Dear Jeanne, dear Craig,

this is my presentation on β2-bungarotoxin as I gave it on Nov 30, 05. The explanatory text is also written in the "note column" under every slide in the powerpoint file "beta2 bungarotoxin MMP 113005.ppt".

I hope everything is fine. If there should be further questions please feel free to write me an email.

Best wishes

Martin

Slide 1:

Introduction:
As we should try to fit the presentation on a time scale of 15 to 20 minutes I had to set priorities concerning the content. I decided to give an introduction to the source and the evolutionary development of the venom and just some few remarks on the physiology and anatomy of the „place of action", the neuromuscular junction. Sorry Jeanne, that I didn't include the pathological consequences in terms of action potentials, but I thought this could be too much for some of the fellow-students, that don't know how it is created. Furthermore I wanted to show some features about the quality of the structure. I wanted to describe the function of the two subunits and their peculiarities resulting of their structure. From my point of view there are 4 interesting sites on the protein:
1.) the dimerization surface
2.) the phospholipase A2 active site of subunit a
3.) the specific Shaker type potassium channel binding site of subunit b, the Kunitz subunit
4.) the protease inhibitory loop, which is arranged in a way that it has no protease inhibitory function

Because of the lack of time I decided to concentrate structurally on the (1.)) dimerization surface, because it is contains much more charged interactions than other proteins, which usually have more hydrophobic interactions. Because I considered this as a new structural concept. For the phospholipase A2 activity (2.)) I just wanted to show how nicely the fatty acid, which is one of the „products" of the cleavage of a phospholipid, fits in the active side in bovine pancreatic phospholipase and that the fatty acid collides sterically with the Trp19 in the case of the beta2-bungerotoxin. Unfortunately there has been done no research on the binding motif of the subunit b to the Shaker type potassium channel. The certainly interesting point 4.) about the protease inhibitory loop was left out because of time deficiencies. Sorry Jeanne, I know this would have been interesting to you. A consideration of the b-factors shows an increased mobility of the residues on the surface of the protein as usual in crystal structure. But as long as there is nothing specific about them I excluded them from the presentation. The same situation occurs for distance measurements. As I didn't have a structure of bet2-bungarotoxin bound to the Shaker type potassium channel nor the fatty acid distance measurements of the interesting interaction surfaces are futile.

The structure, which I presented was solved by Kwong and co-workers and explained in this publication:
Kwong PD et al. **Structure of 2-bungarotoxin: potassium channel binding by Kunitz modules and targeted phospholipase action**. Structure. 1995. 3:1109-1119

Remark: The pymol-files 14a, 14b, 16a and 16b contain a structure that is named 1G4I_BPTI… This is the bovine pancreatic PLA2, not the bovine protease trypsin inhibitor (BPTI), which could be assumed by the same abbreviated.

Slide 2:

Source:
This snake injects a venom cocktail into the victim, which acts synergistically. We distinguish between two different classes of venoms, one that destroys the muscle tissue and the other that acts on the neuromuscular junction. Bungarotoxins belong to the second group and this group is further divided (see slide 4)
I will concentrate on the alpha subunit PLA2

Slide 3:

Anatomy of nerve signal conduction from the brain through cerebro-spinal cord to the muscle:
See pictures

Slide 4:

Magnification from last slide: the neuromuscular junction:
Short explanation on membrane potential (was left out in presentation, because of time and complexity):
In the unexcited cell there is an equilibrium of small anions and cations and macromolecular anionic structures. This equlibrium is shifted to a negative potential of -60 mv to -90 mV that is provided by an energy-dependent process by the Na^+/K^+-ATPase, that transports K+ in the cell and Na^+ out of the cell (in the ratio 2:3) against an concentration gradient. If there is a change in the potential because of an incoming signal down the axon, voltage-dependent ion channels open and Na^+ enters the cell which leads to a hyperpolarisation. The slower K^+ ion channels open later to counteract the Na^+ influx with an K+ efflux with the concentration gradient, which is called repolarisation. Before the repolarisation, the potential shot up over +20 mV which leads to an action potential mediated by Ca^{2+}. The result is a fusion of the acetylcholine contending vesicle at the nerve terminal into the neuromuscular junction (= synapsis). This neurotransmitter binds the acetylcholine receptor on the postsynaptic side and leads to a Ca^{2+} dependent contraction of the muscle. The acetylcholine diffuses from its receptor and is cleaved by acetylcholine esterase. The „cleavage-products“ are taken up again by the nerve terminal and new acetylcholine is synthesized and stored in vesicles.

