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Structural Basis for Epibatidine Selectivity at Desensitized Nicotinic Receptors

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Nicotinic ACh receptor agonist selectivity

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List of abbreviations:

nAChR nicotinic acetylcholine receptor; AChBP acetylcholine binding protein; HEK 293 Human embryonic kidney 293; RESP restrained electrostatic potential; ESP electrostatic potential.

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Abstract-

The agonist binding sites of the foetal muscle nicotinic acetylcholine receptor are formed at the interfaces of α subunits and neighbouring γ and δ subunits. When the receptor is in the non-conducting desensitised state, the αγ site binds the agonist epibatidine 200-fold tighter than does the αδ site. To determine the structural basis for this selectivity we constructed γ/δ subunit chimeras, co-expressed them with complementary wild-type subunits in HEK 293 cells, and determined epibatidine affinity of the resulting complexes. The results reveal three determinants of epibatidine selectivity: γ104-117/δ106-δ119, γ164-171/δ166-177, and γP190/δA196. Point mutations reveal that three sequence differences within the γ104-117/δ106-δ119 region are determinants of epibatidine selectivity: γL104/δY106, γS111/δY113 and γY117/δT119. In the δ subunit, simultaneous mutation of these residues to their γ equivalent produces high affinity, γ-like epibatidine binding. However, converting γ to δ affinity requires replacement of the γ104-117 segment with δ sequence, suggesting interplay of residues in this region. The structural basis for epibatidine selectivity is explained by computational docking of epibatidine to a homology model of the αγ binding site.

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The structure of the nicotinic acetylcholine receptor (nAChR) agonist binding sites has been the subject of intense investigation for more than 20 years. Functional and biochemical data indicate that there are two binding sites for ACh located within the $\alpha_2\beta\delta\gamma$ oligomeric structure of the receptor found in embryonic muscle, and early affinity labelling studies and mutagenesis experiments provided strong evidence that the α subunits play a major role in ligand binding. In particular, three loops of the α subunit, centered around residues Y93, W149 and C192/C193 respectively (loops A-C) were proposed to form the core of the agonist binding site. However, it has become clear in recent years that the nAChR agonist binding sites are formed at the interfaces of the α subunits with their neighbouring γ/ε and δ subunits (reviewed in Sine, 2002). One of the main lines of evidence that supports an interface model is the two binding sites are not identical, but differ in their affinities for different types of ligands. For example, in the resting state of the receptor, the agonists carbamylcholine and acetylcholine bind around 30-fold more tightly to the $\alpha\delta$ than to the $\alpha\gamma$ binding site (Prince and Sine, 1996), whereas curariform antagonists bind more tightly to the $\alpha\gamma$ than to the $\alpha\delta$ site (Blount and Merlie, 1989; Sine and Claudio, 1991). This pharmacological non-equivalence of the nAChR agonist binding sites, together with affinity labelling studies (Czajkowski and Karlin, 1995; Martin *et al.*, 1996 ;Chiara and Cohen, 1997; Chiara *et al.*, 1998; Chiara *et al.*, 1999) has led to a model in which the binding sites contain residues from the γ and δ subunits in addition to the α subunit, and in which differences in ligand affinity owe to sequence differences between the γ and δ subunits (Sine, 2002).

Previous studies in our laboratories probed the structure of the nAChR using chimeric subunits to identify amino acid differences between γ and δ that determine agonist and antagonist selectivity in the resting, activatible state of the receptor (Sine, 1997; Bren and Sine, 1997; Prince and Sine, 1996; Sine, 1993; Molles *et al.*, 2002b). The emerging overall picture showed that four loops from the γ and δ subunits (loops D-G), well-separated along the primary sequence, contribute to each binding site

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interface. Over the last few years, our understanding of the nAChR binding sites has been greatly extended by the atomic structural determination of an ACh binding protein (AChBP) derived from snail glial cells (Brejc *et al.*, 2001). AChBP has striking homology with the N-terminal extracellular domains of the nAChR subunits and has confirmed many aspects of earlier models. More recently AChBP has been co-crystalized with carbamylcholine and nicotine, providing insights into agonist docking to the receptor (Celie *et al.*, 2004). However, many questions about the structure of the nAChR agonist binding sites are still unanswered. In particular, how the structure of AChBP relates to the various conformational states of the nAChR remains unknown.

The present study extends our use of γ/δ subunit chimeras to identify determinants of selectivity for the agonist epibatidine in foetal nAChRs in the desensitised state. To date, chimera studies on receptors in the desensitised state have not been possible because classical agonists such as ACh and carbamylcholine do not distinguish between the two agonist binding sites of desensitised receptors, and competitive antagonists do not appreciably stabilise the desensitised state. However, we previously showed that epibatidine binds with higher affinity to the α-γ binding site than to the α-δ site of muscle nAChRs i.e. with opposite selectivity to carbamylcholine and ACh, and further, the binding site selectivity of epibatidine is maintained when the receptor changes functional state from activatible to desensitized (Prince and Sine, 1998b;Prince and Sine, 1998a). Taking advantage of the unique site- and state- selectivity of epibatidine, we show that three regions of the N-terminal domain of the γ and δ subunits determine (-)-epibatidine selectivity in the desensitised state, and identify individual residues that confer this site-selectivity. The identified selectivity determinants are explained by computational docking of epibatidine to a structural model of the high affinity αγ binding site.

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Materials and Methods-

Materials: [^{125}I]-labelled α-bungarotoxin was purchased from Amersham-Pharmacia. Human embryonic kidney 293 cells (HEK 293) were purchased from the American Type Culture Collection. (-)-Epibatidine, carbamylcholine, proadifen, foetal calf serum and BSA were purchased from Sigma. Dulbecco's MEM, penicillin and streptomycin were purchased from Invitrogen. All other chemicals were obtained from BDH (Poole, Dorset). The sources of the nicotinic nAChR subunits were as previously described (Sine, 1993).

Mutagenesis: nAChR subunits were subcloned into the cytomegalovirus-based expression vector pRBG4 as described previously (Prince and Sine, 1998b). Subunit chimeras of the form $\gamma_n\delta_{225}\gamma$ were constructed by oligo-bridging mutagenesis, as previously described (Sine, 1993). $\gamma_n\delta_{225}\gamma$ indicates a chimera that contains γ sequence for the first n amino acids followed by δ sequence to amino acid 225 (the start of the first transmembrane domain) and γ sequence thereafter. Point mutations were installed using either oligo-bridging or using the Quikchange site-directed mutagenesis kit (Stratagene). All mutations were verified by DNA sequencing and restriction mapping.

Cell culture and receptor expression: HEK 293 cells were grown in Dulbecco's MEM containing 10% foetal calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. At around 40% confluency, cells were transiently transfected using calcium phosphate precipitation as previously described (Prince and Sine, 1998b). In order to express subunit-omitted pentamers of the form $\alpha_2\beta\chi_2$ (where χ is γ, δ or chimera), cells were transfected with human α, mouse β and mouse χ cDNAs in the ratio 2: 1: 2. In all experiments, 13.5 μg of α subunit cDNA was used per 10 cm tissue culture plate of cells. Cells were incubated at 37 ºC for 24 h after transfection followed by 48 h at 31 ºC before use in ligand binding assays. Complexes containing all mouse subunits were produced as described previously (Prince and Sine, 1996a).

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Radioligand binding assay: Cells were harvested from tissue culture plates and resuspended in potassium Ringer's solution (140 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, 25 mM HEPES 30 mg/l BSA pH 7.4 with NaOH). Agonist binding was determined as described previously (Prince and Sine, 1998b). Briefly, cells were equilibrated with agonist and 100 μM of the desensitising agent proadifen for 45 min before the addition of 5 nM [^{125}I]-labelled α-bungarotoxin. Proadifen locks the receptor in the high affinity desensitised state and thus allows ligand binding studies to be performed on a fixed conformational state of the receptor. The cells were then incubated for a further 30-40 min to allow occupancy of at most, 50% of the binding sites by [^{125}I]-labelled α-bungarotoxin. The total number of binding sites was determined by incubating with 25 nM [^{125}I]-labelled α-bungarotoxin for 30-40 min. Non-specific binding was determined by subtracting a blank determined in the presence of 10 mM carbamylcholine. The cells were harvested onto Whatman GF-B filters using a Brandel cell harvester and counted in a γ counter.

