Chapter 3

Targeted Disulfide Crosslinking in the Nicotinic Acetylcholine Receptor

3.1 Introduction:

Target disulfide crosslinking is a technique that is very amenable to the study of ion channels. The technique provides distance information on where amino acids are in relation to each other in a protein. The technique is based on the fact that thiols in a protein can crosslink when they are between 4-9 Å of each other. Cysteines are systematically incorporated into a protein and then oxidized to reveal information on the distance relationship between amino acids.

This method was used to study the internal pore-lining residues of the nicotinic acetylcholine receptor (nAChR). Several cysteine mutants were made using mutagenesis and then studied in functional channels expressed in *Xenopus* oocytes. The channels were then exposed to oxidizing agents, and the ability of these mutant channels to form disulfide bonds was evaluated.

3.2 Structure of the nACh receptor:

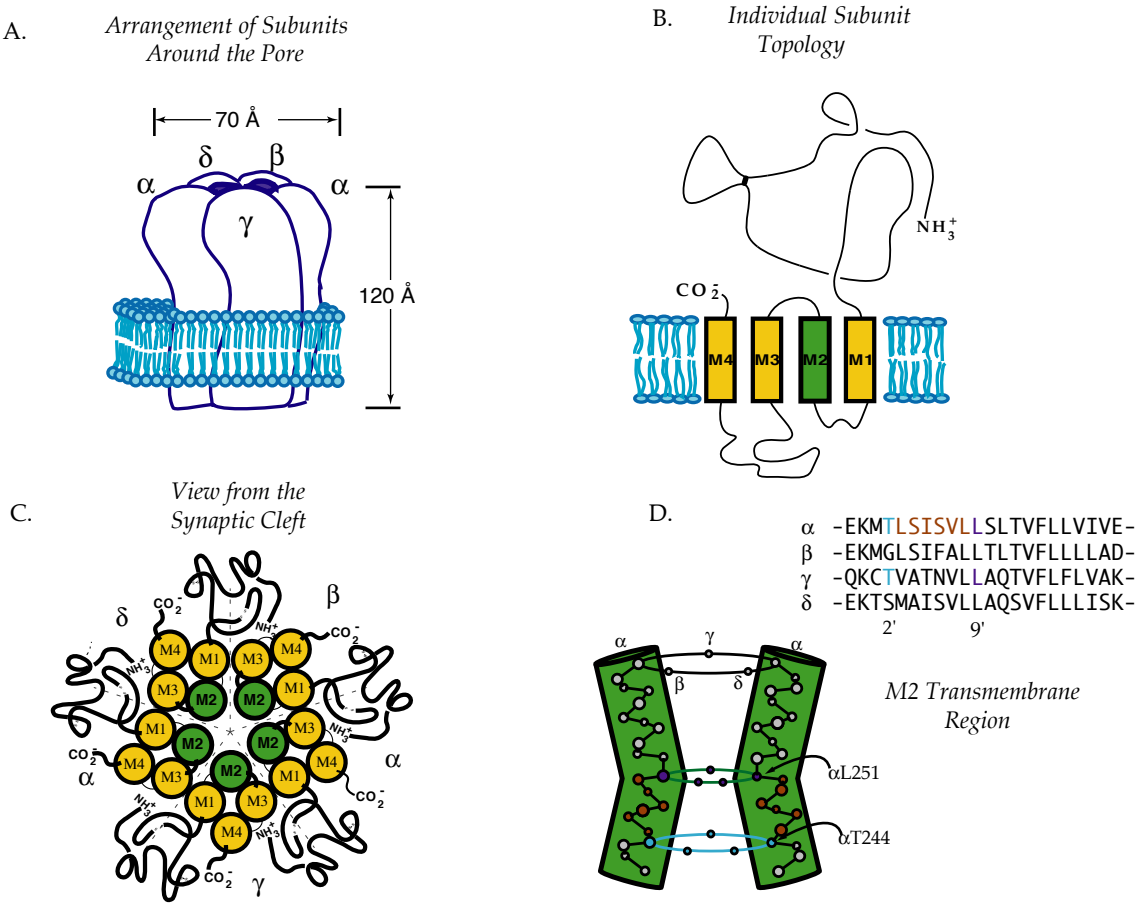
The nAChR is a ligand-gated ion channel. Ligand-gated ion channels gate ion movements and generate electrical signals in response to specific chemical neurotransmitters. The nAChR is one member of the Cys-loop superfamily of ligand-gated ion channels that includes γ-aminobutyric acid (GABA), glycine (Gly), and 5-hydroxytryptamine (5HT) channels (1). nAChRs are cationic channels found in the muscle-axon junction and in the brain, and whose agonist is acetylcholine (ACh). Nicotine also activates the receptor (1-3).

There is a large diversity of nAChRs in the nervous system. To date there are 17 different nAChR subunits (10 α, 4 β, δ, ε, and γ subunits) (4). These subunits form multiple functional homopentamers (α_7, α_8, α_9) or heteropentamers containing one or more different β subunits (1, 4). The subunits arrange around a fivefold axis of pseudosymmetry,

which forms a central pore perpendicular to the plane of the membrane (1, 3). Numerous biochemical studies have shown that the principal part of the ligand-binding pocket is composed of the □ subunit with residues from the adjacent subunit also contributing to the binding site (5-9). Only the nACh receptor at the axon-muscle junction will be discussed further.

All nACh subunits exhibit significant sequence homologies. All subunits are composed of a hydrophilic amino terminal domain which usually has one or two glycosylation sites and a rather small C terminal domain believed to be extracellular (10, 11). All subunits are composed of four hydrophobic domains referred to as M1-M4, which have been shown to transverse the membrane (1, 12). (Figure 3.1)

The muscle type nACh receptor is composed of five subunits with a stoichiometry of either $\square_2\square\square$ or $\square_2\square\square$. The □subunit is expressed in embryonic muscle while □ is expressed in adult muscle (1, 12). The subunits are arranged □, □, □, □, □around a central pore (13). The receptor has been shown to exist in various functional states - resting, active, and desensitized - which are in rapid equilibrium and are affected by reversible binding of ligands (14). A closed channel without agonist characterizes the resting state. The open state is considered to be the open conformation of the channel with two agonists bound. The desensitized state is characterized by high affinity for acetylcholine and a closed channel. The dynamics of the nAChR are very complex and are best described in (14), which describes the open, closed, and five desensitized states.



A. Schematic of the muscle nicotinic aceylcholine receptor. The distance measurements are taken from reference (22). B. A diagram of an individual nAChR subunit with the transmembrane domains labeled as M1-M4. C. A schematic of the arrangement of the transmembrane domains around the central pore. D. The primary sequence of the M2 domain with the αT244 and αL251 labeled as 2' and 9'. The schematic illustrates where these amino acids lie in the transmembrane region of M2 .

Figure 3.1

In addition, biochemical techniques have revealed several aspects of the three dimensional structure of the transmembrane region of the receptor. The transmembrane spanning regions of M3 and M4 have been shown to line the nACh receptor lipid protein interface (15-18). The substituted cysteine accessibility studies (SCAM) first done by Arthur Karlin and colleagues revealed that the top of the N-terminal third of M1 and M2 transmembrane regions line the pore of the channel (4, 19, 20). His SCAM studies will be discussed in further detail later in the chapter. In addition, the maximum diameter of the pore is believed to be 6.4 Å, by organic ion permeability studies (21).

3.3 Gate:

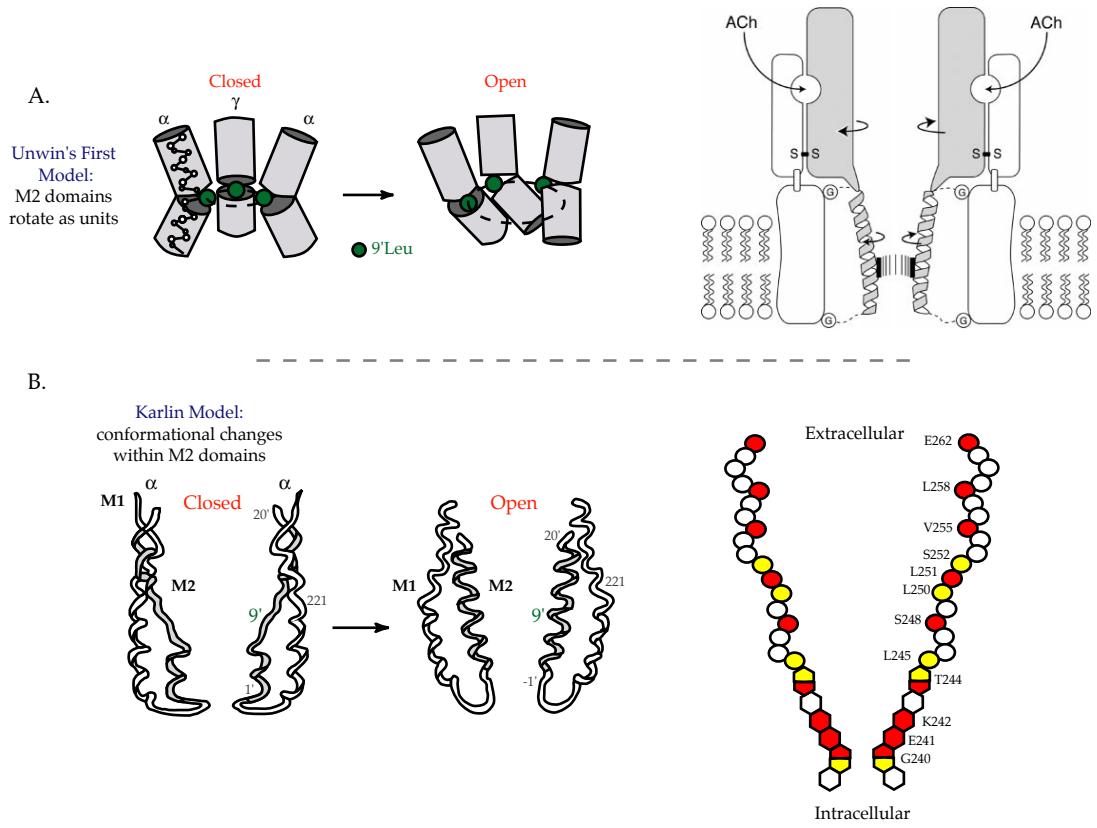
The nACh receptor is believed to open after the binding of acetylcholine elicits a conformational change in the protein. This conformation change can be imagined as the opening of a gate or the most constricted area within the pore, which allows the influx of positive ions through the pore. There are two different models on where this gate is located.