The diverging roles of Bungarotoxins are depicted in the slide

Slide 5:

Enzymatic action of the beta2-bungerotoxin subunit a phospholipase A2:

Phospholipids, in this case Phosphotidylcholine (=Lecithin), are hydrolyzed at the 2nd position ester bond of the Glycerol by Phospholipase A2. The „cleavage products“ are a fatty acid and a „destructed“ phospholipid that cannot build up a bio membrane any more. Therefore the plasma membrane of the nerve terminal is corrupted and destroyed, which leads to the collapse of the membrane potential and the inability of recepting and conducting nerve signals. The neurotransmitters are not released from their vesicles any more and no neurotransmitter can therefore bind to its receptor at the postsynaptical side. This results in no contraction of the muscles, but a relaxation, which is in first instance lethal for breathing, because the lung muscles can‘t contract any more. (As the heart has another kind of muscle tissue (we distinguish between striated, smooth and heart muscle) this is not the primary reason for death).

Slide 6:

Quality of crystal structure analysis:
The structure was determined by structure solved by molecular replacement and MIR. The resolution is high enough for recognizing the different residues in the electron density map. The discrepancy in the R-factor, which itself is good as long as it‘s smaller than 0.2, and the free R-value is obvious. Which leads me to the conclusion that the structure is perhaps overrefined as the difference should be no more than 0.03. The occupancy is 1 for every atom, which is perfect. The B-factors are small. The solvent content nearly fits Jeanne‘s thumb rule: resolution [no unit] * number of solvent molecules < amino acids (2.45 * 81 = 198.45 > 181 (17.45 „too much“)
Water is important for quartenary structure

Slide 7:

Ramachandran plot:
The phi- and psi- angle relation for the amino acids is within the allowed areas in the Ramachandran plot. Only one amino acid is in the unallowed and three are in the partially unallowed zone. This is a good result!

Slide 8:

Structural composition of β-bungarotoxins:
All β-bungarotoxins consist of two subunits. This is peculiar for toxins because most other snake venoms like from Naja naja and *Bungarus caeruleus* consist of one „optimized“ peptide chain that includes all necessary functions, mostly a combination of a binding site and an enzymatic activity. There exist at least 6 (beta 1 to 6) and 3 further related β-bungarotoxins, which only differ in the combination of different subunit a and b. These different Subunits have different specificities. In the case of the subunit a, the PLA2, the turnover time and perhaps even the substrate specificity (as there are different phospholipids in bio membranes) is different. In the case of subunit b, the Kunitz subunit, their binding ability is different. Perhaps even the protease inhibitory function is still active in some subunits b. To confirm this considerations further work has to be done to clearly define the differences in the subunits.
The subunits function and structure is described later.

The subunits are structurally and functionally distinct. Each subunit for itself is quite globular as can be seen in „8 und 9 bunga subunits joining.pse“ F1 and F2, but bound to each other they have an extended form (F3), which is presumably necessary for the arrangement of the active site in subunit A relative to the potassium channel binding site in subunit b.

Slide 9:

The most obvious structural feature in the interaction of the subunits is a disulfide bond between Cys15 of subunit a PLA2 and Cys55 of subunit b Kunitz as can be seen in „8 und 9 bunga subunits joining.pse (F4). These cysteines are unique to β-bungarotoxins. In all monomeric venoms and in homologous enzymes in the gastro-intestinal tract of the digestive system these cysteines are missing, because there is no necessity for them to build up a disulfide bond on their surface.
Furthermore inter- and intra-peptide chain disulfide bonds, which are both existing in the β-bungarotoxins, are typical for extracellular proteins, because the reductive milieu in the cell prevents their formation.

Slide 10:

A closer look on the binding interface of the subunits shows that not only the disulfide bond but also other charged interactions support the binding. The hydrophobic interactions that usually dominate large scale interactions are minimal but essential. As stated in the lectures hydrophobic residues on the surface are suspicious for interactions, because this arrangement is entropically unfavourable. This discovery of new structural principles could evoke new considerations about this „hydrophobicity rule“. The charged interactions are mostly mediated by water. This reminds the crystallographer again of the fact that the solvent in which the crystal is grown should be as physiological as possible. If the crystallization process was perform in e.g. organic solvent this binding interface perhaps would not have occurred like this and wrong conclusions could have been drawn.