Data analysis: The following equations were fitted to our data using Prism 3.0 (GraphPad software):

$$1\text{-fractionaloccupancy}=1-\frac{[L]^{nH}}{[L]^{nH}+K^{nH}} \qquad \text{Equation 1}$$

$$1\text{-fractional occupancy} = 1-P.\frac{[L]}{[L]+K_1}-(1-P)\frac{[L]}{[L]+K_2} \qquad \text{Equation 2}$$

where $[L]$ is the concentration of competing ligand, *Kd* is the dissociation constant, *nH* is the Hill coefficient, K_1 and K_2 are dissociation constants and *P* is the fraction of sites with dissociation constant K_1. To normalise data between experiments we calculated the value log(Kd mutant/Kd $\alpha_2\beta\gamma_2$). Statistical comparisons were made on log Kd values using one-way ANOVA with Tukey's post-test. Differences were considered significant when $P<0.05$.

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Homology modelling and epibatidine docking- A structural model of extracellular portions of the α-γ subunit pair of the foetal mouse receptor was constructed by using the homology modelling software JACKAL 1.5 (obtained at http://trantor.bioc.columbia.edu) based on sequence alignment with acetylcholine binding protein (AChBP) determined by scanning mutagenesis of the acetylcholine receptor ε subunit (Sine *et al.*, 2002). JACKAL 1.5 generates the structural model using an artificial evolution strategy, which considers the protein to be modelled a combination of mutagenesis, insertion and deletion operations performed on the template protein. It then determines side chain orientation using a coordinate rotamer library (Xiang and Honig, 2001), loop prediction (Xiang *et al.*, 2002) and employs four levels of structural refinement and then energy minimization. The output from JACKAL was then further energy minimized using the SANDER module of AMBER 7 (Pearlman *et al.*, 1995). Partial atomic charges were then assigned to each atom of the α and γ subunits using the restrained electrostatic potential (RESP) charge model of AMBER 7. Partial atomic charges for protonated epibatidine were obtained from the electrostatic potential (ESP) fitted charges derived according to the HF/6-31* quantum mechanics calculation provided in the GAUSSIAN 98 software package (Frish *et al.*, 1998). Docking simulation of epibatidine was done using AUTODOCK 3.0.3 (Morris *et al.*, 1996), which uses the Lamarckian genetic algorithm, and grid sizes of 40 x 40 x 40 (grid spacing 0.375Å) were used. Ten docked ligand-receptor complexes were produced, and the predominant epibatidine conformation was selected as the most probable docking orientation in the αγ binding site.

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Results-

To simplify interpretation of our results we performed experiments with subunit-omitted complexes that form pentamers of the form $\alpha_2\beta\chi_2$ where χ is γ, δ or chimera (Sine and Claudio, 1991). Unlike native heteropentamers, the two binding sites in subunit-omitted complexes are formed by α and identical non-α subunits (Sine, 1993). To maximise our signal, we took advantage of the 5- to10-fold increase in expression conferred by the human α subunit (αH) when combined with mouse non-α subunits (Prince and Sine, 1996). As described previously, αH increases expression without altering binding site selectivity for carbamylcholine. We found that in the presence of the desensitising agent proadifen (100 μM), $\alpha H_2\beta\gamma_2$ complexes bound epibatidine with around 200-fold higher affinity than $\alpha H_2\beta\delta_2$ complexes (Fig. 1). This degree of binding site selectivity is similar to that observed between the α-γ and α-δ binding sites in all-mouse $\alpha_2\beta\gamma\delta$ pentameric receptors (Prince and Sine, 1998b)

To identify residues in the γ and δ subunits that confer epibatidine selectivity we constructed a series of γ-δ chimeras, co-expressed each as subunit omitted complexes with αH and mouse β, and measured epibatidine affinity in the presence of 100 μM proadifen. Our first chimera, $\gamma 100\delta 225\gamma$, contained γ sequence for the first 100 amino acids followed by δ sequence until amino acid 225 (start of the first transmembrane domain) and γ sequence thereafter. Complexes with composition $\alpha H_2\beta(\gamma 100\delta 225\gamma)_2$ bind epibatidine with low affinity characteristic of complexes containing the wild type δ subunit (Figs. 1 and 2). Thus the major determinants of epibatidine selectivity are located between residues 100 and 225 of the subunits.

Our next chimera, $\gamma 117\delta 225\gamma$, yielded a dramatic increase in epibatidine affinity (Figs. 1 and 2) indicating that one or more selectivity determinants lie between positions 100 and 117. However, complexes containing $\gamma 117\delta 225\gamma$ bound epibatidine around four-fold less tightly than $\alpha H_2\beta\gamma_2$ complexes, suggesting that additional determinants are present C-terminal to position 117.

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The region between residues 100 and 117 contains four known determinants of ligand selectivity at nAChR binding sites (see Fig. 4 for alignment of the N-terminal extracellular domains of the γ and δ subunits). γS111 (aligned position is δY113) is a major determinant of conotoxin M1 selectivity (Sine, et al., 1995), γC115 (δY117) contributes to carbamylcholine selectivity (Prince and Sine, 1996), while γI116 (δV118) and γY117 (δT119) are determinants of metocurine selectivity (Sine, 1993). We therefore reasoned that one or more of these residues likely contribute to epibatidine selectivity. To test this hypothesis we constructed additional chimeras in which the chimera junction stepped through this region (Fig. 2). We first examined γ103δ225γ, and found that it conferred similar epibatidine affinity to that of $\alpha H_2\beta\delta_2$ complexes. However, introducing additional γ sequence in a C terminal direction, with γ104δ225γ, increased epibatidine affinity ~30-fold relative to γ103δ225γ, suggesting that γL104 and its equivalent δY106 are major determinants of epibatidine selectivity.

To confirm that γL104 and δY106 contribute to agonist selectivity we constructed subunits containing point mutations of these residues (Fig. 3). Both γL104Y and δY106L produced clear changes in epibatidine affinity, although these were somewhat smaller than the changes observed with chimeric subunits. The collective findings suggest the contributions of γL104/δY106 may also depend on interactions with other residues in the N-terminal domain.

Our next two chimeras, γ110δ225γ and γ112δ225γ, revealed a further increase in epibatidine affinity when γ sequence was introduced at residues 111 and 112 (Fig. 2). Unfortunately the intervening chimera, γ111δ225γ, did not express, preventing assessment of the contributions of individual amino acids using chimeras. To probe further the contributions of residues γ111-112/δ113-114, we introduced point mutations at these positions. The first pair of mutations, γS111Y and δY113S, markedly altered epibatidine affinity (Fig. 3), consistent with our measurements using chimeras. The second pair of candidate determinants, γP112/δD114, revealed a significant change in epibatidine affinity with γP112D, but the affinity conferred by

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the converse mutation, δD114P, did not differ from that conferred by wild-type δ. Thus the residue pair γS111/δY113 is a clear determinant of epibatidine affinity and the pair γP112/δD114 may contribute to selectivity in a subunit specific manner.

Our final chimera in this region, γ117δ225γ conferred 3-fold higher affinity compared to γ116δ225 and γ112δ225γ (Fig. 2). Thus, residue γY117 and its equivalent δT119 appear to contribute to epibatidine selectivity of the two binding sites. This hypothesis was confirmed by constructing the corresponding point mutant subunits: γY117T reduced epibatidine affinity ~4-fold, while the converse mutation, δT119Y, increased affinity ~8-fold (Fig. 3).

