopy revealed that the fibrils grown from the seeds under near-physiological conditions were structurally indistinguishable from those grown under denaturing conditions. Attempts to seed amyloid growth under these conditions using monomeric filament seeds proved fruitless.
Chapter 7
Background

7.1 Amyloid Fibrils

Amyloid fibrils are unbranched polymeric aggregates of proteins. These aggregates assume a characteristic cross-β sheet structure, irrespective of the native structure of the aggregating protein. A great number of otherwise dissimilar proteins are able to form these aggregates. While amyloids are most readily identified with diseases such as Alzheimer’s disease or Parkinson’s disease (Kelly 1996; Chiti and Dobson 2006), more recent evidence shows that they can also be biologically useful, as in the tight packing of peptide hormones into secretory granules (Fowler et al., 2005).

The cross-β sheet motif that is common to these aggregates comes from the ordered repetition of β strands perpendicular to the length of the fibril (see Figure 7.1A). The backbone amide N-H groups and C=O groups of a protein chain enter intermolecular hydrogen bonds with other protein chains, creating a β-sheet structure. The side chains of the proteins intertwine in a steric zipper, as two β-sheets come together (Eisenberg and Jucker, 2012) (see Figure 7.1B).

The hydrogen bonding between the chains is quite insensitive to the side chains, so the interstrand spacing is very close to 4.8 Å, regardless of the protein. The structure of the steric zipper that holds the two β-sheets together does depend on the sidechains, so the intersheet spacing can vary about the typical value of approximately 10 Å.
Figure 7.1 Cross-β Structure of Amyloid Fibrils

(A) A schematic of the characteristic x-ray diffraction pattern of amyloid fibrils. The β-strands run perpendicular to the length of the fibril, with an interstrand spacing of 4.8 Å. This spacing is hydrogen bonding between the protein backbones, and is remarkable conserved among amyloid fibrils regardless of the protein. The intersheet spacing of about 10 Å depends on the interactions of the side chains, and shows more variation. (B) The steric zipper is created by the interdigitating side chains of the proteins. (C) Water, indicated by the red + signs, is excluded from the space between the β-sheets. (Figure from Eisenberg and Jucker, 2012)

7.2 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is a widely employed technique that has many applications in not only protein structure analysis, but also nanoparticle characterization,
compound identification, and many other fields (Schwinte, et al. 2002; Boccuzzi et al. 2001; Thenmozhi et al. 2011).

Infrared absorption spectroscopy can reveal important structural information about molecules. The energies of the absorbed radiation are readily associated with specific molecular vibrations for molecules containing only two or three atoms, but analysis becomes substantially more complicated in the case of large molecules such as proteins. Thankfully, certain repeated motifs emerge, allowing for the assignment of absorption peaks to specific structural features.

Molecular vibrations arise from stretching or bending of the bonds between atoms. In stretching vibrations, the distance between atoms varies. In the case of more than two atoms, stretching vibrations can be either symmetric, in which identical bonds increase and decrease their lengths in unison, or asymmetric. Bond angles change during bending vibrations in one of four different ways: rocking, scissoring, wagging, or twisting. A diagram of these six types of vibrations is shown in Figure 7.2 (From Serdyuk, et al. 2007).

An oscillating electric dipole can absorb electromagnetic radiation at its normal mode frequency, but not all vibrations lead to dipole oscillations. For example, the vibrations of a linear molecule, A-B-A, include both a symmetric and an asymmetric stretching mode. In the symmetric mode, the bonds increase and decrease in length simultaneously, and therefore the dipole moments created by each of the two A-B bonds are equal in magnitude, but opposite in direction. The net dipole moment is constant during this vibrational mode, so the symmetric stretching mode is IR-inactive. When the two A-B bonds vibrate out of phase, in the asymmetric mode, however, the net dipole moment does vary with the stretching vibration, and the mode is IR-active. Infrared radiation at the frequency of this mode can be absorbed, exciting the asymmetric stretching vibration (Serdyuk, et al. 2007).
In general, a non-linear molecule of $N$ atoms will have $3N-6$ normal modes of vibration (Hiramatsu and Kitagawa 2005), but a polypeptide chain will often have repeated units that share a common configuration and environment. This greatly reduces the number of independent peaks, and gives rise to relatively strong absorption bands. These bands are named amide A, amide B, and amide I-VII, and arise from vibrations in the CONH groups that are common to proteins. Typical wavenumber ranges and dominant vibrational modes for the amide bands are shown in Table 7.1. The exact wavenumber boundaries of the bands vary slightly throughout the literature (Serdyuk, et al. 2007; Kong and Yu 2007; Schweitzer-Stenner 2006)
Table 7.1 Amide Bands
The typical frequency ranges and vibrational modes for the nine amide bands.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Approximate frequency (cm$^{-1}$)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amide A</td>
<td>3300</td>
<td>NH stretching</td>
</tr>
<tr>
<td>Amide B</td>
<td>3100</td>
<td>NH stretching</td>
</tr>
<tr>
<td>Amide I</td>
<td>1600-1690</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>Amide II</td>
<td>1480-1575</td>
<td>CN stretching, NH bending</td>
</tr>
<tr>
<td>Amide III</td>
<td>1229-1301</td>
<td>CN stretching, NH bending</td>
</tr>
<tr>
<td>Amide IV</td>
<td>625-767</td>
<td>OCN bending</td>
</tr>
<tr>
<td>Amide V</td>
<td>640-800</td>
<td>Out-of-plane NH bending</td>
</tr>
<tr>
<td>Amide VI</td>
<td>537-606</td>
<td>Out-of-plane C=O bending</td>
</tr>
<tr>
<td>Amide VII</td>
<td>200</td>
<td>Skeletal torsion</td>
</tr>
</tbody>
</table>