The first model is Unwin's model which is derived from his 4 Å electron micrograph of the nAChR (22, 23). His work describes all four transmembrane regions as α helixes. M2 lines the pore and tilts towards the central axis of the receptor, while the remaining helixes tilt tangentially around the central axis and give rise to left-handed coiling (24). He depicts the gate of the channel to be a hydrophobic "girdle" made up of the hydrophobic residues in M2 between αF256, αV255, αL251, and αS252 (24). This hydrophobic "girdle" creates a hydrophobic plug in the middle of the channel that does not allow ions to permeate through the channel. He considers this region to be the narrowest region of the pore and states that the distance between the central axis to the nearest van der Waals surface is close to 3 Å at αL251 and αV255 (24). Many of the above residues have

been shown in biochemical studies to be involved in the open sensitivity of the channel (25, 26).

Using the recent acetylcholine binding protein (AChBP) crystal structure and his electron micrograph data (27, 28), Unwin has created a model for the opening mechanism for the nAChR(24). The ligand-binding domain of the nAChR is the N-terminus portion of the receptor and is made up of mostly the subunit. The binding domain is made of predominantly sheets. Unwin states that there is a link between the ligand-binding domain in the subunit and the extracellular portion of its M2 helix at positions S269 and P272(24). During binding, the binding domains 1/2 loop in the N-terminus transmit a rotation through the above residues. This rotation twists the M2 hydrophobic plug out of the channel and against the outer protein wall (24).

The other model for gating is derived from data using substituted cysteine accessibility studies (SCAM) done by Arthur Karlin and colleagues (4, 19, 29). Using site directed mutagenesis, amino acids are converted to cysteine sequentially throughout the protein. The mutant channels are then exposed to polar and charged thiosulfonate reagents. Cysteines that react with these reagents are considered to be at water-accessible regions of the protein. SCAM was used to reveal the gate of the nAChR by converting the amino acids down the M2 and M1 regions of the receptor sequentially to cysteine and then exposing the channels to thiosulfonate reagents in the presence and absence of acetylcholine (29). The reaction was monitored by electrophysiology using the reduction or total loss of ACh-induced current has an indicator of reactivity. The difference in exposure of residues to the reagents in the absence and presence of acetylcholine correlates with the difference between closed state and the open or desensitized state. In addition, SCAM reagents were applied intracellularly and extracellularly to determine more precisely where the gate of the receptor is located (29). Thiosulfonate reagents are water soluble; therefore, it is believed that they are traversing down the pore.



A. A schematic of Unwin's first gating model, where the hydrophobic plug around αV255 and αL251 (represented as the green ball) moves out of the pore after the binding to ACh, and the domains rotate as a unit. In Unwin's new model (depicted to the right) ACh is thought to induce rotations in the α subunits, which are transmitted to the gate through the M2 helix (24). However, even in Unwin's new gating model the hydrophobic plug or gate is the same. B. A schematic of Karlin's model of gating. The diagram to the right depicts the SCAM results on αM2. The circles represent residues treated with MTSEA (methane thiosulfonate ethyl ammonium) extracellularly. Diamonds represent residues treated with MTSEA both extracellularly and intracellulary. The top of the diamond refers to the extracellular application and, the botton of the diamond refers to the the intracellular application. Yellow areas are residues which react thiosulfonate reagents without ACh. Dark red areas are residues in which the rate of the reaction is faster in the presence of ACh (29).

Figure 3.2

Karlin used SCAM on the mouse embryonic form of the nicotinic acetylcholine receptor on δM1, δM1, and δM2 domains(19, 20, 29). The top third of M1 in both the δ and δ subunits was shown to react with extracellularly applied thiosulfonate reagents. In addition, many residues in the M2 region were shown to react with thiosulfonate reagents. In Karlin's model, there is a ring of the five subunits surrounding the channel composed of alternating M1 and M2 segments (29). During gating, these segments move relative to one another. Residues between δT244-δK242 did not react with SCAM agents applied intracellularly or extracellularly; therefore, Karlin considers this stretch of residues to be the gate (4, 29).

3.4 Targeted disulfide crosslinking:

The location and the nature of the gate in the nAChR are highly debated. Another biochemical technique must be used to give even higher-resolution data than Unwin's structure and Karlin's SCAM data. Targeted disulfide crosslinking could be used to reveal fine structural aspects of proteins. In the targeted disulfide crosslinking method, cysteine residues are introduced at various locations throughout the protein and oxidized to see whether disulfide bond formation can occur, typically catalyzed by $CuSO_4$ and (1,10-phenanthroline)$_3$ (30-34). For a disulfide bond to be formed between two cysteines, their β carbon atoms must be within 4-9 Å of each other and the adjoining peptide backbone must also be in proper orientation so that all five bond rotations between the side chain atoms can adopt favorable conformations (35). Cysteines that are in close proximity in a protein collide through thermal motion and then can form disulfide bonds. The rate of disulfide formation depends critically on the average distance between the disulfide-forming thiol pair as well as on the amplitude of their thermal motion. The method has recently been

used on several membrane bound proteins to investigate the proximity of amino acids to each other (30-32, 36, 37).

Disulfides do not form spontaneously between thiols, even those within 5 Å of each other, unless there is an appropriate electron acceptor present (35). The classic oxidant has been oxygen, especially in the presence of trace amounts of metal ions such as Cu^{2+}, Fe^{2+}, Co^{2+}, and Mn^{2+} (35, 38). The mechanism for copper oxidation is not well understood but probably involves the generation of reactive oxygen species (superoxide radicals, hydroxyl radicals) during the redox cycling of copper. The complex between Cu^{2+}(phenanthroline)$_3$ has a redox potential E^0 for Cu^{2+}/Cu^{+} =0.17 V (35).

This report discusses the work done to date on using targeted disulfide crosslinking in the mouse embryonic form of the nicotinic receptor. Amino acids in the subunit in the M2 region have been converted into cysteines one at a time (244T to 251L) using site directed mutagenesis. In addition, two gamma cysteine mutants were made T253C and L260C. All receptors have an HA epitope in the M3 -M4 loop in the subunit between the 347-348. The HA epitope is an antigenic peptide (YPYDVPDYA) derived from hemagglutinin on the coat of the influenza virus (39, 40). The epitope is used to visualize the receptor on Western blots or immunoprecipitation. The HA epitope in the wild-type receptor gave no change in EC_{50} (41). Messenger RNA was injected in a 2:1:1:1 ratio of :: subunits and expressed in *Xenopus* oocytes. Electrophysiology experiments were then done on the mutant receptors before and after being exposed to different concentrations of crosslinking reagents. The protein was then isolated from oocytes by either a physical separation from intact cells by dissecting away the membrane or crushing the oocytes and then isolating the protein by immunoprecipitation.

3.5 Electrophysiology results:

All electrophysiology was done on *Xenopus* oocytes held at a constant voltage (-80 mV) using a two electrode voltage clamp. Oocytes were perfused with recording solution (96 mM $NaCl_2$, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.5) during recording. Oocytes expressing nACh receptors were exposed to two brief applications of acetylcholine (ACh) before and after application of copper reagents to compare current before and after copper. Two minutes of washing with recording solution occurred after each application of acetylcholine or crosslinking reagent, unless noted otherwise. All time values are measured from when the copper treatment is removed. $Cu(phen)_3$ stands for Cu(1,10-phenanthroline)$_3$ solution. Inhibition of current due to application of crosslinking reagents or thiosulfonate reagents is defined as:

$$1 - I_{\text{After appl.}} / I_{\text{Before appl.}} \text{ X } 100 = \text{Percent of Inhibition}$$

I = acetylcholine induced current

3.5.1 MTSEA results:

As a control, a solution of 2.5 mM of methanethiosulfoante ethyl ammonium (MTSEA) with 100 μM acetylcholine was applied to wild-type receptors, αT244C, αT244C/αT253C, αL251C, and αL251C/αL260C mutant receptors for two minutes. All of the mutant receptors were inhibited (Table 3.1), which agrees with Karlin's work (19, 29, 42, 43).