While residues γ104-117 account for much of the ~200-fold selectivity of epibitidine between the γ and δ binding sites, our results indicate that at least one more determinant lies between residue 117 and the beginning of the first transmembrane domain. Contained within this segment are selectivity determinants for metocurine (γS161/δK163), carbamylcholine (γF172/δI178) and α-conotoxin M1 (γF172/δI178) (Sine, 1993; Prince and Sine, 1996; Sine, et al., 1995). To identify amino acids in this region that contribute to epibatidine selectivity we extended the γ/δ chimeric boundary in a C-terminal direction (Fig. 5). The first three chimeras γ131δ225γ, γ156δ225γ and γ163δ225γ produced epibatidine affinities very close to that produced by γ117δ225γ, but extending the chimeric boundary to form γ171δ225γ increased affinity to within 2-fold of that produced by the wild-type γ subunit. In the region γ164-171/δ166-177 there is little homology between the subunits and the δ sequence contains an insertion of four amino acids relative to γ. This lack of homology makes it impossible to produce a meaningful alignment of this section of the subunits and for this reason we did not attempt to probe this region with further chimeras or point mutants.

Moving further in the C terminal direction, we found that the chimeras γ177δ225γ, γ186δ225 and γ189δ225γ gave essentially identical epibatidine affinity to γ171δ225γ (Fig. 5). However, when residue γP190 (equivalent to δA196) was surpassed, by generating γ190δ225γ, a small ~2-fold increase in affinity was noted,

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yielding a Kd identical to that conferred by the wild-type γ subunit. To assess the importance of γP190 and δA196 we made the corresponding point mutations. As predicted from the chimera experiments, γP190A slightly decreased epibatidine affinity, while δA196P slightly increased affinity (Fig. 5). Thus, the residue pair γP190/δA196 is a potential minor determinant of epibatidine selectivity.

Effects of previously identified ligand selectivity determinants: Previous studies in our laboratories used chimeras to examine the site-selective ligands metocurine, α-conotoxin M1, carbamylcholine and Waglerin, and identified determinants of binding selectivity in the γ, δ and ε subunits of the nAChR (Bren and Sine, 1997; Prince and Sine, 1996; Sine *et al.*, 1995; Sine, 1993; Molles *et al.*, 2002a). In the present study, two known ligand selectivity determinants γS111/δY113 (an α-conotoxin M1 determinant) and γY117/δT119 (a metocurine determinant) also influenced the binding of epibatidine and we were interested to know if other residues that determine the binding site selectivity of α-conotoxin M1, carbamylcholine and metocurine also affected epibatidine selectivity. To test this possibility, we expressed a series of point mutant γ or δ subunits as $\alpha H_2\beta\chi_2$ complexes and determined epibatidine affinity under desensitising conditions (Fig. 6). The first pair of point mutations we considered was the carbamylcholine and Waglerin determinant γC115Y/δY117C. As predicted from our chimera experiments, neither mutation produced a significant change in epibatidine selectivity. Next, we examined the metocurine selectivity determinants γI116/δV118 and γS161/δK163. Point mutations at either of these positions (γI116V/δV118I and γS161K/δK163S) produced essentially the same effects: a small (2-3 fold) decrease in epibatidine affinity with the γ mutations and no effect with the δ mutations. Finally we examined the residue pair γF172/δI178 which is a major determinant of α-conotoxin M1 selectivity. Again, we noted a small decrease (2-fold) in affinity with γF172I but found that the corresponding δ point mutation also decreased affinity. Coupled with data from the present chimera experiments, these findings indicate that none of the ligand selectivity determinants

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examined in this series of experiments make major contributions to epibatidine selectivity.

Subunits containing multiple point mutations: The preceding chimera experiments identify three pairs of residues as major determinants of epibatidine selectivity. Individually, none of these residues fully converts γ to δ affinity and *vice versa,* so we reasoned that some combination of these residues must be necessary. We therefore constructed a series of γ subunits containing point mutations at two or more candidate residues and determined epibatidine affinity when expressed as $\alpha_2\beta\gamma_2$ complexes. Our first construct was the double point mutant γL104Y+Y117T, which decreased epibatidine affinity by approximately the sum of the contributions of the corresponding single point mutations (Fig. 7). Next we added γS111Y to form the triple point mutant γL104Y+S111Y+Y117T, but this yielded similar affinity to that of γL104Y+Y117T. To probe further potential contributions by our most C-terminal residue pair γP190/δA196 we also constructed the quadruple point mutant subunit γL104Y+I116V+Y117T+P190A. However, expression levels yielded by this construct were too low to determine epibatidine affinity. Next, we examined the effects of mutating the curare selectivity determinants at positions γ116 and γ161. Although these residues were not highlighted by our chimera study and did not increase affinity when introduced into the δ subunit, we reasoned that they might have either a γ-subunit specific effect on epibatidine affinity, or they might require other determinants in order to influence epibatidine selectivity. Synergistic interactions between selectivity determinants have been noted in several previous studies (Sine *et al.*, 1995; Sine, 1993). To test this hypothesis we constructed γL104Y+I116V+Y117T, γL104Y+Y117T+S161K and γL104Y+I116V+Y117T+S161K. None of these constructs conferred epibatidine affinity significantly different from that of γL104Y+Y117T, confirming our initial hypothesis that γI116 and γS161 do not play a major role in determining epibatidine selectivity. Finally, we examined the effects of replacing the entire γ104-117 segment with δ sequence. This construct conferred an affinity for epibatidine that approached

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within 2-fold of that conferred by wild-type δ, suggesting that additional determinants within the γ104-117/δ106-119 region contribute to low affinity δ-like binding (Figs. 7 and 8).

To further test our identified epibatidine selectivity determinants, we constructed a series of multiple point mutations in the δ subunit. As shown in Figs 7 and 8, the double point mutation δL106Y+T119Y markedly increased epibatidine affinity to closely approach that conferred by the wild-type γ subunit. However, consistent with our results from γ subunit constructs, addition of δY113S, V118I, K163S to form the triple point mutants δL106Y+Y113S+T119Y, δL106Y+V118I+T119Y and δL106Y+T119Y+K163S, and the quadruple point mutant δL106Y+Y113S+T119Y+K163S produced only modest effects that did not significantly increase epibatidine affinity. Our final construct in this series, δL106Y+Y113S+T119Y+A196P, yielded essentially identical affinity to that produced by the triple mutant δL106Y+Y113S+T119Y. This result suggests that, at least in the δ subunit, the role of the residue pair γP190/δA196 may be more limited than suggested by our data from chimeras and single point mutant constructs.

Expression with mouse α subunit: Previous studies in our laboratories showed that the symmetrical binding sites of triplet receptors have similar ligand selectivity properties to the corresponding binding sites in native pentamers. (Sine and Claudio, 1991; Sine, 1993). Further, we have demonstrated that substitution of the human α subunit for its mouse counterpart increases expression, but does not alter ligand selectivity (Prince and Sine, 1996). However, previous studies using these model systems have only addressed ligand selectivity in the resting state of the receptor. Thus, to confirm that the epibatidine selectivity determinants identified in this study are also relevant in native pentamers we expressed a range of point mutant subunits with complementary wild-type mouse subunits as full pentameric receptors. As expected, γL104Y, γS111Y and γY117T increased the *Kd* of the high affinity component of pentamer binding curves but were without affect on the low affinity component. Conversely, δY106L, δY113S and δT119Y all decreased the *Kd* of the

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low affinity component of the pentamer curve without affecting the high affinity component (Figure 9, Table 1). Overall, the magnitudes of the affinity changes observed in this series of experiments were slightly lower than predicted from our results with subunit omitted complexes but nonetheless, these data provide strong support for the involvement of key residues in the region γ104-117/δ106-119 in the epibatidine selectivity of native pentamers.

