

Supporting Information to

Molecular characterization of monoclonal antibodies that inhibit acetylcholinesterase by targeting the peripheral-site and backdoor regions

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Supplemental Experimental Procedures

Materials.

The prepacked Superdex-200 HR-10/30 and HL-26/60 columns and protein-G and protein-A Sepharose HiTrap units (1 ml) and the calibration markers for gel filtration were from GE Healthcare. PEG-6000 was from Hampton Research and Na cacodylate from Fluka. The molecular weight standards for SDS-PAGE and all others biochemical reagents were from Sigma-Aldrich.

Protein purification and preparation.

Elec403, Elec408 and Elec410 were purified from the ascitic fluids in a single step of affinity FPLC on HiTrap protein-G (GE Healthcare) equilibrated with 20 mM NaP, pH 7.0, and eluted with 100 mM glycine, pH 2.7, with immediate neutralization of the eluant with 1 M Tris, pH 9.0 (55 µL/ml). The purified IgGs were dialyzed against 20 mM NaP, pH 7.0, and concentrated by ultrafiltration.

The Fabs were obtained by papain cleavage of the purified IgGs using papain (25.8 IU/mg) from Sigma-Aldrich, a papain-to-IgG ratio of 1:25 (w/w), and 1 mM EDTA and 1 mM β-mercaptoethanol (12-20 h, 37°C); the reaction was stopped with iodoacetamide 6 mM. The cleavage reactants and products were separated by gel-filtration FPLC on prepacked Superdex-200 (GE Healthcare) equilibrated and eluted with 0.02 M NaP, pH 7.2. The coeluting Fab and Fc fragments were separated through several steps of affinity FPLC on HiTrap protein-A (GE Healthcare) equilibrated in the same buffer, with recovery of the non-retained Fab in the flow through and expulsion of the retained Fc using 100 mM citric acid, pH 5.0. Homogeneity of the purified Fab was assessed by SDS- and native-PAGE and by MALDI-TOF mass spectrometry (cf. below). The Fabs were dialyzed against 50 mM Tris pH 7.5, 50 mM NaCl, 0.01% (w/v) NaN₃, and concentrated by ultrafiltration.

EeAChE, as a mixture of soluble asymmetric forms, was isolated from homogenized electric organs by affinity chromatography and subjected to controlled tryptic cleavage to release the constitutive covalent tetramers [S5]. The tetramers were purified from the tryptic mixture by gel filtration in 100 mM NaP, pH 7.4, 400 mM NaCl, 0.01% (w/v) NaN₃ [S5]. Homogeneity was assessed by SDS- and native-PAGE (cf. below). The enzyme was dialyzed against 50 mM NaP pH 7.4, 50 mM NaCl, 0.01 % (w/v) NaN₃ (buffer A) and concentrated by ultrafiltration; it was stored on ice. Native and deglycosylated HuBChE samples were gifts from Dr. Ashima Saxena (WRAIR, Silver Spring, MD).

Biochemical and functional analyses.

SDS- and native-PAGE used a PhastSystem apparatus (GE Healthcare), homogenous 12.5% and 7.5% gels, respectively, migration towards the anode and Coomassie blue staining. The SDS-PAGE samples were boiled for 5 min in the presence of 2.5% (w/v) SDS with (reducing conditions) or without (non-reducing conditions) 5% (v/v) β-mercaptoethanol. Native-PAGE mobility shift assays used Fab-EeAChE or Fab-HuBChE complexes formed in solution at a ~1:1 molar ratio (3 h incubation, room temperature). Isoelectric focusing used the same apparatus and pI 3-9 gels.

MALDI-TOF MS was performed on a Voyager-DETMRP BioSpectrometer Workstation (Perseptive Biosystems) in the positive linear mode using ~10 pmol/0.5 µl samples mixed with, as a matrix, 0.5 µl of sinapinic acid at 10 mg/ml in TFA/acetonitrile/water 0.1:0.6:0.3 (v/v/v), and the dried-droplet method. The samples were desorbed with a 337 nm nitrogen laser.