*Adapted from Kong and Yu 2007

Until the widespread use of Fourier transform infrared (FTIR) spectroscopy, most infrared spectroscopy was performed by dispersive methods using prisms or diffraction gratings. The increase in computing power over the years opened the doors for a faster, higher throughput method that allows a wide spectrum of IR absorbance to be measured at once. FTIR spectroscopy uses a wide spectrum infrared light source that is passed through a Michelson interferometer (See Figure 7.3 for a schematic). The light is then directed through the sample and into the IR detector.

The Michelson interferometer consists of a beam splitter, a fixed mirror, and a movable mirror. These are arranged such that a collimated light source is divided along two path lengths, each reflecting off of one of the mirrors, and is then recombined. As one mirror in the interferometer is moved by a distance $\frac{1}{2} \Delta L$, the difference in the two optical path lengths...
Figure 7.3 Schematic of Bruker Optik Vertex 70 FTIR Spectrometer

The shading shows the optical path from the mid-infrared source into the interferometer, through the sample, and on to the detector. (Figure from Bruker Optik Vertex 70 Manual; 2007)

changes by ΔL. This path difference leads to interference, and the plot of the detected intensity versus path difference is called an interferogram. For a monochromatic light source with wavelength λ, the interferogram would simply be a cosine function with a frequency of 1/λ, or the wavenumber. For the wide spectrum source used in FTIR measurements, the interferogram is the sum of the contributions from each wavelength component. A typical interferogram is shown in Figure 7.4A. To retrieve the spectral information from the interferogram, a Fourier transform is performed. This converts the data from the spatial regime to the frequency domain. A sample raw spectrum is shown in Figure 7.4B.
**Figure 7.4 FTIR Interferogram and Transmission Spectrum**

An interferogram (A) and the corresponding transmission spectrum (B) measured for a lysozyme amyloid fibril sample. (pH 2; 50 mM NaCl; incubated at 50 °C)

This spectrum will include contributions from not only the sample, but any absorption due to optical components, water vapor, and, in the case of aqueous proteins, the buffer solution. In addition, this detected spectrum will depend on the spectrum of the IR source output itself. To eliminate these unwanted contributions, it is important to take background spectra before measuring the absorbance spectrum of the sample. The absorbance of the buffer solution is particularly important because water absorbs IR light very strongly in the amide I band. To ensure that the water background is properly removed, the thickness of the buffer layer through which the light travels must be the same during the background and the sample spectra. This is accomplished by using an attenuated total reflectance (ATR) sample chamber. At an interface between media of different indices of refraction, the transmitted light is refracted according to Snell’s Law. In the case of light traveling from a region of higher to lower refractive index, it is
easily shown that there exists an angle above which there is no transmitted light. This is the critical angle, $\theta_c$.

$$n_1 \sin \theta_1 = n_2 \sin \theta_2$$

$$\theta_c = \sin^{-1} \left( \frac{n_2}{n_1} \right)$$

While there is no transmitted light during total internal reflection, an evanescent field is produced across the interface. This field drops off exponentially, providing a limited and consistent effective sample thickness. The penetration depth, $d$, depends on the wavelength of the light, the indices of refraction of the crystal and sample solution, and the angle of incidence as shown in the equations below.

$$d = \frac{\lambda}{4\pi \sqrt{n_1^2 \sin^2 \theta - n_2^2}}$$

$$I_z = I_0 e^{-z/d}$$

The sample solution is placed on the silicon crystal of the ATR accessory. The infrared light is reflected from the surface of the sample a number of times to increase the sensitivity, as shown in Figure 7.5 (Image from “FT-IR Spectroscopy - Attenuated Total Reflectance (ATR),” PerkinElmer, Inc., 2005.) Typical ATR cells give a penetration depth on the order of 5 μm (Serdyuk, et al. 2007).