Homology modelling and computational docking of epibatidine- To explain in terms of binding site structure how the identified selectivity determinants contribute to epibatidine binding, we constructed a homology model of the high affinity αγ binding site based on the atomic structure of AChBP and the experimentally-determined sequence alignment (Sine *et al.*, 2002). After aligning the AChBP template sequence with those of the foetal α and γ sequences, we used the modelling program JACKAL (http://trantor.bioc.columbia.edu) to obtain a structural model of the αγ site. To model the 7 residue insertion present in the γ subunit, which has no counterpart in AChBP, we tried several alignments and chose the only one that produced a pair-wise interaction between γK34 and γF172 in the final structure (Fig.10); this pair of residues was shown to interact and be essential for proper subunit folding and low affinity conotoxin MI binding characteristic of the native αγ site (Sine *et al.*, 1995).

We next used AUTODOCK 3.0.3 to dock epibatidine into the resulting structural model of the αγ site, assigning epibatidine a charge of +1. The resulting complex shows the polar face of epibatidine juxtaposed to αW149 in the center of the binding site, and the non-polar face oriented toward γY117, also deep in the binding site. The epibatidine selectivity determinant γL104 contacts αW149 in an apparent hydrophobic interaction, and appears to position the indole ring for optimal contact with both nitrogen atoms of epibatidine; the pyridine nitrogen of epibatidine is close enough to hydrogen bond with the indole nitrogen of αW149, while the azabicyclo nitrogen is positioned over the π-electron cloud of αW149. The selectivity determinant γY117 closely apposes the hydrophobic face of epibatidine, and the position of its phenol side chain is constrained by the third major selectivity

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determinant γS111. Thus a molecular continuum is established from γL104, γW149, epibatidine, γY117 and γS111, yielding a high affinity receptor-ligand complex.

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Discussion-

Previous studies in our laboratory demonstrated a 250-fold affinity difference for epibatidine between the two agonist binding sites of muscle nAChRs (Prince and Sine, 1998b). Here, we delineate the sequence differences between the γ and δ subunits that are responsible for this selectivity. Our results suggest that a minimum of three regions of γ and δ contribute to epibatidine selectivity.

The first and most important region identified in this study is located between residues 104 and 117 of the γ subunit (equivalent to δ 106-119; Fig. 4) and contains three γ/δ sequence differences that contribute to epibatidine selectivity: γL104/δY106, γS111/δY113 and γY117/δT119. That this segment mediates the majority of epibatidine selectivity is perhaps not surprising. This region is rich in previously identified agonist and antagonist selectivity determinants and is the target of several affinity-labeling agents. The most N-terminal epibatidine selectivity determinant identified here, γL104/δY106, was not previously implicated in ligand binding nor in contributing to site-selectivity at muscle type receptors. However, studies on the β_2 and β_4 subunits from neuronal nAChRs show the analogous residue is a determinant of cytisine and TMA affinity (Figl *et al.*, 1992). γS111/δY113, on the other hand, was identified as a determinant of α-conotoxin M1 selectivity, but for conotoxin, this sequence difference contributes to high affinity binding to the αδ interface and low affinity binding at the αγ interface (Sine, et al., 1995); i.e. it confers opposite selectivity to that seen with epibatidine. The final epibatidine determinant in this region, γY117/δT119 was also identified as a major determinant of metocurine selectivity (Sine, 1993), and has been suggested to make direct contact with this competitive antagonist (Fu and Sine, 1994; Gao *et al.*, 2003). Also within this region is γL109/δL111, which was recently shown to be labeled by the competitive antagonist TDBz choline (Chiara *et al.*, 2003), γC115/δY117, which contributes to carbamylcholine (Prince and Sine, 1996) and Waglerin (Molles, et al., 2002b) selectivity, and γI116/δV118 which contributes to metocurine selectivity (Sine, 1993). Overall, our present results, combined with findings from previous studies,

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demonstrate a major role of γ104-117/δ106-119 in conferring ligand binding selectivity at the nAChR.

In the δ subunit, mutations of δY106, δY113 and δT119 to their γ subunit equivalents results in an almost full conversion to high affinity αγ-like binding, suggesting that they represent the major determinants of epibatidine selectivity. However, introducing the equivalent δ residues into the γ subunit has more modest effects, and we found it necessary to replace the entire γ104-117 segment with δ sequence to effect a full γ to δ affinity conversion. One explanation for the asymmetric effects of mutations of epibatidine determinants is that the low affinity conferred by the δ subunit may result from interactions between its residues and other binding site components, primarily in the α subunit. In the δ subunit, replacing discrete residues with γ sequence might abolish such interdependent interactions and lead to γ-like affinity. By contrast, in the γ subunit, replacing discrete residues with δ sequence might not restore the interactions that cause low affinity, perhaps because of subtle differences in the three-dimensional scaffold due to local residue differences. Introduction of δ sequence between residues 104 and 117 may therefore be required to correctly orient the selectivity determinants within the binding site.

To explain our findings in terms of binding site structure, we generated a structural model of the nAChR αγ binding site and docked epibatidine to it. We hypothesize that because the template upon which our model is based, AChBP, has been suggested to have been crystallised in a desensitized-like conformation (Fruchart-Gaillard *et al.*, 2002), our computationally derived structure should provide useful information about binding site structure in the desensitized state. Consistent with previous structural models of the receptor (Sine *et al.*, 2002; Le Novere *et al.*, 2002; Molles et al., 2002b; Chiara *et al.*, 2003; Le Novere, 2003), the agonist binding sites in our model are formed by the convergence of a series of mostly aromatic residues from the α subunit (Y93, W149, Y190, C192, C193 and Y198) and a series of complementary residues from the non-α subunit. In our epibatidine docked complex, this non-α contribution comprises several residues contained within an

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extended hairpin structure, formed by residues γN94-S127, which passes diagonally through the extracellular domain of the subunit. Docking epibatidine to our structural model reveals that the ligand orients such that both its azabicyclo- and pyridine-nitrogens are positioned to interact with αW149, with the hydrophobic face of epibatidine closely apposed to the epibatidine selectivity determinant γY117. Additional hydrophobic contacts from the γ subunit include γL109 and γL119. The overall findings are consistent with previous mutagenesis and affinity labeling studies: αW149 is a strong candidate for stabilizing the quaternary ammonium moeity of nicotinic agonists, and is labelled by the competitive antagonist affinity probe p-N,N-(dimethylamino)phenyldiazonium fluoroborate (Dennis *et al.*, 1988; Zhong *et al.*, 1998; Dennis *et al.*, 1988). Similarly, γL109, γL119 and γY117 have been identified as candidate binding site residues by affinity labelling (Wang *et al.*, 2000) or mutagenesis studies (Chiara *et al.*, 1999; Sine, 1993; Sine, 1997). Further, the results from our docking studies, both in terms of ligand orientation and the roles of individual amino acids residues, are in close agreement with the crystal structure of nicotine-bound AChBP (Celie *et al.*, 2004).

The two other epibatidine selectivity determinants identified in this study do not appear to contact the agonist directly. γL104 is at the periphery of the binding site, but appears to make hydrophobic interactions with αW149, perhaps positioning its indole ring optimally for interaction with epibatidine. Likewise, S111 is at the periphery of the binding site, being located close to the apex of the γN94-S127 hairpin, but is positioned very close to γY117 and thus may govern the orientation of the phenol side chain to stabilize the uncharged face of epibatidine through hydrophobic contacts. Thus, the selectivity of the αγ and αδ for epibatidine can be rationalized in terms of a series of direct and indirect interactions between receptor and ligand, with high affinity achieved through a molecular continuum between epibatidine and γL104, αW149, γY117 and γS111.

We also uncovered two further determinants that make relatively small contributions to epibatidine selectivity. The first of these is in the segment γ164-171,

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close to γS161/δK163 which is a determinant of metocurine selectivity (Sine, 1993). Unfortunately, a four residue insertion in δ within this region makes sequence alignments very difficult and we were therefore unable to identify the precise residue mediating epibatidine selectivity. The second minor determinant is the sequence difference γP190/δA196, and is the most C-terminal non-α subunit residue thus far implicated in ligand selectivity for the nAChR. In our three-dimensional model of the receptor, both the γ164-171/δ166-177 segment and γP190/δA196 are distant from the putative ligand docking site and are located on the outside face of the subunit, midway between the two neighboring subunits. Thus, although these determinants are unlikely to participate in its final docking site, they may regulate the entry of epibatidine into the agonist binding cleft. Alternatively, long distance allosteric effects may be responsible for their contributions to selectivity.