AChE activities were recorded for 5 min in duplicate or triplicate on a UNICAM 8700 spectrophotometer (Thermo Optek) using 10 pM EeAChE, 1.25 mM acetylthiocholine iodide (~10 x Km) and 0.33 mM dithiobis(2-nitro-benzoic acid) in 100 mM sodium phosphate, pH 8.0, 0.1mg/ml BSA (λ = 412 nm) [S16]. The Fab/AChE mixtures were

incubated overnight (equilibrium analysis) or for selected time intervals (kinetics analysis) under mild agitation at room temperature before recording of fractional activity. Data analysis used GraphPad Prism 4.0.

N-linked carbohydrate removal.

Deglycosylation of the EeAChE tetramer (~3 mg/ml) in native conditions was performed in buffer A using PNGaseF (1,800,000 U.mg⁻¹) from BioLabs, a PNGaseF-to-tetramer ratio of 1:200 (w/w) and overnight incubation at 25°C. A control sample for protein integrity was incubated in the absence of PNGaseF. A control sample for total deglycosylation was prepared on denatured and reduced EeAChE: briefly, the EeAChE tetramer (~20 µg in 6.25 µl buffer A) was boiled for 10 min in the presence of 40 mM DTT and 0.5% (w/v) SDS, cooled down, added with 1% (v/v) Nonidet P-40, then incubated in the presence of PNGaseF (~2 µg, 3600 U) for 1 h at 37°C. The native and reduced deglycosylated samples were analyzed comparatively with the unaltered native enzyme by SDS-PAGE in reducing conditions and native-PAGE.

Crystallization of Fab408 and data collection.

Crystallization was achieved at 20°C by vapor diffusion using Fab408 at 10 mg/ml, 1 µl hanging drops and a protein-to-well solution ratio of 1:1. Plate crystals grew spontaneously within 2 days with 18% (v/v) PEG-6000 in 100 mM Na cacodylate, pH 6.0, as the well solution. Crystals were flash-cooled in the nitrogen gas stream after successive short soaks in the well solution supplemented with 7.5%, 15% and 25% (v/v) glycerol, and were stored in liquid nitrogen. Diffraction data were collected at 100 K at the ESRF (Grenoble, France), processed with XDS [S17], and scaled and merged with SCALA. Despite the numerous attempts, no suitable crystals were obtained from Fab403 and Fab410.

Supplemental references to the Introduction

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Supplemental Table

Table S1. Data collection and refinement statistics.

	Fab408
Data collection ^a	
Space group	C222 ₁
Cell parameters (Å)	a = 69.95, b = 129.11, c = 99.26
Beamline (ESRF)	ID14-EH2
Resolution range (Å)	30.0 - 1.9
Total observations	211 893
Unique reflections	35 565
Multiplicity	6.0 (5.4)
Completeness (%)	99.5 (97.3)
<(I)/σ(I)>	15.0 (3.8)
R _{sym} ^b	6.6 (44.9)
B Wilson (Å ²)	31.7
Refinement ^c	
R-factor / R-free (%)	20.8 (28.2) / 25.3 (37.2)
R.m.s.d. ^d	
Bonds (Å) / Angles (°)	0.0097 / 1.34
Chiral volume (Å ³)	0.089
Mean B-factors (Å²)	
Main / Side chains	41.8 / 43.8
Solvent	40.4
Ramachandran plot statistics ^e	
% Residues in favored/outlier regions	97.4/0.2
PDB accession code	2YMX

^a Values in parentheses are those for the highest resolution shell.

^b $R_{sym} = \sum_{hkl} (\sum_i |I_{hkl} - \langle I_{hkl} \rangle|) / \sum_{hkl} \langle I_{hkl} \rangle$.

^c R-factor = $\sum_{hkl} |F_o| - |F_c| / \sum_{hkl} |F_o|$. Rfree is calculated for 2% of randomly selected reflections excluded from refinement.