The vibrational modes accessible through FTIR spectroscopy are excellent probes of the secondary structure in proteins. While proteins show absorbance in many amide bands with wavenumbers varying from approximately 3,300 cm$^{-1}$ to 200 cm$^{-1}$ (Table 2), two bands, Amide I and Amide II, are of particular interest. The Amide II band (approx. 1,600 cm$^{-1}$ to 1,480 cm$^{-1}$) is mostly due to vibrations from in-plane NH bending and CN stretching (Kong and Yu 2007). Although this band does provide some conformational information, the Amide I band
Figure 7.5  ATR Crystal
Schematic of an attenuated total reflectance sample chamber, highlighting the multiple reflections at the sample interface. (Image from “FT-IR Spectroscopy - Attenuated Total Reflectance (ATR),” PerkinElmer, Inc., 2005.)

(approx. 1,700 cm\(^{-1}\) to 1,600 cm\(^{-1}\)) is a more sensitive indicator of secondary structure. The Amide I band arises from C=O stretching vibrations, and is composed of a number of overlapping peaks which are associated with \(\alpha\)-helices, \(\beta\)-sheets and \(\beta\)-turns, and unordered structures. The exact positions vary among different proteins, but the peaks corresponding to the two secondary structures in which we are most interested, \(\beta\)-sheets and \(\alpha\)-helices, generally fall near 1,620 cm\(^{-1}\) and 1,655 cm\(^{-1}\) wavenumbers, respectively (Schwinte, et al. 2002).

7.3 Intrinsic Fluorescence Spectroscopy

Fluorescence spectroscopy on natively occurring tryptophan (Trp) residues is a reliable and non-invasive way to monitor the melting of a protein’s tertiary structure. Both the peak position and magnitude of the emission spectrum of tryptophan depend strongly on its local environment, particularly the exposure to water.

Lysozyme contains six tryptophan residues (Trp28, Trp62, Trp63, Trp108, Trp111,
Trp123), with the majority of the emission coming from Trp62 and Trp108 (Nishimoto, et al. 1998). Trp108 is mostly shielded from water in native lysozyme, and its emission peak is near 342 nm. Under denaturing conditions, the protein partially unfolds, exposing the Trp108 residue and shifting its emission maximum to longer wavelengths. The tryptophan at residue 62, however is almost fully exposed, even in the native state. Its emission maximum near 352 nm is largely insensitive to unfolding events. By exciting the fluorescence and monitoring the shift of the emission spectrum, which is dominated by a superposition of the spectra from Trp62 and Trp108, one can track the partial unfolding of the protein sample.
8.1 Protein Sample Preparation

Twice recrystallized, dialyzed, and lyophilized hen egg white lysozyme (HEWL) was purchased from Worthington Biochemicals (Lakewood NJ) and was used for all experiments. All chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and were reagent grade or better. All solutions were prepared using 18 MΩ water from a reverse osmosis unit (Barnstead E-pure, Dubuque, IA).

HEWL was dissolved at twice its final concentration in either 25 mM KH$_2$PO$_4$ at pH 2 or 20 mM HEPES at pH 7 buffer and was briefly placed in a water bath at 45 °C to help dissolve preformed clusters. Samples were consecutively filtered through 220-nm and 50-nm pore size syringe filters. This concentrated HEWL stock was mixed 1:1 either with a NaCl /25 mM KH$_2$PO$_4$ pH 2 or with a NaCl /20 mM HEPES pH 7 stock solution, with NaCl concentrations in this salt/buffer stock adjusted to twice their final concentrations. Actual lysozyme concentrations were determined from UV absorption measurements at 280 nm ($\alpha_{280} = 2.64$ ml mg$^{-1}$ cm$^{-1}$).

Typically, samples were prepared at 20 mg/mL (1.4 mM) lysozyme concentration. These solutions were placed in standard glass cuvettes (Starna Cell, Atascadero, CA) and were incubated at 50 °C (pH 2) or 65-70 °C (pH 7) for as little as 4 h and as long as 5 days using a dry bath or the themostated cuvette holder of a dynamic light scattering unit (Zetasizer S, Malvern Instruments, Worcestershire, UK). For accelerated growth of monomeric fibrils at pH 2, fibril
seeds were grown at pH 2 in 100 mM NaCl for 2.5 days. A freshly prepared lysozyme solution was then mixed with 3% of the seeding solution and was incubated for 24 h under the same growth conditions. Accelerated growth of pH 2 oligomeric samples within just hours, in turn, was achieved by raising NaCl concentrations to 325 mM.