Table 3.1: Percent of Current Inhibition after MTSEA and ACh Application

	Two Minute Wash	Four Minute Wash
Wild-type	24 ± 2 %	26 ± 2 %
αT253C	80 %	72 %
αT244C/αT253C	90 ± 1 %	86 ± 1 %
αL260C	57 %	52 %
αL251C	84 %	74 %
αL251C/αL260C	53 ± 2 %	43 %

3.5.2 Results with copper:

Two different concentrations of copper crosslinking reagents were used. The first copper application studied was 3 mM $CuSO_4$ and 9 mM 1,10-phenanthroline in recording solution (37, 44). Mutant receptors were incubated in the solution for one minute without ACh. Results can be seen in Table 3.2. The percent of current in αS246C mutant was similar to that observed in wild-type receptors, and αL245C current inhibition was also similar to wild-type after a three minute wash. Only αS248C and αT244C receptors' current was inhibited approximately 30 % more than wild-type after a three minute wash. Two unresolved issues are that wild-type receptors are affected by the copper treatment and that continuous washing diminishes inhibition, which is inconsistent with covalent disulfide bond formation.

Table 3.2: Percent of Current Inhibition after the Application of 3 mM $Cu(phen)_3$

	One Minute Wash	Three Minute Wash
Wild-type	28 ± 2 %	28 ± 2 %
αS248C	74 ± 1 %	67 ± 2 %
αS246C	32 ± 8 %	29 ± 6 %
αL245C	68 ± 4 %	38 ± 6 %
αT244C	71 ± 4 %	52 ± 4 %

Cysteine mutants were studied using a four minute application 100 μM $CuSO_4$ and 300 μM 1,10-phenanthroline with and without ACh. These conditions have been used in targeted disulfide crosslinking in other ion channels (31, 45). Results with acetylcholine can be seen in Table 3.3. Results without acetylcholine can be seen in Table 3.4. In addition, four new mutant nACh receptors were αT253C, αT244C/αT253C, αL260C, and αL251C/αL260C.

Table 3.3: Percent of Current Inhibition after Application of 100 μM $Cu(phen)_3$ with ACh

	Four Minute Wash	Six Minute Wash
Wild-type	24 ± 4 %	20 ± 3 %
αT244C	66 ± 3 %	58 ± 3 %
αT253C	30 ± 5%	28 ± 4%
αT244C/αT253C	82 ± 1 %	79 ± 1%
αL251C	51 ± 9 %	
αL260C	27 ± 8%	24 ± 7%
αL251C/α260C	50 ± 6 %	45 ± 6 %

Table 3.4: Percent of Current Inhibition after Application of 100 μM $Cu(phen)_3$

	1 Minute Wash	3 Minute Wash	5 Minute Wash
Wild-type	13 ± 2 %	5 ± 2 %	
αT244C	72 ± 2 %	40 ± 1 %	
αV249C	3 ± 0.5 %		
αL250C	14 ± 2 %	13 ± 4 %	
αL251C	43 ± 2 %	16 ± 2 %	14 ± 2 %

The electrophysiology data indicates that a disulfide bond could be forming in the αT244C/αT253C, αT244C, αL251C/αL260C, and αL251C mutants when acetylcholine and 100 μM Cu(phenanthroline)$_3$ is applied for four minutes. The αT244C/αT253C and

δT244C mutant currents are inhibited by 58% and 42% respectively more than wild-type. In the δL251C/δL260C and δL251C mutants, current is inhibited 26% and 27% respectively more than in wild-type. In addition, the current inhibition of receptors with cysteine mutations only in the δ subunit is equivalent to wild-type receptors. In Arthur Karlin's work, a pore accessible cysteine mutant is considered to react with a thiosulfonate reagent when 35-40% of the current is inhibited after adding the SCAM reagent (19, 42, 43).

The inhibition could be occurring either as a result of the formation of a disulfide bond or by residual copper blocking the pore. All copper in the bath solution should wash out in the one-minute wash or five-minute wash before acetylcholine is applied. In addition, 1,10-phenanthroline has a high affinity for Cu^{2+} (K_{assoc}= $5X10^{-10}$ sub-nanomolar affinity)(40). However, a copper ion or $Cu(phen)_3$ could be blocking the pore. A way to test this hypothesis is to use a positive voltage jump during recording after copper application. The change to a positive potential within the oocyte should force the copper out of the pore.

3.5.3 Positive voltage jumps:

A positive voltage step was done on oocytes expressing either the δL251C or δT244C/δT253C mutant. The oocytes were first exposed to the 100 μM $CuSO_4$ and 300 μM 1,10-phenanthroline solution with acetylcholine present for four minutes before the positive voltage jump. No difference was seen in the percent of current inhibition after the positive voltage jump.

3.5.4 DTT reduction:

A technique widely used in disulfide bond crosslinking studies to confirm disulfide bond formation is to reverse the reaction with dithiothreitol (DTT) reduction (45, 46). This

can easily be assayed in ion channels by measuring the increase in current after DTT application. Both in sodium and potassium channels $Cu(phen)_3$ has been used to catalyze the formation of disulfide bonds in the pore of the channel, which inhibits the current. In both studies, after an application of DTT the original current (before copper treatment) is restored (31, 45). The same experiment could be done in the nACh receptor mutant channels. However, there are several barriers to the use of DTT on the nAChR. The agonist-binding pockets in the δ subunits have a strained disulfide between δ192C-δ193C that is easily reduced. The reduction of this disulfide leads to loss of function in the receptor (47-49). Sixty percent of the current is lost in wild-type receptors after incubation with 5 mM DTT for four minutes. However, this disulfide bond has been shown to be protected against reduction when acetylcholine is present (~ 80% decrease in rate of reduction with ACh present) on *Torpedo* electroplagues (50). There are other extracellular disulfide bonds in all subunits that correspond to δ128C - δ142C, which could be reduced in higher concentrations of DTT.

Some studies have been done using DTT to try to recover current in mutant receptors after copper application. A range of results was found using 300 μM to 10 mM of DTT. With wild-type nAChR, little to zero reduction in current was seen after a 2 minute application with 300 μM DTT in the presence of 50 μM ACh. However, when concentrations of 5 mM to 10 mM DTT were used decreases in current (up to 30%) were seen after incubation with DTT and ACh. With mutant receptors, DTT application gave varied results. Four different batches of oocytes expressing δL251C and δT244C nAChR were tested with DTT. In δL251C nACh receptors, a 300 μM DTT with 50 μM ACh application gave a 36 ± 20 % increase in current after a 2 minute application. While in δT244C mutants 5 mM DTT with 50 μM ACh gave a 14 ± 8% increase in current after a 2 minute application. Due to the high variation in the results with DTT, I abandoned the DTT recovery experiments. However, now looking back on the data I think I should have tried more experiments. Our new instrument the Opus Express might allow for this work to be

re-evaluated. The Opus Express allows one to record from eight oocytes at once, which allows one to get more data points in a shorter amount of time. The inability of DTT when applied with ACh to reduce the wild-type nAChR is an encouraging result and perhaps with a higher concentrations of DTT or a longer incubation complete reversal of disulfide crosslinking could have been achieved.

3.6 Western blot results:

By the electrophysiology results on mutant receptors exposed to 100 μM $Cu(phen)_3$ with ACh, a disulfide could be forming in the αT244C, αT244C/αT253C, αL251C, and αL251C/αL260C receptors. However, visualization of a dimer band in a Western blot would increase the assurance that the disulfide bond did form.

After electrophysiological recordings, oocytes expressing mutant nACh channels were either physically stripped of their membranes or completely homogenized in order to isolate nAChR protein to analyze by Western blotting. All oocytes used for Western blots were expressing between 5-11 μA maximal current. After isolation and treatment of receptors, the protein was electrophoresed on 10-12% polyacrylamide gels before being transferred to nitrocellulose paper. The nitrocellulose paper was then blotted using a mouse anti-HA antibody and subsequently treated with a goat anti-mouse secondary antibody conjugated to horseradish-peroxidase for detection by chemiluminescence. Dimer bands would be 104 kDa (α-α) or 113 kDa (α-β) (12). The α *in vitro* monomer standard is on all gels and runs slightly above the 46 kDa molecular weight marker.

Many different manipulations of the protocol described above were done to try to visualize dimer formation due to copper crosslinking. The expression of nAChR in injected oocytes is approximately 2-3 fmol, estimated by using radio-labeled nicotine or α-bungratoxin binding (51). In addition, the nAChR is a highly hydrophobic protein. Several different techniques were used to isolate, purify, and solublize the protein. In addition,

crosslinking reagents were applied to the receptor in many different ways. The different manipulations to the technique are described below.

Isolation of receptor was first accomplished by dissecting the oocytes after treatment with hypotonic solution, which fuses the vitelline membrane and plasma membrane. The membranes were then treated using the procedure of Kaback and colleagues, who work on site directed cysteine crosslinking in lactose permease (32, 52). The procedure involves incubation of the protein in either a 1 mM $Cu(phen)_3$ or 0.5 mM I_2 as oxidants for 20 minutes at room temperature. The oxidant is removed after centrifugation and 2 X gel-loading buffer (120 mM Tri-Cl pH 6.8, 12% sucrose, 4% SDS, .001% bromophenol blue) is added. Blot 3.1 shows a Western blot utilizing this procedure. Treated samples have no crosslinked bands and seem to contain less protein than untreated samples. This even occurs in the wild-type control lanes. After the protein is treated with copper or iodine, apparently it becomes insoluble or has an incorrect conformation for antibody binding. Different pre-loading treatments were applied on samples to try to elevate a solubility problem.