Slide 11:

Additionally see slide 1

Slide 12:

Subunit b, the Kunitz „protease inhibitor“:
This subunit like subunit a is stabilized by disulfide bonds and essentially lacks in most parts secondary structure. It just has a short alpha-helix that positions the Cys55 for the inter-subunit disulfide bond and a twisted antiparallel beta-sheet (see „12 Kunitz disulfide.pse“ F1). This can be explained by the fact that this subunit includes 3 interaction areas in just 61 amino acids. As these interaction areas are by definition mostly made up by loop regions the lack of secondary structure is a tribute to the existence of 3 interaction surfaces.
A homologous member of the Kunitz protease inhibitor superfamily is bovine pancreatic trypsin inhibitor (BPTI), which is present in the digestive tract of cattle. It differs mainly to subunit b in its three interaction areas (not compared in pymol):

1.) The protease inhibitory function of subunit b is lost, which is mostly due to the structural rearrangement of the antiprotease loop (aa 13-18). As I explained in the introduction I had to leave this structural aspect out, but if you are deeply interested please refer to Kwong PD et al. **Structure of 2-bungarotoxin: potassium channel binding by Kunitz modules and targeted phospholipase action**. Structure. 1995. 3:1109-1119 page 1111, Fig. 2.
2.) the binding surface to subunit a differs in subunit b from BPTI, which makes sense, because BPTI does not multimerize.
3.) see slide 13

Slide 13:

Potassium channel binding of subunit b:
All toxin members of the Kunitz superfamily, differing from the non-toxin members, like BPTI, bind to voltage-sensitive ion channels. The structural mode of this binding has not been elucidated, presumably because there is no structure of an ion channel with beta-bungarotoxins, but Kwong assumes that the beta-turn built up by residue 27 to 30 is involved in this ion channel binding. Usually the bound toxins block the ion channel, which results in the inability of the channel to let ions diffuse over the membrane. In the case of a potassium channel block on the nerve terminal this leads to a facilitation of the creation of an action potential. Because the voltage-gated potassium channels do not counteract the Na+ influx, the depolarisation is stronger and presumably longer. Therefore more neurotransmitter (here: Acetylcholine) will be released in the neuromuscular junction.
Differing from this concept β-bungarotoxin binds specific Shaker type potassium channels, but does not block the channel in a relevant manner. Counteracting the later occurring destruction of the plasma membrane and resulting inability of neurotransmitter release by subunit a, the binding first facilitates the neurotransmitter release by partial ion channel blocking. This blocking can be considered as a rudiment, an evolutionary rest function of the original channel blocking ability.
Because of the inability to block the potassium channel, this function of subunit b is thought to be just responsible for binding the Shaker type potassium channel specifically for positioning subunit a correctly for a specific destruction of nerve terminal membranes. (see slide 16)

Slide 14:

Subunit a, the Phospholipase A2:
As subunit b, subunit a is stabilized by disulfide bonds (see „14a PLA2 comparison to pancreas.pse" F2). The ratio of disulfide bonds to number of amino acids is the same for both subunits (b: 3 to 61; a: 6 to 120). Subunit a shows more secondary structure than subunit b. Additionally 3 alpha-helices are stabilizing subunit a (see „14a PLA2 comparison to pancreas.pse" F1). Another structural feature is the complexation of a Ca2+ ion, which is important for the enzymatic activity of the PLA2. Unfortunately the Ca2+ was not present in the crystallization, but is surely there (Kwong) and perfectly fits like in the structure of bovine pancreatic PLA2 (see "14b PLA2 comparison to pancreas with all features for alignment.pse" F1
A structural comparison of subunit a with bovine pancreatic PLA2, that is homologous and belongs to the PLA2 family too, shows that both peptides have a highly similar structure. This can be seen already by a first simple view on both peptides positioned in the same way („14a

PLA2 comparison to pancreas.pse" F3). A representation of the structural differences is shown in "14b PLA2 comparison to pancreas with all features for alignment.pse" F1:
The yellow residue Cys15 in subunit a builds up the disulfide bond with Cys55 of subunit b and is not present in bovine pancreatic PLA2.
The blue residues (58 to 66 in subunit a and 58 to 71 in bovine pancreatic PLA2) form the substrate binding loop. Major differences in secondary structure and positioning of the loop can be seen. They become even more obvious, when the structures are aligned (type: align 1b, 1g) (see „14b PLA2 comparison to pancreas with all features for alignment.pse"). Kwong does not describe the consequences of this difference, but it can be assumed that either the substrate or the affinity to the substrate differs between both proteins.
The alignment also confirms that the complexation of Ca2+ is the same in both proteins.