8.2 FTIR Spectra Measurements

Attenuated total reflection Fourier-Transform Infrared (ATR- FTIR) spectroscopy was performed on a Bruker Optik Vertex 70 (Ettlingen, Germany) spectrometer with a mid-infrared source and pyroelectric DLATGS (deuterated L-alanine doped triglycine sulphate) room temperature detector. Usually, 25-40 μL of protein solution was placed on the thermostated silicon crystal of a BioATRcell II (Harrick Scientific Products, Inc.; Pleasantville, New York) attenuated total reflectance (ATR) accessory. All spectra were taken at 24 °C with an aperture setting of 8 mm and a scanner velocity of 10 kHz. In order to minimize water vapor contamination of the spectra, we employed the atmospheric compensation algorithm within the OPUS software analysis package (version 6.5, Bruker Optik). The background spectrum of the buffer was recorded over 200-800 scans at 2 cm⁻¹ resolution and subtracted from the sample spectra. Typically, three to twelve such runs were averaged prior to data analysis.

For the FTIR spectra of some of the aggregated samples, we used ultracentrifugation to lower the concentration of the monomers and their contribution to the spectrum. Typically, a 1.5 ml centrifuge tube (Polyallomer, Beckman) was filled with 750 μL of aggregated sample and subjected to 24 hours of ultracentrifugation at 90,000 g (Avanti J-30I, JA-30.50T rotor, Beckman Coulter). The supernatant was decanted and the remaining pellet diluted 3-fold into salt/buffer solutions prior to FTIR spectral analysis.
FTIR spectra were acquired between 4,000 cm\(^{-1}\) and 1,000 cm\(^{-1}\) wavenumbers. The Amide I and Amide II bands (1,700 cm\(^{-1}\) - 1,500 cm\(^{-1}\)) were simultaneously subjected to peak and deconvolution analysis. Peak positions in the Amide I band of the spectra were identified using the Fourier Self-Deconvolution (FSD) (Bandwidth 6 cm\(^{-1}\); Enhancement 2.4) and second derivative (13 smoothing points) algorithms within the OPUS software analysis package (version 6.5, Bruker Optik). After a horizontal baseline correction, Gaussian curves were fitted from 1,700 cm\(^{-1}\) to 1,500 cm\(^{-1}\). We accounted for the overlap of the Amide I and Amide II band by fitting the entire Amide I band together with the high-frequency portion of the Amide II band. Peak positions were fixed to the values identified by FSD and second derivative algorithms while the intensity and widths of the Gaussian curves were optimized using the Levenberg-Marquardt Algorithm. To avoid unreasonable broadening of Gaussian peaks observed for some spectra, peak widths were restricted to values near those obtained with native lysozyme.

To compare spectra from different fibril samples, the spectra were first corrected by a horizontal baseline. These spectra were then normalized to the integral over the Amide I band (1710 cm\(^{-1}\) to 1590 cm\(^{-1}\)), and the difference spectra were obtained by subtracting the normalized reference spectrum of monomeric lysozyme from the spectra of the aggregated samples. These difference spectra were then rescaled to the magnitude of the β-sheet peak near 1620 cm\(^{-1}\).

### 8.3 Intrinsic Tryptophan Fluorescence Spectroscopy

Tryptophan (Trp) fluorescence measurements were performed using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ). Intrinsic Trp fluorescence was excited at 280 nm and emission spectra were measured from 300 nm to 400 nm. HEWL samples were prepared at 1 mg/mL in HEPES buffer (pH 7; no salt), and 1 mL aliquots were placed in a quartz
cuvette with a screw cap to minimize evaporation. The samples were raised from 25 °C to 85 °C in 3 - 5 °C increments, allowing five minutes for equilibration at each temperature step. Five spectra were taken at each temperature step and averaged. The peak position was found by first fitting a quadratic function to the region of the spectrum within 10 nm above and below the maximum, and then finding the maximum of this fit.
9.1 Introduction