The solubilization of membrane proteins is a very empirical technique. In many cases, boiling samples induces proteins to aggregate and precipitate or adhere to the sample tube. In other cases, only by boiling the samples do membrane bound proteins solubilize. In Blot 3.2 the Kaback treatment of samples was still used, but most samples were pre-treated before loading the gel by either a two-minute sonication or by two minutes of boiling (32, 52, 53). Membranes were treated with either 1 mM $Cu(phen)_3$, 0.1 mM *o*-PDM, or 0.1 mM *p*-PDM in buffer for twenty minutes at room temperature. *o*-PDM (N,N-*o*-phenylenedimaleimide) and *p*-PDM (N,N-*p*-phenylenedimaleimide) are commercial bi-functional thiol crosslinkers, which include 6 Å and 10 Å rigid crosslinkers, respectively (32, 52, 53). Therefore, thiols between 4 to 10 Å should have been able to crosslink with either $Cu(phen)_3$, *o*-PDM, or *p*-PDM. However, there is still no evidence for a dimer band in mutant nAChR sample lanes. Reducing agent was included in some sample buffers. In

fact, bands appear around 97.4 kDa in samples with reducing agent in the sample buffer (including the wild-type sample and uninjected sample). It still appears that when the membranes encounter copper or iodine the protein vanishes. The pretreatment seemed to not inhibit the reduction of protein in treated samples. I decided to try treating intact oocytes with crosslinking reagents before removing the membrane.

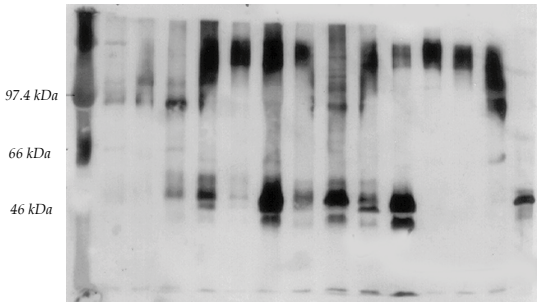
In Blot 3.3, intact oocytes were incubated for twenty minutes in either recording solution alone or in 3 mM $Cu(phen)_3$ in recording solution. The oocytes were then washed and physically stripped of their membranes. Samples were then solubilized in 4% SDS detergent. As can be seen by the gel, the loss of protein after treatment with copper seems to be eliminated. The same result was seen in wild-type receptor and uninjected oocytes control gel. The procedure was tried on oocytes expressing αL251C/βL260C mutant receptors and αT244C/βT253C mutant receptors (Blot 3.4). This blot was the first encouraging result. Both double mutant samples lanes had dark streaks when copper was applied, which implied that oligomerization could be occurring. In addition, stripped membranes were treated directly with 3 mM $Cu(phen)_3$ in buffer A for 30 minutes at 37°C (Blot 3.5). The reaction was stopped with 2X stop buffer containing 37.5 mM NEM or containing 200 mM DTT and 37.5 NEM. Samples treated with DTT were allowed to sit at room temperature for one hour before SDS-PAGE was performed. However, in the absence of further purification, dimer bands could not be definitely assigned. This led to the use of a technique involving a membrane impermeant biotin derivative for purification.

Sulfo-NHS-biotin is sulfosuccinimidyl 6-(biotinamido) hexanoate, which reacts with lysines at pH 7 or 9 (54). Sulfo-NHS-biotin is highly water soluble, therefore is believed to react with proteins only on the surface of the membrane. After treatment, residual biotin-NHS esters are quenched with excess glycine. The oocytes are homogenized, yolks removed, and the remaining supernatant is incubated with streptavidin-agarose beads. The protein is then released from the beads by adding gel-loading buffer directly to the beads and heating (54).

fact, bands appear around 97.4 kDa in samples with reducing agent in the sample buffer (including the wild-type sample and uninjected sample). It still appears that when the membranes encounter copper or iodine the protein vanishes. The pretreatment seemed to not inhibit the reduction of protein in treated samples. I decided to try treating intact oocytes with crosslinking reagents before removing the membrane.

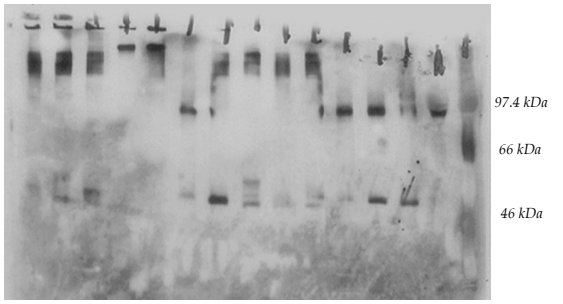
In Blot 3.3, intact oocytes were incubated for twenty minutes in either recording solution alone or in 3 mM $Cu(phen)_3$ in recording solution. The oocytes were then washed and physically stripped of their membranes. Samples were then solubilized in 4% SDS detergent. As can be seen by the gel, the loss of protein after treatment with copper seems to be eliminated. The same result was seen in wild-type receptor and uninjected oocytes control gel. The procedure was tried on oocytes expressing αL251C/βL260C mutant receptors and αT244C/βT253C mutant receptors (Blot 3.4). This blot was the first encouraging result. Both double mutant samples lanes had dark streaks when copper was applied, which implied that oligomerization could be occurring. In addition, stripped membranes were treated directly with 3 mM $Cu(phen)_3$ in buffer A for 30 minutes at 37°C (Blot 3.5). The reaction was stopped with 2X stop buffer containing 37.5 mM NEM or containing 200 mM DTT and 37.5 NEM. Samples treated with DTT were allowed to sit at room temperature for one hour before SDS-PAGE was performed. However, in the absence of further purification, dimer bands could not be definitely assigned. This led to the use of a technique involving a membrane impermeant biotin derivative for purification.

Sulfo-NHS-biotin is sulfosuccinimidyl 6-(biotinamido) hexanoate, which reacts with lysines at pH 7 or 9 (54). Sulfo-NHS-biotin is highly water soluble, therefore is believed to react with proteins only on the surface of the membrane. After treatment, residual biotin-NHS esters are quenched with excess glycine. The oocytes are homogenized, yolks removed, and the remaining supernatant is incubated with streptavidin-agarose beads. The protein is then released from the beads by adding gel-loading buffer directly to the beads and heating (54).



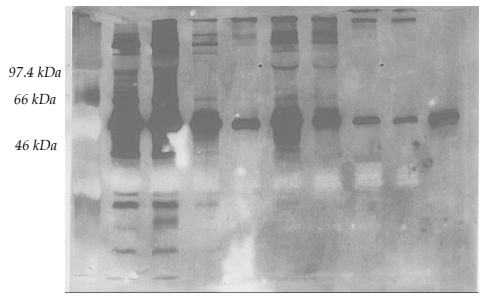
Number of oocytes in each lane is given as (#).
2X sample buffer was added to all samples. Samples that are labeled "reducing agent" were treated with 4 mM β–mercaptoethanol

Blot 3.1



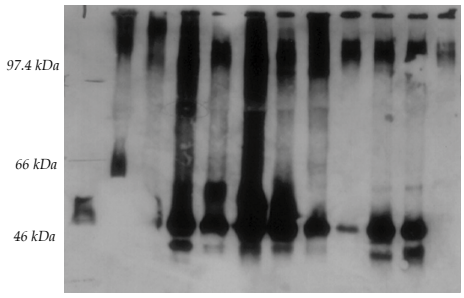
Number of oocytes per lane is depicted as (#). Reducing agent stands for 4 mM of β-Mercaptoethanol, which was in the 2X sample buffer. *p*-PDM and *o*-PDM are N,N-*p*-phenylenedimaleimide and N,N-*o*-phenylenedimaleimide respectively. Both are homobifunctional thiol crosslinkers.

Blot 3.2



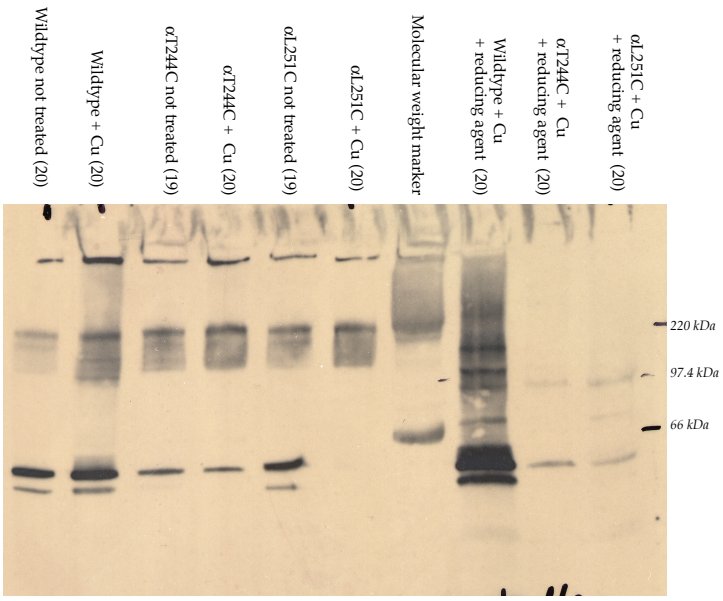
Number of oocytes in each lane is indicated by (#). All oocytes were stripped of their membranes.

Blot 3.3



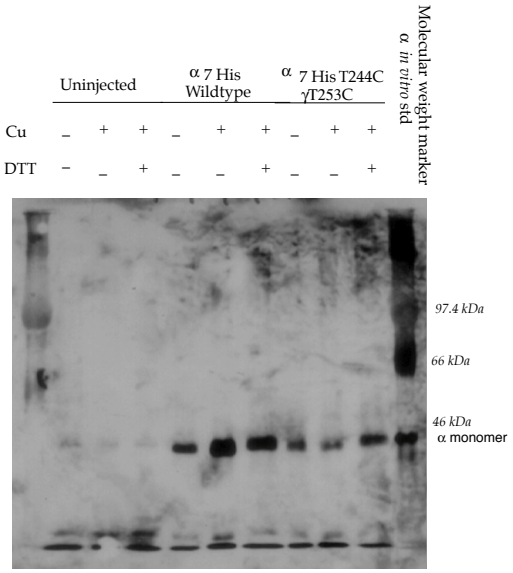
Number of oocytes in each lane is indicated by (#)
+ Cu - treated intact oocytes for 20 min in recording solution with 3 mM $CuSO_4$ and 9 mM 1,10 - phenanthroline
+ I_2 - treated intact oocytes for 20 min in 0.5 mM I_2 solution for 20 min
Then oocytes were stripped.