In the adult muscle nAChR the ε subunit replaces γ. In our original studies, we demonstrated that epibatidine also selects by 200-300 fold between the binding sites of the adult receptor with the αε interface displaying high affinity and αδ site low affinity (Prince and Sine, 1998b). Interestingly, ε diverges from γ at all three epibatidine determinants within the 104-117 region (Fig. 4). Like δ, ε has tyrosine at residues 104 and 111 while at residue 117, ε has a serine. This sequence divergence strongly suggests that the elements conferring high epibatidine affinity to the αε site differ from those responsible for high affinity at the αγ interface. Similarly, whereas γI116, γY117 and γS161 mediate the high affinity binding of metocurine to the αγ interface (Sine, 1993), εI58 and εD59 mediate high affinity binding to the αε interface (Bren and Sine, 1997). In the latter study, it was suggested that at both interfaces, metocurine docked with one of its quaternary nitrogens stabilized by α subunit residues, but that the orientation of the second quaternary group differed between the αγ and αε interfaces such that it was stabilised by either γY117 or εI58 and εD59. Likewise, epibatidine may dock in different orientations in the αγ and αε binding sites.

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In summary, our results indicate that three segments of the nAChR γ and δ subunits contribute to epibatidine selectivity in the desensitized state. The most N-terminal of these regions, located between residues 104 and 117 of the γ subunit (δ106-119), accounts for most of the affinity difference between the αγ and αδ binding sites. Examination of homology models of nAChR binding sites reveals that γ104-117/δ106-119 is located close to the predicted docking site for agonists and suggests that residues within this segment of the non-α subunits may influence epibatidine binding via interaction with residues from the α subunits as well as by direct contact with the ligand.

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Figure Legends

Figure 1. Binding of epibatidine to subunit-omitted receptor complexes. Epibatidine binds with low affinity to $\alpha H_2\beta\delta_2$ (∇) and $\alpha H_2\beta(\gamma 100\delta 225\gamma)_2$ (◇), high affinity to $\alpha H_2\beta\gamma_2$ (□) and intermediate affinity to $\alpha H_2\beta(\gamma 117\delta 225\gamma)_2$ (△) complexes. The binding measurements were performed as described in *Materials and Methods* and are representative of at least 3 similar experiments. The curves are fits of equation 1 to the data and are described by the following parameters: $\alpha H_2\beta\gamma_2$ Kd 1.06 ± 0.11 nM, nH 1 ± 0.1; $\alpha H_2\beta(\gamma 100\delta 225\gamma)_2$ Kd 1092 ± 130 nM, nH 0.96 ± 0.1; $\alpha H_2\beta\delta_2$ Kd 258 ± 26 nM, nH 0.87 ± 0.07; $\alpha H_2\beta(\gamma 117\delta 225\gamma)_2$ Kd 6.7 ± 0.5 nM, nH 0.97 ± 0.06.

Figure 2. Dissection of epibatidine determinants in the region γ100-γ117. Chimeras of the form γnδ225γ were constructed as described in *Materials and Methods* and were expressed as subunit omitted complexes in HEK 293 cells. Epibatidine affinity is expressed relative to that conferred by wild-type γ. Thus, γ affinity corresponds to the y-axis position. The data are the means of at least three experiments with the error bars indicating the SEM.

Figure 3. Point mutation of epibatidine determinants in the region γ100-γ117. Point mutant subunits were constructed as described in *Materials and Methods* and were expressed as subunit omitted complexes in HEK 293 cells. Epibatidine affinity is expressed relative to that conferred by wild-type γ. Thus, γ affinity corresponds to the y-axis position while δ affinity is indicated by the dashed line . The data are the means of at least three experiments with the error bars representing the SEM.

Figure 4. Alignment of the N-terminal domains of the γ and δ subunits. Shaded residues are those identified as binding site determinants in previous chimera or affinity labeling studies. Bold, underlined residues are putative epibatidine selectivity determinants identified in the present study.

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Figure 5. Dissection of epibatidine determinants in the region γ131-γ190. Chimeras of the form γnδ225γ and point mutant subunits were constructed as described in *Materials and Methods* and were expressed as subunit omitted complexes in HEK 293 cells. Epibatidine affinity is expressed relative to that conferred by wild-type γ. Thus, γ affinity corresponds to the y-axis position and wild-type δ affinity is indicated by the dashed line. The data are the means of at least three experiments with the error bars representing the SEM.

Figure 6. Point mutation of α-conotoxin M1, metocurine and carbamylcholine selectivity determinants. Point mutant subunits were constructed as described in *Methods and materials* and were expressed as subunit omitted complexes in HEK 293 cells. Epibatidine affinity is expressed relative to that conferred by wild-type γ. Thus, γ affinity corresponds to the y-axis position while δ affinity is indicated by the dashed line. The data are the means of at least three experiments with the error bars representing the SEM.

Figure 7. Binding of epibatidine to subunit omitted complexes harboring multiple point mutations. Epibatidine affinity was determined as described in *Materials and Methods* and is expressed relative to that conferred by wild-type γ. Thus, γ affinity corresponds to the y-axis position while δ affinity is indicated by the dashed line. The data are the means of at least three experiments with the error bars representing the SEM.

Figure 8. Binding of epibatidine to desensitized subunit-omitted complexes containing multi-point mutant complexes. Binding of epibatidine was measured as described in the legend to Figure 1. The data are the mean ± SEM of 3 experiments and the curves are fits of equation 1: $\alpha_2\beta\gamma_2$ (□); $\alpha_2\beta\delta_2$ (▽); $\alpha_2\beta(\gamma 103\delta 117\gamma)_2$ (■); $\alpha_2\beta(\delta L106Y+T119Y)_2$ (▼).

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Figure 9. Binding of epibatidine to desensitized full-pentamer receptors containing point mutations at epibatidine selectivity determinants. The data are representative of at least 3 similar experiments and the curves represent fits of Equation 2 to the data: wild-type $\alpha_2\beta\delta\gamma$ (□), $\alpha_2\beta\delta$+γY117T (△), $\alpha_2\beta\gamma$+δT119Y (▽). Parameters from multiple experiments are given in Table 1.

Figure 10. Structural model of the foetal $\alpha\gamma$ binding site and predicted docking orientation of epibatidine. Receptor subunits are rendered as secondary structures, with the α subunit highlighted in *magenta* and the γ subunit in *orange*. Side chains of key binding site residues are shown in stick representation with contact surfaces shown in *light blue*. Note the polar surface of epibatidine faces αW149 and the hydrophobic surface faces γY117, and the molecular continuum formed by the highlighted residues and bound epibatidine.

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Table 1. Binding of epibatidine to desensitized full pentameric receptors containing all mouse subunits.

Wild Type Subunits	Mutant Subunit	K_1 (nM)	K_2 (nM)	P
$\alpha_2\beta\delta\gamma$		0.49± 0.04	201±36	0.66± 0.03
$\alpha_2\beta\delta$+	γL104Y	0.84± 0.09	168± 60	0.62± 0.04
	γS111Y	0.66± 0.12	180± 18	0.42± 0.03
	γY117T	1.92± 0.04	184± 59	0.47± 0.05
$\alpha_2\beta\gamma$+	δY106L	0.48± 0.1	59± 6.8	0.55± 0.06
	δY113S	0.36± 0.03	83± 11	0.534± 0.09
	δT119Y	0.61± 0.2	71± 25	0.58± 0.1

The parameters (K_1, K_2, dissociation constants; P, proportion of sites with affinity K_1) are derived from fits of equation 2 to our data and are expressed ± SE. Data are the mean of 3-5 experiments.