In previous experiments, our group has shown that heat-induced denaturation of hen egg white lysozyme at acidic pH results in amyloid fibril formation along two clearly distinct assembly pathways (Hill, et al. 2011). Lysozyme is a small enzyme (14.3 kD), the human variants of which are implicated in systemic forms of amyloid diseases (Booth, et al. 1997; Gillmore et al. 1999; Pepys et al. 1993). At low salt concentrations (≤ 150 mM), a long lag period is followed by a prominent nucleation event which indicates the formation of monomeric filaments of two distinct lengths. Three of these monomeric filaments can later cross-assemble into thicker fibrils. As ionic strength exceeds 150 mM, assembly abruptly switches over to oligomeric fibril growth. In this latter path-way, compact oligomers begin to form at a steady rate upon the onset of incubation, which eventually results in nucleation of oligomeric protofibrils (i.e. curvilinear polymers of oligomers). Protofibrils then increase in length and occasionally cross-assemble pairwise into mature fibrils. (Hill, et al. 2011) Atomic force microscopy images of late stage fibrils grown under these two pathways are shown in Figure 9.1. Similarly distinct assembly pathways have been described for a variety of structurally and functionally distinct amyloidogenic proteins including β2-microglobulin, Aβ(1-40) and the yeast prion Sup35 (Goldsbury et al. 2005; Gosal et al. 2005; Jahn and Radford 2008; Hess et al. 2007).
Figure 9.1 AFM Images of Monomeric Filaments, Oligomers, and Oligomeric Protofibrils
Atomic force microscopy phase images of hen egg white lysozyme fibrils and intermediates incubated at pH = 2 and T = 50 °C in the presence of (A) 50 mM NaCl or (B and C) 175 mM NaCl. (A) Monomeric filaments grown under low salt conditions for 2 days are very straight and, hence, mechanically stiff. Long and short filaments and wider, cross-assembled fibrils are shown. (B) The oligomeric intermediates that appear prior to protofibril nucleation are shown after 4 hours of incubation. (C) Protofibrils grown at elevated salt concentrations for 25 hours are much more curvilinear than the monomeric filaments despite their larger height. False color overlay indicates aggregate heights, with orange indicating heights of less than or equal to 3 nm and green representative of taller aggregates (>4 nm). The images have not been corrected for tip dilation effects. The scale bars in all images are 250 nm. The images were recorded by Mentor Mulaj.

By analyzing the FTIR spectra of lysozyme during aggregation along these two pathways, we can gain insight into the structural changes that occur during this assembly process.

9.2 Changes in Secondary Structure Upon Amyloid Fibril Formation

Although we are concerned only with changes arising from aggregation, much of the protein in a given sample is still in its unaggregated, monomeric form. FTIR spectra represent the algebraic sum of all species in solution, and therefore, spectral contributions of aggregated species are easily "washed out" by the predominant monomeric background. We used ultracentrifugation to separate aggregated species from the monomeric background and, thereby,
isolate their contributions to the FTIR spectra. The supernatant was decanted and the remaining pellet diluted 3-fold into salt/buffer solutions prior to FTIR spectral analysis.

Figure 9.2 displays the Amide I peak spectra for monomeric lysozyme at the onset of incubation vs. late stage fibril aggregates, subjected to ultracentrifugation and slight dilution, grown in the presence of either 50 mM NaCl (oligomer-free filamentous assembly) or 175 mM NaCl (oligomeric assembly). Inspecting the raw spectra several overarching trends are noticeable. First, both filamentous and oligomeric fibril assembly result in the emergence of a prominent $\beta$-sheet peak around 1,620 cm$^{-1}$, consistent with $\beta$-sheet peak positions associated with the intermolecular hydrogen bonds resulting from amyloid fiber formation (Zandomeneghi et al. 2004). In addition, the native $\alpha$-helix content dramatically declines but does not disappear. Dynamic light scattering measurements on the pelleted fibril samples (not shown) confirm that residual monomeric content in the resuspended pellets is negligible. Hence, the persistent $\alpha$-helical content in these spectra is not simply due to contamination by monomers but implies that lysozyme fibrils preserve some secondary structure elements of their native monomers. This matches NMR determinations of the structure of mature lysozyme fibrils obtained under comparable growth conditions (Frare et al. 2004). Finally, the region of the Amide-I peak associated with disorder (1,700–1,660 cm$^{-1}$) is more elevated and prominent for late-stage protofibrils than for the monomeric filament assembly pathway. This would be consistent with our previously proposed model in which the strong charge repulsion prevailing at low salt concentrations promotes the formation of more organized filamentous structures while the slightly weaker charge repulsion at intermediate salt concentration permits the formation of more disordered, oligomeric intermediates (Hill et al. 2011).
Figure 9.2 Infrared Spectroscopy of Late Stage Fibrils shows characteristic $\beta$-sheet Peaks

Amide I peak in H$_2$O for (A) native lysozyme at pH 2.0, and mature fibrils obtained after ultracentrifugation and incubation under (B) oligomer-free (50 mM NaCl) or (C) oligomeric (175 mM NaCl) growth conditions. Light gray lines represent the decomposition of the spectra into $\alpha$-helix, $\beta$-turn, and $\beta$-sheet peaks, with the composite spectra superimposed onto the (solid) raw spectra. (D-F) Bar graphs to the right represent the relative amplitudes for the $\alpha$-helix and $\beta$-sheet peaks in the spectra.