Blot 3.4



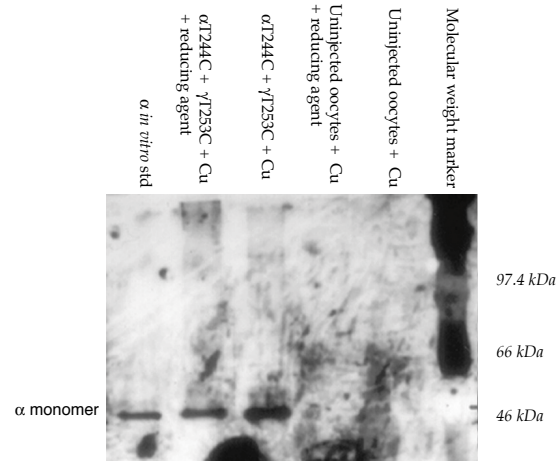
Number of oocytes in each lane is given as (#).
2X sample buffer was added to all samples. Samples that are labeled "reducing agent" were treated with 200 mM DTT.

Blot 3.5



All samples have 24 membranes.
Reducing agent - 4 mM β-mercaptoethanol in 2X sample buffer.

Blot 3.6



NHS biotin labeling. Copper treatment on the bead.
Fifty - three oocytes used per lane.

Blot 3.7

Both Blots 3.6 and 3.7 used the sulfo-NHS-biotin to isolate protein prior to PAGE. In Blot 3.6, the oocytes were incubated in 3 mM $Cu(phen)_3$ for 20 minutes before treatment with sulfo-NHS-biotin (1 mg per 40 - 50 oocytes) for ten minutes. The residual NHS-biotin esters were quenched with excess glycine. The oocytes were then washed, homogenized, pelleted to remove the yolk, and incubated with streptavidin beads for an hour at 4°C. The protein was removed from the beads by addition of gel-loading buffer and boiling for three minutes before loading the gel. No band was evident at either 104 kDa (□ -□ dimer) or 113 kDa (□ - □dimer). The streaking was eliminated, but there was concern that after all of the manipulation of the membranes the disulfide bonds could have become reduced. By applying the copper solution directly to the beads after the protein is bound, subsequent manipulation was minimized (Blot 3.6). The oocytes in Blot 3.6 were washed 5 times and then treated with sulfo-NHS-biotin (1 mg per 40 - 50 oocytes) for 10 minutes. The 1 mM $Cu(phen)_3$ solution was added directly to the protein attached to streptavidin beads. The samples were rocked at 4°C for 45 minutes. The copper solution was removed and the beads are washed before adding gel-loading buffer. Again, no dimer band appeared at 104 kDa or 113 kDa.

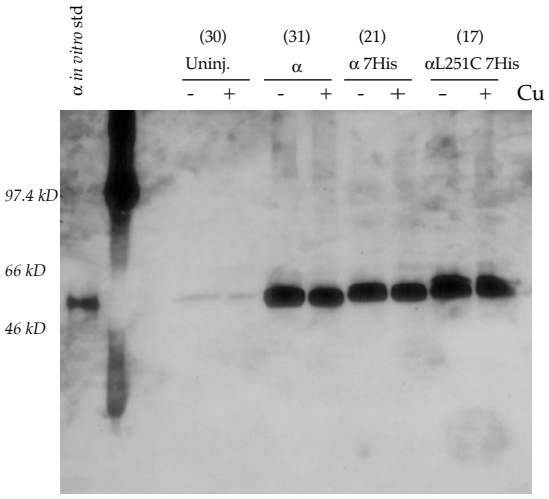
3.6.1 Ni^{2+} Purification and N-Ethyl Maleimide:

As discussed earlier, oocytes do not express a high quantity of protein. Therefore, it is critical to find an efficient way to isolate and purify as much protein as possible from each oocyte. One technique often used to isolate protein is His tag purification (55-57). The technique works by cloning six or more histidines into the protein of interest. These histidines can then be bound to Ni^{2+} beads, which are used to isolate the protein. A seven histindine tag was cloned into □ wild-type, □T244C, and □L251C constructs at the C-terminus. In addition, all of these constructs contained the HA epitope as discussed earlier.

Protein for all of these constructs was expressed in oocytes and used in copper crosslinking studies.

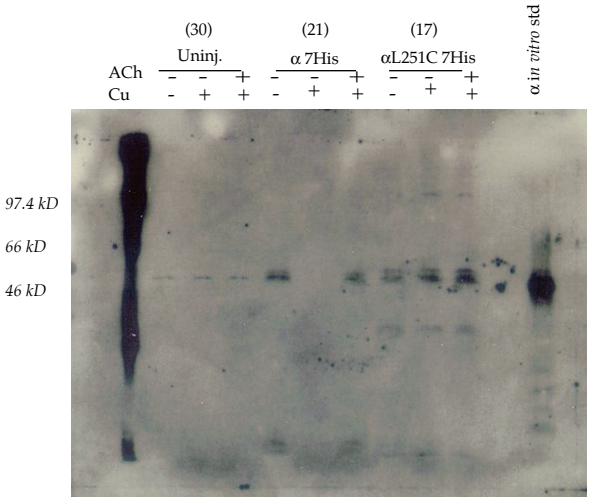
In addition, during this time several papers on disulfide crosslinking revealed that a very important aspect to the technique is the use of n-ethyl maleimide (NEM) (58-60). Oprian and colleagues showed that the use of NEM greatly reduced disulfide bond shuffling and that NEM is required to see dimer formation due to disulfide crosslinking in rhodopsin (58). The gels below use His tag purification to isolate nAChR and NEM to stop the copper crosslinking reaction.

His tag purification was done according to Schmalzing and colleagues (55). They use His tag purification to isolate nAChR and apply crosslinkers to the protein after removing it from the Ni^{2+} beads. In his procedure oocytes are homogenized in a 0.5 % □-dodecyl maltoside and 100 mM sodium phosphate buffered solution (pH 8) (Buffer A). All solutions contain protease inhibitors. The homogenate is centrifuged and the yolk removed. The supernatant is then diluted with binding buffer (Buffer A with 10 mM imidizole) and added to Ni^{2+} beads. The beads are then incubated with supernatant for thirty minutes at room temperature. The beads are then washed four times with 25 to 50 mM imidizole buffer A solution. The protein is then eluted off the beads with a high concentration of imidazole (200 mM – 1 M) solution. A more detailed procedure can be found in (55).



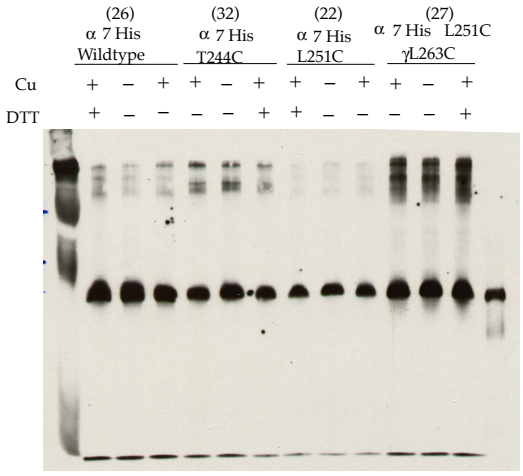
Number of oocytes in each lane is given as (#).

Blot 3.8



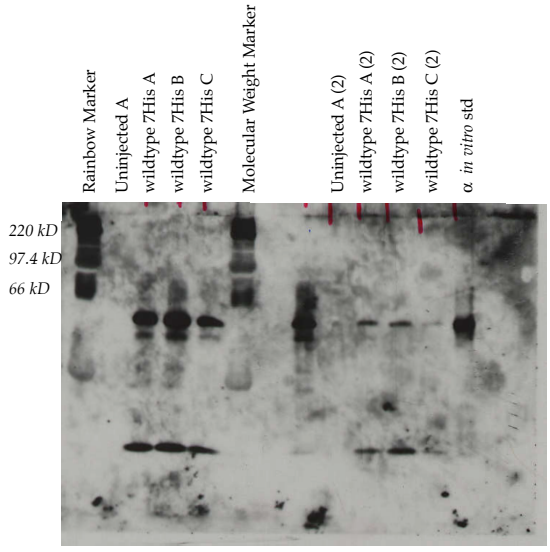
Number of oocytes in each lane is given as (#).

Blot 3.9



Number of oocytes in each lane is given as (#).