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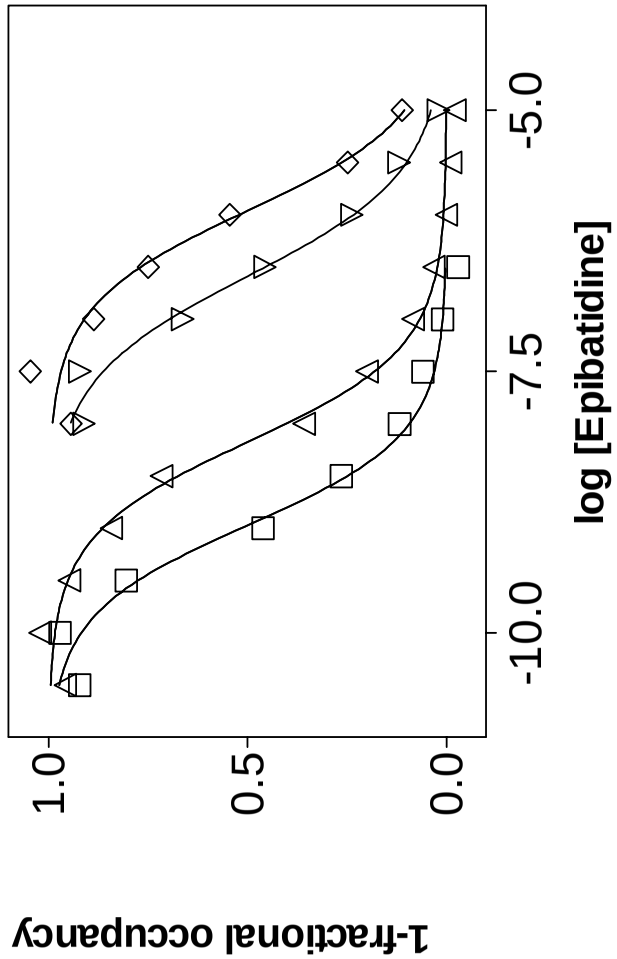

Figure 1

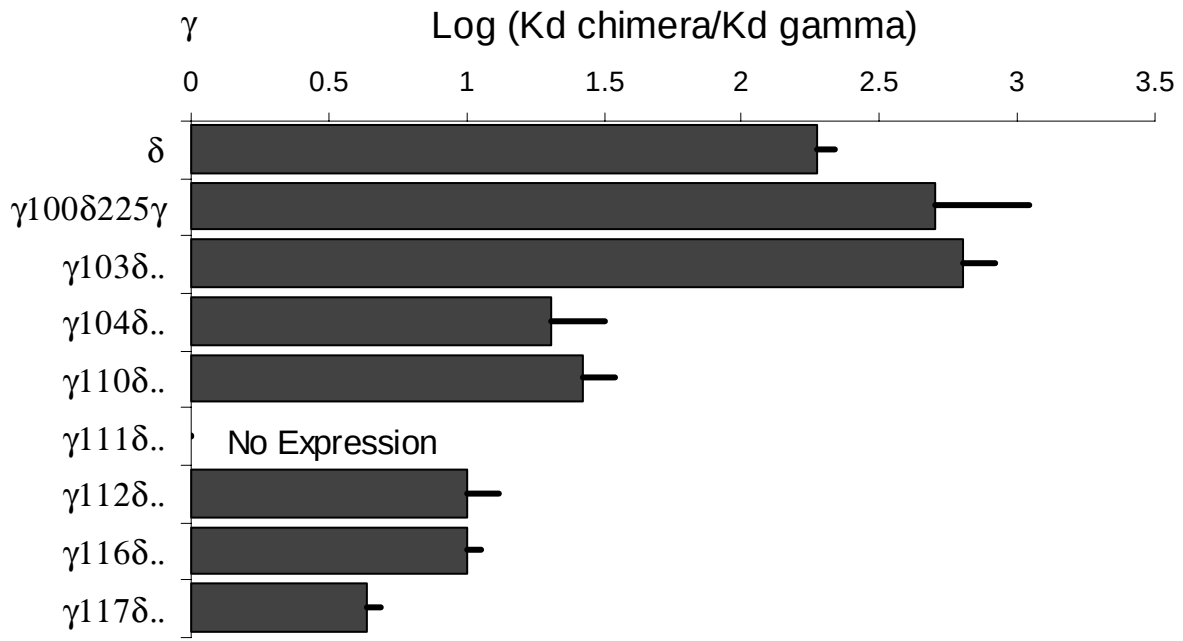

Figure 2

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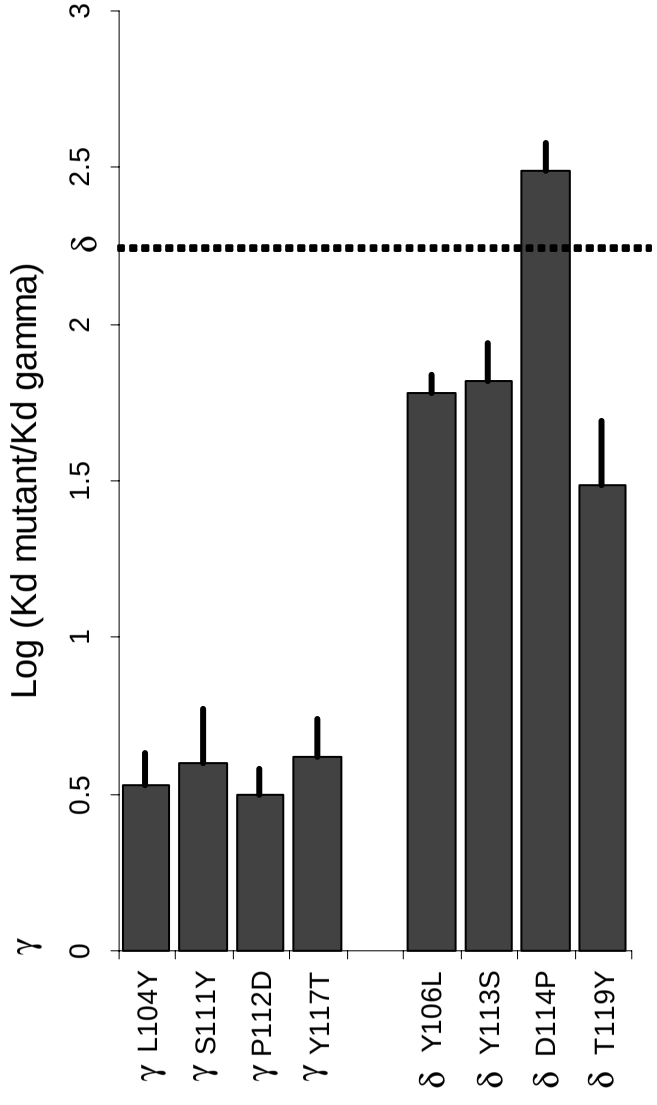