For quantitative analysis, we simultaneously fitted the Amide-I and Amide-II peaks of the spectra using second-derivatives and direct fits. The Amide-II peak was included during fitting since it partially "spills over" into the Amide-I band. The corresponding quantitative
decomposition of the spectra into individual Gaussian peaks and the resulting summed spectra are indicated via gray curves and are superimposed on the original data (See Figure 9.2, black lines). The relative peak area for the α-helix peak (~1655 cm⁻¹) and amyloid β-sheet peak (~1620 cm⁻¹) shown next to each spectrum further illustrates the main feature already seen in the raw spectra: fibril formation in either pathway induces a significant decrease in the α-helix content and concurrent rise of the amyloid-specific β-sheet region (see Figure 9.2D-F). In addition, we consistently found that both the α-helix and β-sheet peaks for fibrils grown in the filamentous pathway where shifted to higher wavenumbers (see dashed lines, Figure 9.2D-F). The corresponding peaks for the protofibril pathway occurred at the location close to that observed with native lysozyme.

9.3 Differences in Secondary Structure Between Oligomeric and Oligomer-Free Pathways

To confirm this pathway-specific difference in the β-sheet position we generated difference spectra of the fibril FTIR spectra and their corresponding monomer spectra, collected under the same solution conditions. Figure 9.3A shows a superposition of the area-normalized FTIR spectra for monomers, late-stage oligomer-free fibrils, and oligomeric fibrils while Figure 9.3B displays the corresponding difference spectra matched to the maxima of their amyloid β-sheet peak (for details of the normalization and subtraction procedure see section 8.2 above). These difference spectra directly yield the peak position for the emerging amyloid β-sheet peak without the detour over spectral decomposition (and its potential artifacts). As seen in Figure 9.3B, the amyloid β-sheet peaks differ by 6 cm⁻¹, with the peak for oligomer-free assembly developing at (1623 ± 1) cm⁻¹, while oligomeric assembly induced a peak at (1617 ± 1) cm⁻¹. The additional difference spectra in Figure 9.3B were obtained from samples subjected to
Figure 9.3 Difference Spectra for Monomeric Filaments and Oligomeric Protofibrils
(a) Amide I FTIR spectra for native (black), monomeric amyloid filaments (red) and oligomeric protofibrils (blue). The three spectra were normalized by the area for comparison. (b) Corresponding FTIR difference spectra of the Amide I region for oligomer free fibrils (red curves) and for oligomeric protofibrils (blue curves) at pH = 2.0. The two difference spectra for oligomer-free fibril growth are for samples either incubated for multiple days (solid red curve) or derived from rapid growth (<12 hours) via seeding with pre-formed fibrils (short-dashed red curve). Similarly, oligomeric difference spectra are shown for growth with 175 mM NaCl (solid blue curve), during rapid growth (<3 hours) in 325 mM NaCl (short dashed blue curve), or for growth at pH 7/65 °C (blue dotted curve).
accelerated fibril growth or from fibrils grown at pH 7. For all these different growth conditions
the difference spectra yielded subtle but reproducible structural differences in the FTIR spectra
of oligomer-free filaments and oligomeric protofibrils which, in turn, correlated with the
pronounced differences in their respective fibril morphologies.

9.4 Time-Course of the Structural Evolution in the Oligomeric Pathway

![Figure 9.4](image)

**Figure 9.4 Time-Course of Difference Spectrum for Lysozyme Solution Undergoing Oligomeric Fibril Growth**

The FTIR difference spectra of a lysozyme solution undergoing oligomeric fibril growth shows
an early onset of the prominent features present in late stage fibrils. Spectra were measured from
aliquots removed at different times during the incubation and at the end-stage of the incubation
process, and subtracted from the initial monomer FTIR spectrum at 25 °C.