Blot 3.10

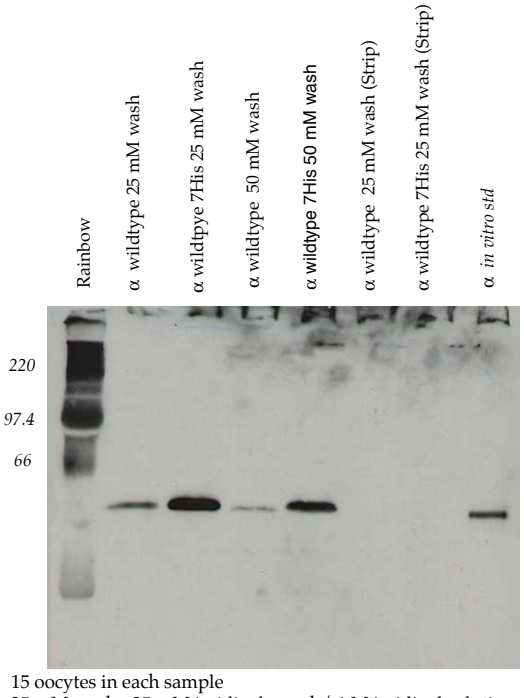


Homogenized oocytes containing wildtype nACh receptors , containing an α subunit with the HA epitope and 7 histidines, were treated with Ni^{2+} beads. Three different washing and eluding buffers were used in treating the Ni^{2+} beads. These procedures are labeled as A, B, and C. In procedure A, three 25 mM imidizole wash steps and two 200 mM imidizole elution steps were used. The sample from the first elution is labeled as "wildtype 7His A" and the sample from the second elution is labeled "wildtype 7His A (2)". In procedure B, three 50 mM imidizole washs and two 1 M imidizole elution washes were done. In procedure C, three 25 mM imidizole washes and two 1 M imidizole elution washes were done. All samples from the second elution are labeled with a (2).

Blot 3.11

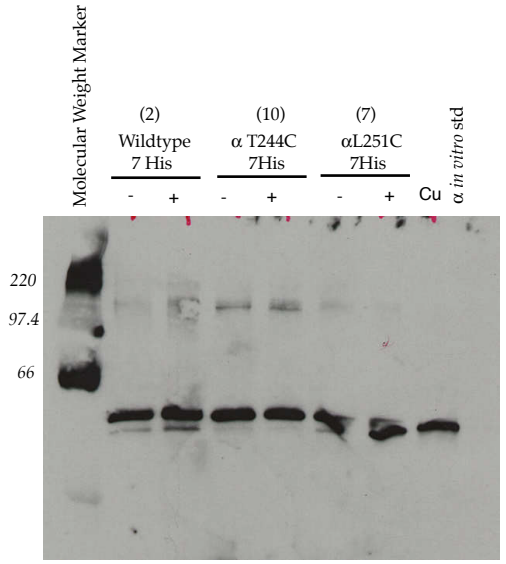
Blots 3.8, 3.9, and 3.10 are the first attempts to purify nAChR using Ni^{2+} beads. The number of oocytes per lane is written in the caption of the gel. The beads were washed with 25 mM imidazole and protein was eluted with 200 mM imidazole. After elution, samples were incubated in 100 mM sodium phosphate buffer (pH 8) with 3 mM $Cu(phen)_3$ for 20 minutes at 37°C. The reaction was stopped with 2X sample buffer containing NEM. In gel 3.8, the final concentration of NEM in the sample was 4.1 mM. A second elution of the protein from the Ni^{2+} beads with 200 mM imidazole buffer A was done, and the results can be seen in Blot 3.9. This gel shows that there is a significant amount of protein still left on the beads after one 200 mM imidazole elution step. In addition, based on the □ wild-type lane in gel 3.8, it seems that proteins not containing the His tag are binding to the beads. The His tag purification was tried again with a greater final concentration of NEM and some of the samples were treated with DTT. In Blot 3.10, a final concentration of 12 mM NEM and 50 mM DTT were used in the indicated samples. There is a large increase in high molecular weight staining in most lanes, and this staining is not reduced by DTT addition.

The His tag purification needs to be improved. There are a significant amount of high molecular weight bands in previous gels. I am assuming that these high molecular weight bands are due to non-specific antibody binding and not to the nAChR. Higher imidazole washes were used in Blots 3.11 and 3.12 to elute off non-specific binding of protein on Ni^{2+} beads. In addition, different concentrations of imidazole were used in the final elution. The gels reveal that the use of 1 M imidazole allows for all of the protein to be eluted off of the beads in one elution step. In addition, the blots reveal what occurs when different concentrations of imidazole are used in the wash step and elution steps. Washing the beads with 50 mM imidazole removes most of the non-specific binding of □ wild-type protein. Based on these two blots, 50 mM imidazole was subsequently used in the Ni^{2+} bead wash buffer and 1 M imidazole in the elution buffer.



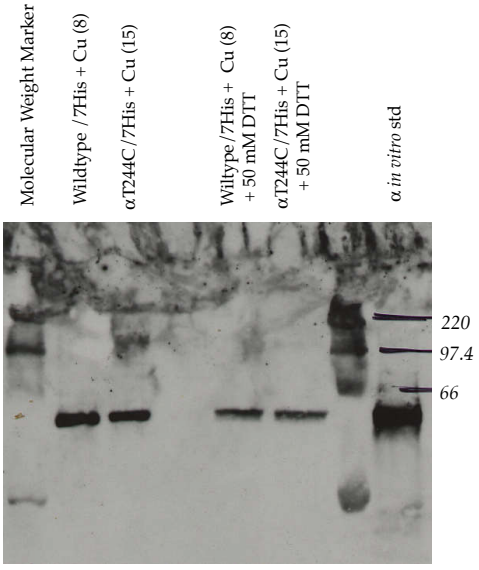
15 oocytes in each sample
25 mM wash - 25 mM imidizole wash/ 1 M imidizole elution
50 mM wash - 50 mM imidizole wash/ 1 M imidizole elution
Strip - 10 oocytes stripped membranes/ 25 mM imidizole wash/ 1 M imidizole elution

Blot 3.12



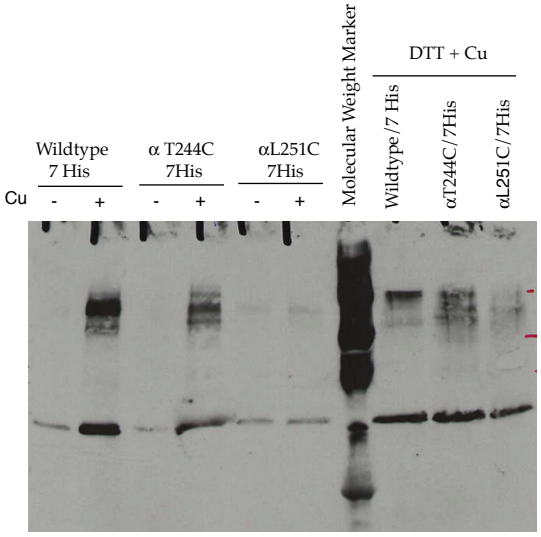
The number of oocytes in each sample is above the lane.

Blot 3.13



Number of oocytes is indicated by (#).

Blot 3.14



Blot 3.15

Blot 3.13 is a blot with protein homogenized from oocytes that were His tag purified using 50 mM imidazole wash buffer and 1 M elution buffer. After elution, the samples were treated with 3 mM $Cu(phen)_3$ in a 100 mM sodium phosphate buffer (pH 8) for 30 minutes. Again high molecular weight bands were seen in all lanes, and these bands were not dependent on the introduction of copper to the sample. Could the high concentration of imidazole be interfering with the reaction? Blot 3.14 is a blot of copper mediated disulfide crosslinking done on the Ni^{2+} bead. The instructions discussed above were followed. The beads were washed twice with 50 mM imidazole in buffer A and then washed overnight in buffer A without imidazole. The beads were centrifuged and the supernatant removed. The beads were then incubated in 500 □l 3mM $Cu(phen)_3$ in buffer A for 30 minutes at room temperature. The reaction was stopped with 500 □l of buffer A containing 25 mM NEM. The beads were then removed and the protein eluted with 1 M imidazole in buffer A. Is the higher molecular weight band in the □□244C/7His treated with copper a dimer band? The experiment was repeated, and some of the samples were treated with 50 mM DTT (Blot 3.15). High molecular weight bands are seen in all lanes, and DTT treatment does not remove them.

3.6.2 DMS control gels:

DMS (3,3'-dimethylsuberimidate) is a biofunctional crosslinking reagent which reacts with external lysines on the nAChR (55, 61). It has been shown to create intramolecular crosslinks between subunits in the nAChR. DMS crosslinking was tried twice on nAChR (with the HA epitope in the □ subunit) expressed in *Xenupos* oocytes. In the first experiment, oocyte membranes were physically dissected and then treated with 5 mM DMS in 0.2 M triethanolamine and 10% TRITON-100 solution (55). In the second, membranes were physically dissected off the oocytes and then treated with 5 mM DMS in

0.2 M triethanolamine and 0.5% dodecyl β-maltoside solution (55). Oocyte membranes were incubated for one hour in this solution before applying gel-loading buffer. No crosslinking bands were ever seen.