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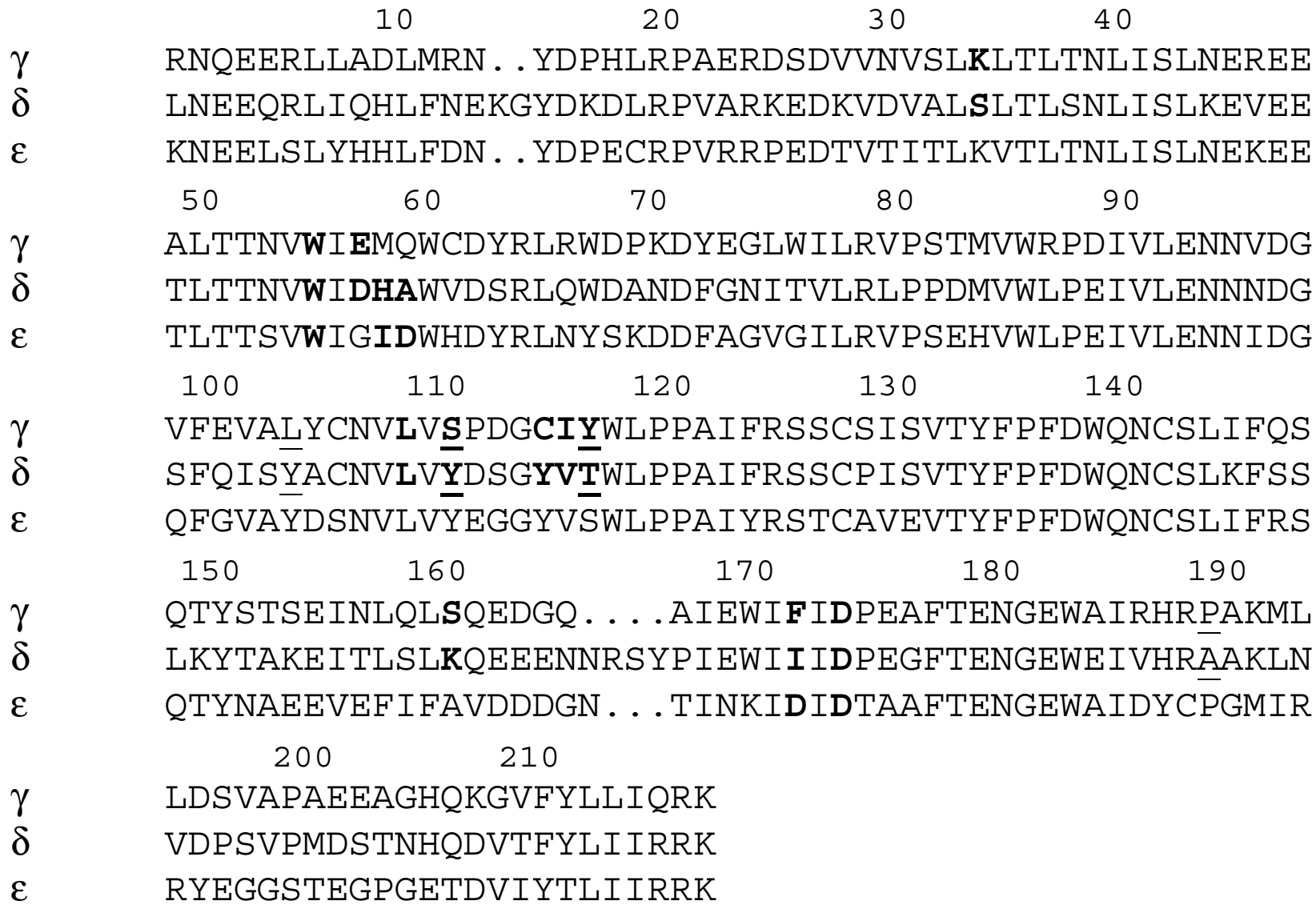

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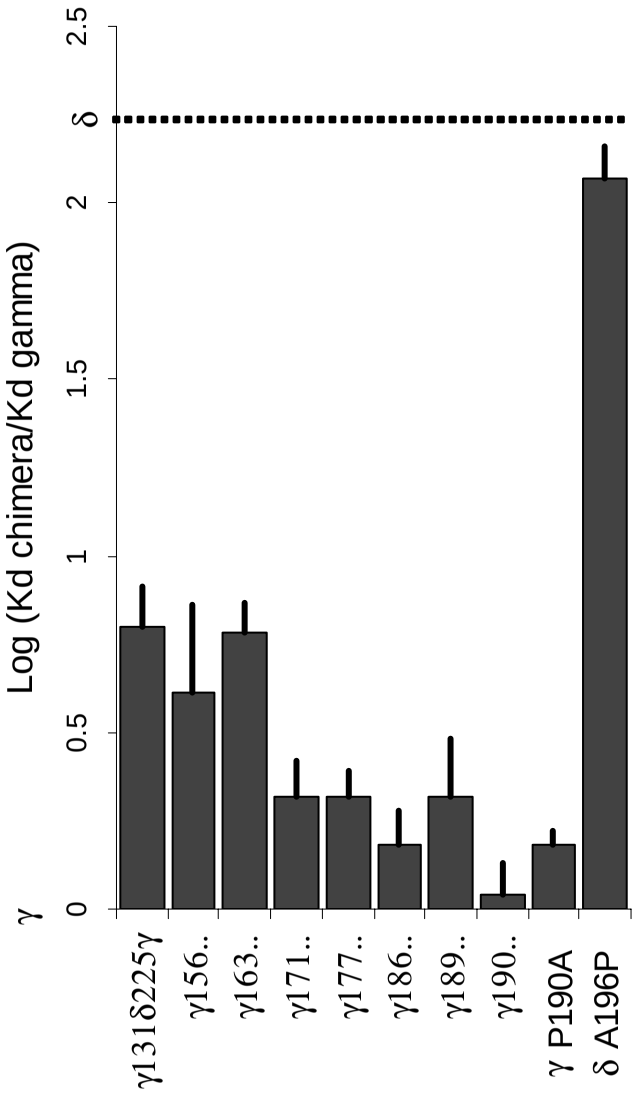
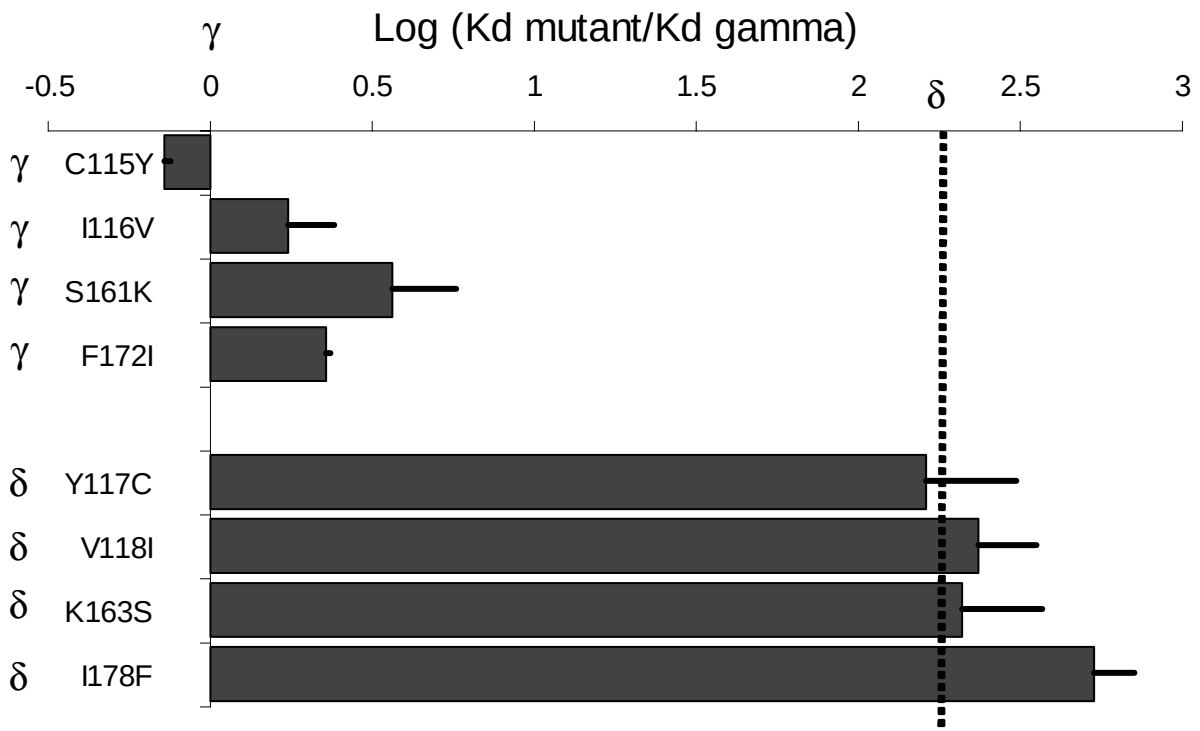