Slide 15:

Active site:
For getting a clue, where the active site of PLA2 or other active sites could be, the pdb file was analyzed by hotpatch for concavities. „16a PLA2 active site comparison to pancreas.pse" shows the result. The red concavity at the interface of subunit a and b shows a deep red colour, but it is known that this interface does not provide any further function or binding than the connection of both subunits. Ignoring the other small concavities, one big concavity can be clearly made out. In the middle of subunit a a deep „hole" can be seen (red arrow). Kwong already found out that this concavity is the active site of the hydrolysis of phospholipids in PLA2.

Slide 16:

The function of PLA2 was already described in slide 5. What's peculiar about the PLA2 of β2-bungarotoxin is that it does not unspecifically hydrolyses all phospholipids and therefore plasma membranes. The homologous Bovine pancreatic PLA2 just hydrolyses all phospholipids that get into the small intestine and helps digesting the remaining components of the food (e.g. bio membranes from devoured cells in plants and meat). This inability to unspecifically act on all phospholipids is due to subunit a's weak enzymatic activity, when not bound to a membrane or separated from subunit b. Why? A comparison between bovine pancreatic PLA2 and the subunit a of β2-bungarotoxins („16a PLA2 active site comparison to pancreas.pse" F1) shows that not only the substrate binding loop (see slide 14) differ. Residue 19 is a „small" leucine in the case of bovine pancreatic PLA2, but a „bulky" tryptophane in subunit a. Unfortunately there is no crystal structure of β2-bungarotoxin and a phospholipid or a fatty acid. Therefore I tried to model in a fatty acid (which is one of the cleavage products) which was cocrystalized with 1TC8 (, a Krait-Venom Phospholipase A2) (see „16b fatty acid modelled in.pse"). Even though it is difficult to see, within the modelling procedure it became logical for me that the Trp19 really is colliding with at least one of the carbon atoms of the fatty acid, like Kwong reports. Kwong further describes that the Trp19, which is occluding the active site can fulfil a torsional rotation to the most favourable sterically allowed rotamer conformation and stick the indole-ring of its tryptophane into the plasma membrane. This acts as a lipophilic anchor and additionally stabilizes the binding of β2-bungarotoxin to the plasma-membrane near a Shaker type potassium channel.

Slide 17:

Summary (1):
The targeted phospholipase β2-bungarotoxin specifically binds the Kv1.2 Shaker type potassium channel, which is preliminary expressed on nerve terminals with its subunit b, which is a member of the Kunitz protease inhibitor superfamily, but lost its protease inhibitory function. The subunit a, a phospholipase A2, is bound over an unusually charged interaction that‘s mostly water-mediated. Its occluded active site makes sure that the phospholipase does not unspecifically destroy bio membranes, likes its homologues in the digestive system, but only attacks the bio membrane when already bound nearby, so that the occluding Trp19 can perform a sterically allowed conformational change and integrate into the bio membrane.

Slide 18:

Summary (2):
The hydrolysis of the phospholipid leads to a destruction of the nerve terminal and the inability of the neuromuscular junction to transduce nerve impulses. The primarily reason for death is respiratory failure.

Slide 19:

Relevance:
The close examination on the structure and function of snake venoms bears several advantages. As snakes are present in many regions of the world a lot of humans but also breeding cattle are endangered by poisoning. To prevent serious health consequences or even death that occur after a snake attack (mostly defence...) the development of new antidotes can be accelerated by a precise knowledge of the venom‘s action and function for the design.
All secondary metabolites or molecules from other species with intrinsic action in humans or mammals in general have developed their specificity in an evolutionary process over thousands, sometimes millions of years. Research can use this target specificity to identify it‘s target, as this was done with alpha-bungarotoxin for the acetylcholine receptor, or as a lead compound for the derivation of peptidomimetics.
A very fancy approach is to separate the two subunits from each other and combine them with other subunits with different function. One idea was to fuse subunit a with its occluded fatty acid binding site to a module that targets the construct to viruses. As Viruses have no own biosynthesis machinery they can until today just be fought when they are entering the cell, proliferating in the cell or leaving the cells. With this construct viruses could be targeted before getting in contact with the cells and their bio membrane, if they have one, because most possess at least an additional capsid, could be specifically disintegrated. Unfortunately it is known that the separation of subunit a and b leads to a structural instability.