^d Root-mean-square deviations from ideal geometry.

^e Ramachandran plot statistics have been calculated with the MolProbity server

Supplemental Figures

VL 403

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<----- FR1 - IMGT ----->
1      5      10      15      20
E I F L T Q S P A I I A A S P G E K V T I T C
gaa att ttt ctc acc cag tct cca gca atc ata gct gca tct cct ggg gag aag gtc acc atc acc tg
L
--- --g---

-----> CDR1 - IMGT <-----
25      30      35      40      45
S A S S S V R Y M H W Y Q Q K
c agt gcc agc tca agt gta ... .. cgt tac atg cac tgg tac cag cag aaa
S N
- - - - - a - - - - - a - - - - -

FR2 - IMGT -----> CDR2 - IMGT <-----
50      55      60      65
P G S S P K I W I Y G I S N L A
cca gga tcc tcc ccc aaa ata tgg att tat ggt ata ... .. tcc aac ctg gc

FR3 - IMGT ----->
70      75      80      85      90
S G V P A R F S G S G S G T S F S F T
t tct gga gtt cct ... gct cgc ttc agt ggc agt ggg ... .. tct ggg aca tct ttc tct ttc aca

-----> CDR3 - IMGT <-----
95      100      104
I N S M E A E D V A T Y Y C Q Q R S S Y P P L
atc aac agc atg gag gct gaa gat gtg gcc act tat tac tgt cag caa agg agt agt tac cca ccc ct

--- --t---

T F G A G T K L E L K R
c acg ttc ggt gct ggg acc aag ctg gag ctg aaa cg

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VH 403

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<-----> FR1 - IMGT
1 5 10 15 20
Q V Q L Q Q P G A E L V K P G A S V K L S C
cag gtc caa ctg cag cag cct ggg gct ... gag ttg gta aag cct ggg gct tca gtg aag ttg tcc tgc

AC073939 Musmus IGHV1-64*01 F
-----

-----> CDR1 - IMGT <-----
25 30 35 40 45
K A S G Y T F T S Y W M H W V K Q R
c aag gct tct ggc tac act ttc ... . . . . . acc agc tac tgg atg cac tgg gtg aag cag agg

AC073939 Musmus IGHV1-64*01 F
-----

FR2 - IMGT > CDR2 - IMGT <-----
50 55 60 65
P G Q G L E W I G M A Y S H T T T S R S N
cct gga caa ggc ctt gag tgg att gga atg gct tat tct cat ... . . . . . act act act agt aga tcc aa

AC073939 Musmus IGHV1-64*01 F
-----

----- FR3 - IMGT -----
70 75 80 85 90
E K F K T K A T L T V D K S S S T A Y M Q
t gag aag ttc aag ... acc aag gcc aca ctg act gta gac aaa tct tcc agc aca gcc tac atg caa

AC073939 Musmus IGHV1-64*01 F
-----

-----> CDR3 - IMGT -----
95 100 104
L S S L T S E D S A V Y F C A R G D Y K W Y F
ctc agc agc ctg aca tct gag gac tct gcg gtc tat ttc tgt gca aga ggg gac tat aaa tgg tac tt

AC073939 Musmus IGHV1-64*01 F
-----

D V W G S G T T V T V S S
c gat gtc tgg ggc tca ggg acc acg gtc acc gtc tcc tca

AC073939 Musmus IGHV1-64*01 F

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(Figure S1 A, part 1)

VL 408

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<----- FR1 - IMGT -----
1      5      10      15      20
D I Q M T Q S P A S L S A S V G A T V T I T C
VL408  gac att cag atg acc cag tct cca gct tcc ctg tct gca tct gtg gga gca act gtc acc atc aca tg
AJ235956 Musmus IGHV12-46*01 F  - - -c - - -t - - -c - -a - -t- -a- - - - - - - - -