We further explored whether FTIR spectra supported the conclusion from ThT data (not
shown) that structural features in a given assembly pathway are established early on and remain
preserved through later generations of intermediates. Towards this end, we obtained FTIR spectra of samples at various stages of the assembly process and compared their FTIR spectra. Figure 9.4 displays the difference spectra for a lysozyme samples undergoing oligomeric fibril growth. While the number of time points we were able to sample is limited, the overall trend is obvious: The main structural features observed during the late stages of fibril growth, i.e. the loss of α-helix content and the emergence of a β-sheet peak around 1,620 cm\(^{-1}\), emerge early during the aggregation process. Only the amplitude of these features increases as incubation proceeds and different populations of intermediates are formed. At the same time, the disordered region around 1,720 – 1,680 cm\(^{-1}\) does seem to undergo structural evolution that is distinct at different stages of the aggregation process.

9.5 Summary and Discussion

The FTIR spectra support two conclusions. First, the spectra of monomeric filaments and oligomeric protofibrils both develop a prominent β-sheet peak characteristic for amyloid fibril structures and a concurrent loss in the content of native α-helix content. The difference spectra further suggest that the two fibril pathways do display discernible structural differences both in the position of the β-sheet peak near 1,620 cm\(^{-1}\) and in the region past 1,680 cm\(^{-1}\) associated with multiple secondary structures (turns, disorder and β-sheets). This shift in the β-sheet peak position was independent of growth conditions and highly reproducible. Specifically, fibrils grown for less than 24 h either via seeding (oligomer-free pathway) or via increased salt concentration (oligomeric pathway) showed the same spectral signature as those incubated for long periods. Furthermore, the oligomeric fibrils obtained at physiological pH values showed the same FTIR peak position as those grown at highly acidic pH values. Hence there are noticeable
structural differences among monomeric filaments and oligomeric protofibrils that correlate with their pronounced differences in the morphology of the corresponding intermediates. The overall trend of highly ordered monomeric filaments and more disordered oligomeric protofibrils correlates well with measurements from similar structures obtained with β2-microglobulin by Smith et al. (2003). As shown in Figure 2 in that paper, X-ray diffraction patterns from "long straight" fibrils yielded sharp reflections while the corresponding patterns for worm-like fibrils were much more diffuse.
Chapter 10

Amyloid Growth Under Near-physiological Conditions

10.1 Introduction

The formation of cross β-sheet protein deposits is characteristic of both infectious prion diseases such as Creutzfeldt–Jakob disease and Kuru and human amyloid diseases. The similarity in the amyloid fibrils associated with these diseases implies that molecular mechanisms underlying these disorders may be linked. Stanley Prusiner’s group has recently shown that the Aβ peptide responsible for Alzheimer’s disease displays prion-like infectivity in mouse models of the disease (Stöhr et al. 2012). However, it is still widely accepted that natively folded proteins such as lysozyme require partial unfolding as a prerequisite for fibril growth.

At pH 7, lysozyme readily forms fibrils along an oligomeric pathway when incubated at high, denaturing temperatures (e.g., 65-70 °C), but not at lower temperatures. By seeding a native monomer sample under near physiological conditions (pH 7; 37 °C) with protofibrils and/or oligomers, we have shown that amyloid fibril growth can continue under native conditions with no discernible populations of partially denatured protein. This conversion of natively folded protein into an amyloid state is characteristic of prion self-replication, with the protofibril or oligomer as the prionic species. Amyloid seeds of lysozyme were grown at 70 °C and pH 7 for 1.5 to 6 hours, with dynamic light scattering (DLS) measurements indicating the formation of oligomers, followed by the nucleation of protofibrils (Mulaj, et al., in prep.).
10.2 Temperature-Induced Denaturation of Lysozyme

Figure 10.1 Thermal Denaturation Curve for Lysozyme
Thermal denaturation curve of HEWL at pH 7 measured using either the peak wavelength of Trp fluorescence emission spectra (●) or far-uv circular dichroism signals at 220 nm (□). The solid line represents a fit with a two-state model through the CD data. Arrows indicate the temperatures used for forming oligomer or protofibril seeds (70 °C) and for aggregating native lysozyme in the presence of such seeds (37° C).

To confirm that lysozyme is in its natively folded conformation under the seed propagation conditions, we measured the thermal denaturation curve for lysozyme at pH 7 using intrinsic tryptophan fluorescence. We recorded the emission spectrum for Trp as the temperature was raised from 25 °C to 85 °C. The emission maximum shifts to higher wavelengths as the protein unfolds, exposing partially buried Trp residues to water. As shown by the shift in tryptophan peak fluorescence emission, lysozyme is partially unfolded under the seed growth
conditions, but it is in its natively folded state under the seed propagation conditions (Figure 10.1). Circular dichroism measurements confirm this result.