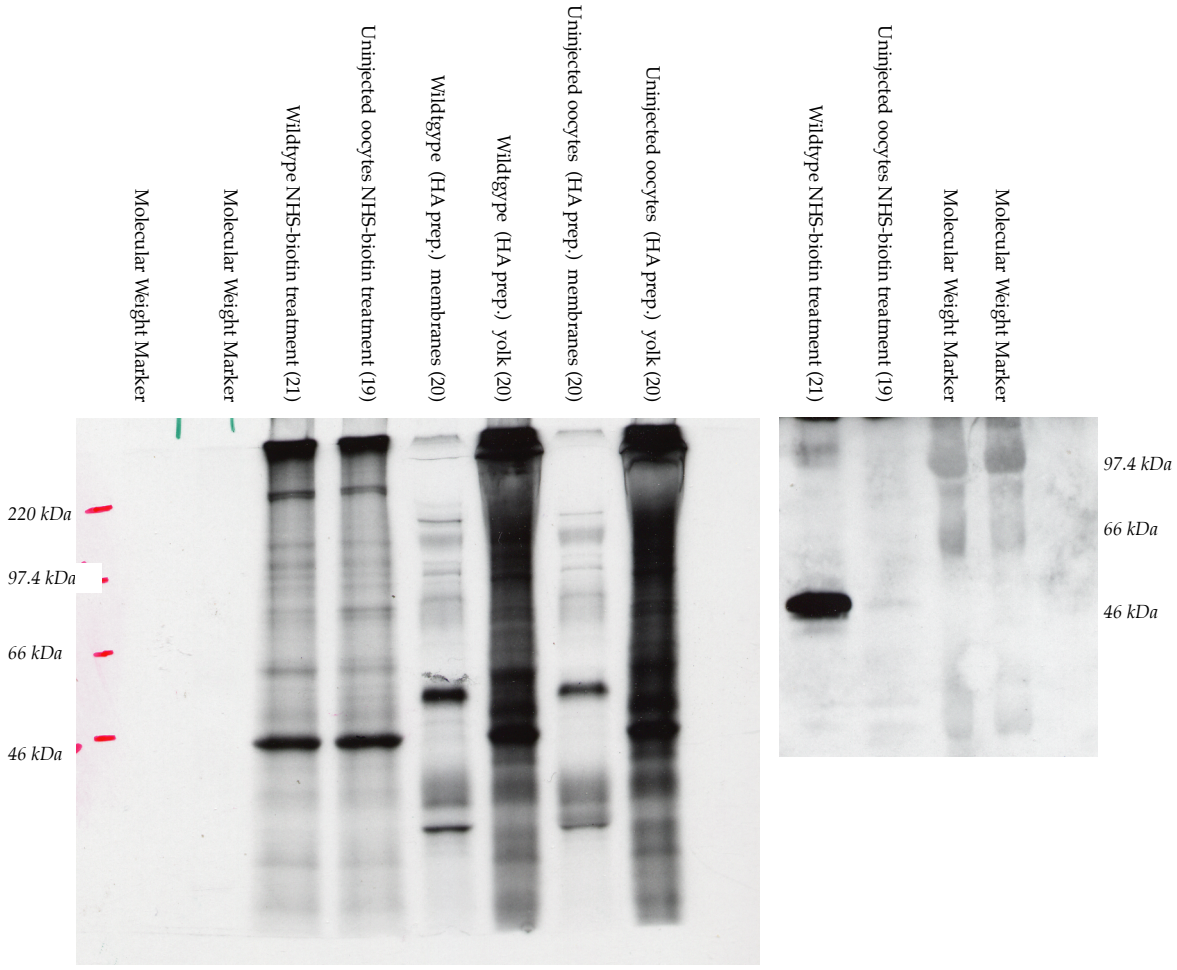
3.7 Radiolabeling:

Another biochemical technique that is used to visualize protein in oocytes is radiolabeling. *In vivo* use of [^{35}S]-methionine labeling is used often in detection and manipulation of small quantities of membrane-bound proteins expressed in oocytes (55, 62, 63). Oocytes are injected with mRNA of a particular protein and then incubated overnight in solution containing [^{35}S]-methionine, which gets incorporated into the expressed protein. The labeled protein is then purified.

[^{35}S]-methionine labeling was tried several times to evaluate if radiolabeling would be an improvement over Western blotting. In most experiments Schmalzing's radio-labeling protocol was used (55). In this protocol oocytes are injected with RNA and then incubated in cold incubating solution for several hours and then incubated in ^{35}[S]-methionine doped solution for 36 hours (0.4 MBq per oocyte). The protein is then purified using either immunoprecipitation or another technique before SDS-PAGE (54, 55, 62, 63). The gel is then dried and exposed to photographic film for minutes to days, which is then developed. Protein containing ^{35}[S]-methionine should be detected as a band on the developed film.

My final radiolabeling experiment illustrates well the problems that were associated with the technique. In this experiment, a Western blot was performed in unison with radiography to analyze the difference in sensitivity between the two. In the experiment, oocytes were injected with wild-type nAChR where the β subunit contained the HA epitope. The oocytes were incubated in ND-96 solution with horse serum overnight (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 5 mM HEPES pH 7.5). They were then incubated in ND-96 solution with 11.56 MBq/ml of ^{35}[S]-methionine. This incubation was

followed with another 3 hour incubation with ND-96 solution alone. Oocytes were then treated two different ways. In half of them, their membranes were dissected using hypertonic solution and the membranes and the remaining yolks were treated separately with G-protein sepharose beads coated with the HA antibody. The immunoprecipitation of both the membranes and the yolks was an attempt to reveal how much of the nAChR was in the cytosol of the cell compared to the membrane. In addition, sulfo-NHS-biotin labeling was used to purify the receptor from the full homogenate of oocytes expressing wild-type nAChR. After labeling, the homogenate was purified using strepavidin beads, and the samples were loaded on to two SDS-PAGE gels. One of the gels was then transferred to nitrocellulose and blotted using the HA-antibody. The other gel was directly exposed to photographic film (radiogram). The blot and the radiogram can be seen below (Radiogram 3.1 and Blot 3.16). In the radiogram there is a non-specific band that occurs in many of the uninjected oocytes lanes that lies at the same molecular weight as the □ subunit. In addition, there is not a new band found in the wild-type lanes compared to the uninjected lanes. The uninjected and the wild-type lanes looked identical. However, the blot reveals clearly the □ monomer band in the wild-type nAChR lane, which is not seen in the uninjected oocytes lane. Based on this experiment and others, at least in my hands, Western blotting is a more sensitive and better technique for revealing the nAChR from SDS-PAGE gels than radiography.



Number of oocytes in each lane is indicated by (#).
Labeling
Membranes: Oocyte membranes were physically dissected and then immunopreciptated using the HA antibody.
Yolk: The remaining yolk from the dissected oocytes were used in these samples. The yolks were also immunopreciptated with the HA antibody.
HA prep: Immunopreciptation using the HA antibody.
NHS-biotin treatment: Whole oocytes were treated with sulfo-NHS-biotin and then homogenized. The homogenate was then treated with strepavidin beads.

Radiogram & Blot 3.16

3.8 Discussion:

3.8.1 Electrophysiology:

The electrophysiology data indicate that a disulfide bond could be forming in the αT244C/βT253C, αT244C, αL251C/βL260C, and αL251C mutants when acetylcholine with 100 μM Cu(phenanthroline)$_3$ is applied for four minutes. The αT244C/βT253C and αT244C mutants currents are inhibited 58% and 42% respectively more than in wild-type. In the αL251C/βL260C and αL251C mutants, currents are inhibited 26% and 27% respectively more than in wild-type. Inhibition of current could be occurring for three reasons; a disulfide bond is forming, copper ions are acting as an open channel blocker, or a high affinity copper binding pocket is formed by the engineered cysteines. If copper ions were simply blocking the pore, then positive voltage jumps should have recovered the original current after copper treatment. Another possibility is that the cysteines form a high-affinity binding pocket for copper (38, 64). Cysteines have been shown in several proteins to chelate various heavy metals (38, 65, 66). However in these experiments, there is probably not a high concentration of free copper ions in the medium. Cu(phen)$_3$ has a sub-nanomolar affinity for Cu^{2+} and is therefore likely to be highly competitive with any Cys binding of Cu^{2+} (38). Thus, disulfide formation is the most probable explanation for the observed inhibition of current. However, inhibition of current is not enough to establish that a disulfide bond truly did form; therefore, Western blots were used to try to visualize the dimer band after copper treatment.

3.8.2 Western Blots:

A dimer band has not yet been seen on any Western blot between either □□□ (104 kDa) or □□□(113 kDa). This could be occurring either because the disulfide bond is truly not forming, the dimer is insoluble, or has an incorrect conformation for antibody binding. The question of whether a disulfide bond is forming is addressed by the

electrophysiological experiments. In addition, in other studies cysteines 6.4 Å apart are routinely crosslinked using $Cu(phen)_3$ (33, 34, 67, 68). The gate or most constricted portion of pore has been shown to be about 6.4 Å (69). I suspect the reason why the dimer has not been seen yet is that the dimer is insoluble.

Many membrane proteins have been shown to be very difficult to solubilize and to detect by PAGE, and the nAChR is a highly hydrophobic protein (32, 46, 52, 55). Also the environment of the epitope is an issue. Based on experiments done by Gabriel Brandt, the HA antibody seems to bind the HA tag when included in the β subunit about four times better than when the epitope is included in α, γ, or the δsubunit (70). This has been shown to occur in both *in vivo* and *in vitro* experiments. In addition, any manipulation done to nAChRs (photocrosslinking experiments and NPG cleavage experiments) have not been able to be detected on a gel (70). I have tried several procedures to try to view the dimer on a gel. Boiling and sonication of samples, different detergents, immunoprecipitation, and employing different copper crosslinking procedures have all been used to try to view the dimer band to no avail. In addition, no crosslinking bands have been seen in gels using 3,3'-dimethylsuberimidate (DMS) in control gels, even though this reagent has been shown previously to create intermolecular crosslinks between subunits in the nAChR (55, 61).