Figure 5



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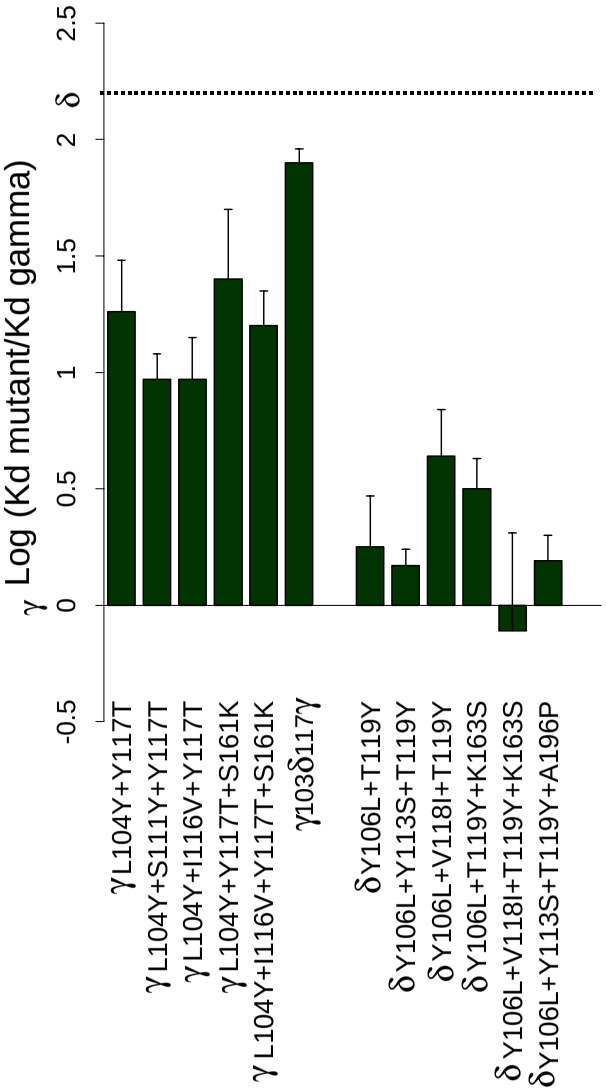

Figure 7

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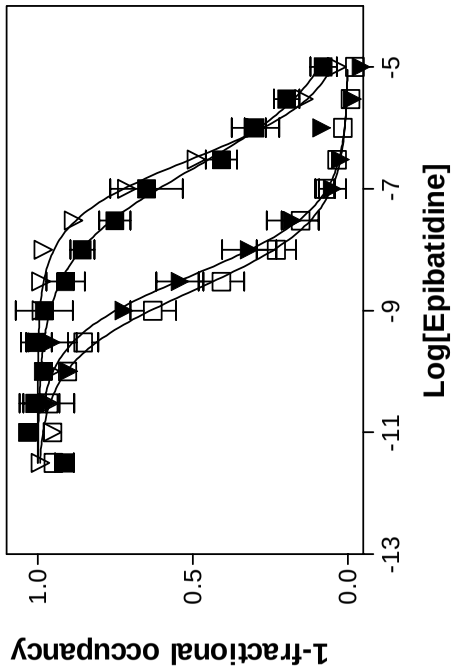

Figure 8

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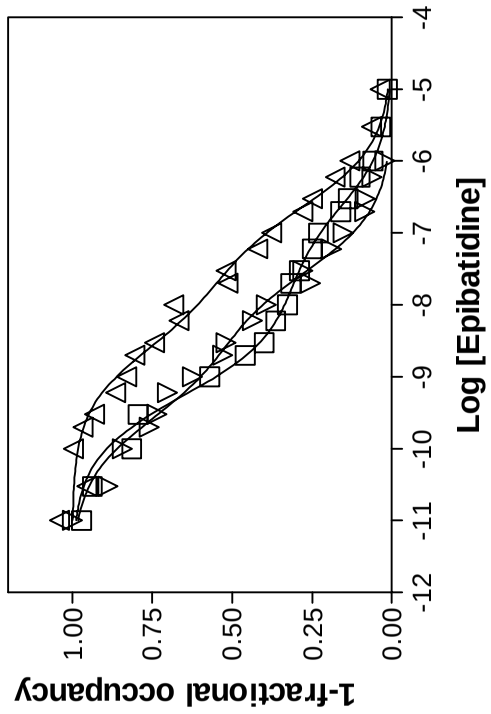

Figure 9

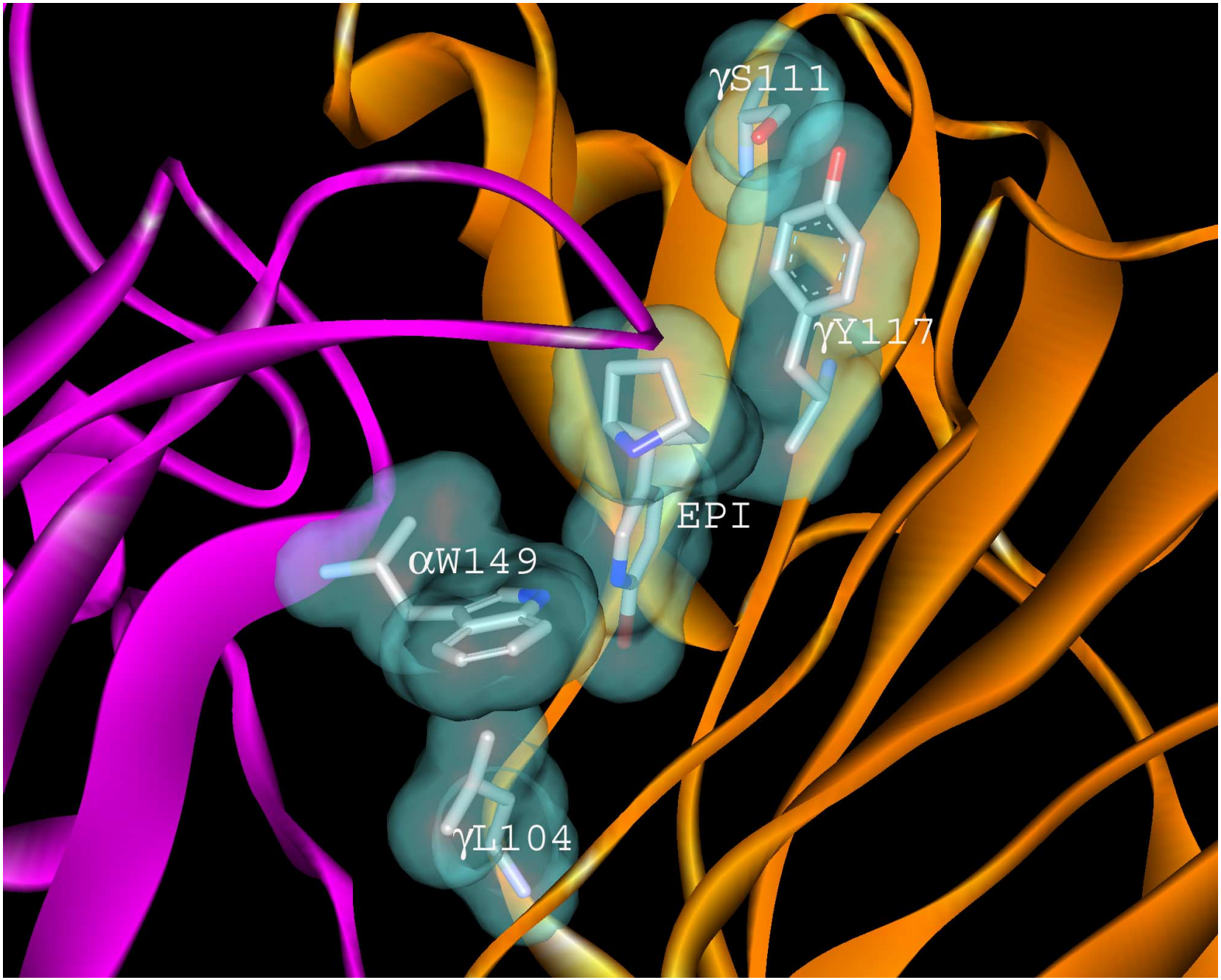

Figure 10