10.3 Secondary Structure of Amyloid Fibrils Grown in Denaturing and Native Conditions

The question still remains, however, whether these amyloid fibrils propagated from the prionic seeds have the same structure as those grown under denaturing conditions. To address this concern, we measured the FTIR spectra of fibrils formed under both conditions. In order to investigate the structure of the fibrils without the contribution from the monomer background, we compared the difference spectra, in which the monomeric spectrum is subtracted from each

![Difference Spectra](image)

**Figure 10.2 Difference Spectra for Amyloid Aggregates Grown Under Denaturing or Near-Physiological Conditions**

FTIR difference spectra within the Amide I band between native lysozyme and late-stage amyloid aggregates formed at pH 7 at 70 °C (red) or 37 °C (blue). Native lysozyme monomers at 37 °C were seeded with preformed protofibrils. Both growth conditions lead to an increase in β-sheet content, and are structurally indistinguishable.
sample spectrum, highlighting only the conformational changes occurring during aggregation.

As seen in Figure 10.2, there is an increase in the β-sheet content accompanied by a decrease in the proportion of α helices. The β-sheet peak matches the peak found in the oligomeric pathway at pH 2. Most importantly, the fibrils grown at 70 °C from denatured monomers and the fibrils grown at 37 °C from natively folded monomers are structurally indistinguishable. This incorporation of native protein into aggregates is a hallmark of prionic propagation.

10.4 Lack of Growth Along Monomeric Filament Pathway Under Near-physiological Conditions

![Graph showing stability of monomeric filament seeds at 37 °C in pH 7 Buffer]

**Figure 10.3 Stability of Monomeric Filament Seeds at 37 °C in pH 7 Buffer**

Long straight monomeric filaments were grown at pH 2, dialyzed to pH 7, and filtered through a 450 nm pore size syringe filter. The resulting seeds were incubated at 37 °C and monitored using dynamic light scattering. The scattering intensity is roughly constant, indicating that the seeds are neither aggregating nor disintegrating.

We have shown that lysozyme oligomers and protofibrils are able to incorporate native monomers into amyloid fibrils, but the question remains as to whether this growth is unique to
the oligomer and protofibril seeds, or if it is simply a generic templating phenomenon. To test if the presence of any amyloid seeds will promote growth from native monomers, the previous experiments were repeated using monomeric filament seeds. Long straight fibrils were grown under acidic denaturing conditions (pH 2; 100 mM NaCl; 50 °C) and dialyzed against pH 7 no salt buffer. The resulting seed sample was then filtered with a 450 nm pore size syringe filter to eliminate any large clusters of aggregates that had formed during dialysis. Additionally, this filtering removed most of the larger amyloid fibrils, leaving the smaller, and therefore more soluble, fibrils. This was particularly important because the net charge on lysozyme is reduced from about 17 to 8 charge units when moving from pH 2 to pH 7 buffer (Kuehner et al., 1999).

![Figure 10.4 Failure of Monomeric Filaments to Seed Amyloid Growth Under Near-Physiological Conditions](image)

A native lysozyme sample (20 mg/mL; pH 7; no salt) was seeded with monomeric filaments grown at pH 2, dialyzed to pH 7, and filtered. The scattering intensity was monitored during incubation at 37 °C. The count rate does not increase, indicating that the seeds are ineffective in promoting amyloid growth.
This loss of charge presents difficulties in keeping the long straight fibrils in solution at pH 7. After filtering, the seeds remained soluble and stable in buffer at 37 °C (Figure 10.3).

Samples of native monomers (20 mg/mL; pH 7; no salt) were seeded with the dialyzed and filtered monomeric filaments and monitored with dynamic light scattering. As shown in Figure 10.4, no amyloid growth is detected. This lack of activity when seeding with long straight fibrils indicates that amyloid growth from natively folded monomers requires more than simply a template. Only the oligomers and protofibrils are able to convert native protein into an amyloid state.

10.5 Summary and Discussion

The partial denaturation of a natively folded protein such as lysozyme is generally considered to be a prerequisite for amyloid fibril growth, but we have shown that growth from native monomers can occur under near-physiological conditions by seeding with oligomers or protofibrils. Furthermore, FTIR spectroscopy shows that the amyloid fibrils grown from native monomers are structurally indistinguishable from those grown from partially unfolded monomers. Finally, the inability of monomeric filaments to seed amyloid growth under the same conditions indicates that this is not simply generic templated growth, but that the oligomers and protofibrils act to convert the natively folded monomers into an amyloid state.
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