3.9 Conclusion:

The work described here is unable to completely verify if the disulfide crosslinking in αT244C and αL251C mutants is occurring. There is some indication that copper is inducing crosslinking in these mutants by the high percentage of inhibition of current after copper application. Another assay must be used to confirm dimer formation. The use of dithiothreitol (DTT) in the confirmation of disulfide bond formation is relatively simple. However, there is the concern that high concentrations of DTT needed to reduce the

disulfide in the channel will also inactivate the receptor by reducing the seven extracellular disulfide bonds. The other direct biochemical assay is the use of Western blots. Currently, the gel assay technique has some fundamental problems in viewing the nAChR. The technique requires more manipulation until the ability to view crosslinking either by copper or 3,3'-dimethylsuberimidate (DMS) is achieved. If the gel assay system could be used to view molecular changes in the protein (oligomers through crosslinking, phosphorylation, or cleavage of the protein) a new area in probing the structure of the nAChR would be open for exploitation.

3.10 Bibliography:

1. Arias, H. R. (1997) *Brain Research Reviews* **25**, 133-197.

2. Galzi, J. L., and Changeux, J. P. (1994) *Current Opinion in Structural Biology* **4**, 554-565.

3. Galzi, J. L., and Changeux, J. P. (1995) *Neuropharmacology* **34**, 563-582.

4. Karlin, A. (2002) *Nature Reviews Neuroscience* **3**, 102-114.

5. Czajkowski, C., and Karlin, A. (1995) *J Biol Chem* **270**, 3160-3164.

6. Stauffer, D. A., and Karlin, A. (1994) *Biochemistry* **33**, 6840-6849.

7. Zhong, W., Gallivan, J. P., Zhang, Y. N., Lintong, L., Lester, H. A., and Dougherty, D. A. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 12088-12093.

8. Li, L. T., Zhong, W. G., Zacharias, N., Gibbs, C., Lester, H. A., and Dougherty, D. A. (2001) *Chemistry & Biology* **8**, 47-58.

9. Nowak, M. W.*, et al*. (1995) *Science* **268**, 439-442.

10. Chavez, R. A., and Hall, Z. W. (1992) *Journal of Cell Biology* **116**, 385-393.

11. Arias, H. R. (1996) *Molecular Membrane Biology* 1-15.

12. Hucho, F., Tsetlin, V. I., and Machold, J. (1996) *European Journal of Biochemistry* **239**, 539-557.

13. Karlin, A., Holtzmann, E., Yodh, N., Lobel, P., Wall, J., and J., H. (1983) *Journal of Biological Chemistry*.

14. Elenes, S., and Auerbach, A. (2002) *Journal of Physiology-London* **541**, 367-383.

15. Blanton, M. P., and Cohen, J. B. (1992) *Biochemistry* **31**, 3738-3750.

16. Blanton, M. P., and Cohen, J. B. (1994) *Biochemistry* **33**, 2859-2872.

17. Blanton, M. P., McCardy, E. A., Huggins, A., and Parikh, D. (1998) *Biochemistry* **41**.

18. Giraudat, J., Montecuco, C., Bisson, R., and Changeux, J. P. (1985) *Biochemistry* **24**, 3121-3127.

19. Akabas, M. H., and Karlin, A. (1995) *Biochemistry* **34**.

20. Wilson, G. G., and Karlin, A. (2001) *Proceedings of the National Academy of Sciences of the United States of America* **98**, 1241-1248.

21. Giraudat, J., Dennis, M., Heidmann, T., Haumont, P. Y., Lederer, F., and Changeux, J. P. (1987) *Biochemistry* **26**, 2410-2418.

22. Miyazawa, A., Fujiyoshi, Y., Stowell, M., and Unwin, N. (1999) *Journal of Molecular Biology* **288**, 765-786.

23. Unwin, N. (2000) *Journal of General Physiology* **116**, 2A-2A.

24. Miyazawa, A., Fujiyoshi, Y., and Unwin, N. (2003) *Nature* **423**, 949-955.

25. Labarca, C., Nowak, M. W., Zhang, H. Y., Tang, L. X., Deshpande, P., and Lester, H. A. (1995) *Nature* **376**, 514-516.

26. Filatov, G. N., and White, M. M. (1995) *Mol Pharmacol* **48**, 379-384.

27. Smit, A. B.*, et al*. (2001) *Nature* **411**, 261-268.

28. Brejc, K.*, et al*. (2001) *Nature* **411**, 269-276.

29. Wilson, G. G., and Karlin, A. (1998) *Neuron* **20**, 1269-1281.

30. Kobertz, W. R., Williams, C., and Miller, C. (2000) *Biochemistry* **39**, 10347-10352.

31. Liu, Y., Jurman, M. E., and Yellen, G. (1996) *Neuron* **16**, 859-867.

32. Sun, J., Kemp, C. R., and Kaback, H. R. (1998) *Biochemistry* **37**.

33. Lynch, B. A., and Koshland, D. E. (1991) *Proceedings of the National Academy of Sciences of the United States of America* **88**, 10402-10406.

34. Stoddard, B. L., Bui, J. D., and Koshland, D. E. (1992) *Biochemistry* **31**, 11978-11983.

35. Creighton, T. E. (1984) *Methods in Enzymology* **107**, 305-329.

36. Wolin, C. D., and Kaback, H. R. (2000) *Biochemistry* **39**, 6130-6135.

37. Yu, H. B., Kono, M., and Oprian, D. D. (1999) *Biochemistry* **38**, 12028-12032.

38. Creighton, T. E. (1993) Proteins: Structures and Molecular Propertieseds. New York: W. H. Freeman,

39. Sato, M. H., and Wada, Y. (1997) *Biotechniques* **23**, 254-256.

40. Canfield, V. A., Norbeck, L., and Levenson, R. (1996) *Biochemistry* **35**, 14165-14172.

41. Brandt, G. S. (1998) Candidacy Report. California Institute of Technology.

42. Zhang, H. Y., and Karlin, A. (1997) *Biochemistry* **36**, 15856-15864.

43. Akabas, M. H., Stauffer, D. A., Xu, M., and Karlin, A. (1992) *Science* **258**, 307-310.

44. Lee, G. F., Burrows, G. G., Lebert, M. R., Dutton, D. P., and Hazelbauer, G. L. (1994) *Journal of Biological Chemistry* **269**, 29920-29927.

45. Benitah, J. P., Ranjan, R., Yamagishi, T., Janecki, M., Tomaselli, G. F., and Marban, E. (1997) *Biophysical Journal* **73**, 603-613.

46. Liu, Y., Holmgren, M., Jurman, M. E., and Yellen, G. (1997) *Neuron* **19**, 175-184.

47. McLaughlin, J. T., Hawrot, E., and Yellen, G. (1995) *Biochemical Journal* **310**, 765-769.

48. Karlin, A., and Bartels, E. (1966) *Biochimica Et Biophysica Acta* **126**, 525-&.

49. Servent, D., Menez, A., and Kessler, P. (1995) *Febs Letters* **360**, 261-265.

50. Damle, V. N., and Karlin, A. (1980) *Biochemistry* **19**, 3924-3932.

51. Anand, R., Conroy, W. G., Schoepfer, R., Whiting, P., and Lindstrom, J. (1991) *Journal of Biological Chemistry* **226**, 11192-11198.

52. Sun, J., and Kaback, H. R. (1997) *Biochemistry* **39**, 11959-11965.

53. Wu, J. H., Hardy, D., and Kaback, H. R. (1998) *Biochemistry* **37**, 15785-15790.

54. Chen, J. G., Liu-Chen, S., and Rudnick, G. (1998) *Journal of Biological Chemistry* **273**, 12675-12681.

55. Nicke, A., *et al*. (1998) *Embo Journal* **17**, 3016-3028.

56. Janknecht, R., Martynoff, G. D., Lou, J., Hipskind, R. A., Nordheim, A., and Stunnenberg, H. G. (1991) *Proceedings of the National Academy of Science U S A* **88**, 8972-8976.

57. Shapovalov, G., Bass, R., Rees, D. C., and Lester, H. A. (2003) *Biophysical Journal* **84**, 2357-2365.

58. Kono, M., Yu, H., and Oprian, D. D. (1998) *Biochemistry* **37**, 1302-1305.

59. Yu, H. B., and Oprian, D. D. (1999) *Biochemistry* **38**, 12033-12040.

60. Zeng, F. Y., Hopp, A., Soldner, A., and Wess, J. (1999) *Journal of Biological Chemistry* **274**, 16629-16640.

61. Watty, A., Methfessel, C., and Hucho, F. (1997) *Proceedings of the National Academy of Science U S A* **94**, 8202-8207.

62. Ivanina, T., Perets, T., Thornhill, W. B., Levin, G., Dascal, N., and Lotan, I. (1994) *Biochemistry* **33**, 8786-8792.

63. Pralongzamofing, D., Yi, Q. H., Schmalzing, G., Good, P., and Geering, K. (1992) *American Journal of Physiology* **262**, C1520-C1530.

64. Burkitt, M. J., *et al*. (1998) *Archives of Biochemistry and Biophysics* **353**, 73-84.

65. Zhu, Z. W., Petering, D. H., and Shaw, C. F. (1995) *Inorganic Chemistry* **34**, 4477-4483.

66. Farrar, J. A., *et al*. (1996) *Journal of the American Chemical Society* **118**, 11501-11514.

67. Careaga, C. L., and Falke, J. J. (1992) *Journal of Molecular Biology* **226**, 1219-1235.

68. Reichert, A., Heintz, D., Echner, H., Voelter, W., and Faulstich, H. (1996) *Journal of Biological Chemistry* **271**, 1301-1308.

69. Unwin, N., Miyazawa, A., Li, J., and Fujiyoshi, Y. (2002) *Journal of Molecular Biology* **319**, 1165-1176.

70. Brandt, G. S. (1998) Unpublished Results. California Institute of Technology