-----> CDR1 - IMGT <-----
      25      30      35      40      45
R T S E N I D S Y L A W Y Q Q R
VL408  t cga aca agt gag aat att ... .. gac agt tat tta gca tgg tat cag cag aga
AJ235956 Musmus IGHV12-46*01 F  - - -g- - - - - - - - - - -t- a- - - -a-

FR2 - IMGT -----> CDR2 - IMGT -----<-----
      50      55      60      65
Q G K S P Q L L V Y A A T N L A
VL408  cag gga aaa tct cct cag ctc ctg gtc tat gca gca ... .. aca aac tta gc
AJ235956 Musmus IGHV12-46*01 F  - - - - - - - - - -t- - - - - - - - - - - - - -

FR3 - IMGT -----> CDR3 - IMGT -----
      70      75      80      85      90
D G V P S R F S G S G S G T Q Y S L K
VL408  a gat ggt gtg cca ... tca agg ttc agt ggc agt gga ... tca ggc aca cag tat tct ctc aag
AJ235956 Musmus IGHV12-46*01 F  - - - - - - - - - - - - - - - - -c - - -

-----> CDR3 - IMGT -----
      95      100      104
I N S L Q S E D V A R Y Y C Q H Y S T T P W T
VL408  atc aac agc ctg cag tct gaa gat gtt gcg aga tat tac tgt caa cat tat tct act act ccg tgg ac
AJ235956 Musmus IGHV12-46*01 F  - - - - - - - - -t- -g- -t- - - - - -t- -gg gg- - -t cc

F G G G T Q L E I K R
VL408  g ttc ggt gga ggc acc cag ctg gaa ata aaa cgt
AJ235956 Musmus IGHV12-46*01 F

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VH 408

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<----- FR1 - IMGT -----
1      5      10      15      20
E V Q L Q Q S G P E L V K P G A S V K I S C
VH408  gag gtc cag ctg cag cag tct gga cct ... gag ctg gtg aag cct ggg gct tca gtg aag ata tcc tg
AC079181 Musmus IGHV1-42*01 F  - - - - - - - - - - - - - - - - - - - - - - - - -

-----> CDR1 - IMGT <-----
      25      30      35      40      45
K A S G Y S F T G Y Y M N W V K Q S
VH408  c aag gct tct ggt tac tca ttc ... act ggc tac tac atg aac tgg gtg aaa caa agt
AC079181 Musmus IGHV1-42*01 F  - - - - - - - - - - - - - - - -g - - -

FR2 - IMGT -----> CDR2 - IMGT -----<-----
      50      55      60      65
P E K S L E W I G E M S P S T G R T T Y N
VH408  cct gaa aag agc ctt gag tgg att gga gag atg agt cct agc ... act ggt cga act acc tac aa
AC079181 Musmus IGHV1-42*01 F  - - - - - - - - -t- a- - - - - -g- - - -

FR3 - IMGT -----> CDR3 -----
      70      75      80      85      90
Q N F K A K A T L T V D Q S S S T A Y M Q
VH408  t cag aat ttt aag ... gcc aag gcc aca ttg act gta gac caa tcc tcc agc aca gcc tac atg cag
AC079181 Musmus IGHV1-42*01 F  c - -g -c - - - - - - - - -a- - - - - - - - -

-----> CDR3 -----
      95      100      104
L K S L T S E D S A V Y Y C A R S V P L T T L
VH408  ctc aag agc ctg aca tct gag gac tct gca gtc tat tac tgt gca aga agt gtc ccc tta act acg tt
AC079181 Musmus IGHV1-42*01 F  - - - - - - - - - - - - - - - - - - - - - - - - -

- IMGT -----
      I E D W Y F D V W G T G T T V T V S S
VH408  a ata gag gac tgg tac ttc gat gtc tgg ggc aca ggg acc acg gtc acc gtc tcc tca
AC079181 Musmus IGHV1-42*01 F

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(Figure S1 A, part 2)

Structures of inhibitory anti-AChE antibodies

VL 410

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<----- FR1 - IMGT -----
1      5      10      15      20
Q I V L T Q S P A I M S A S P G E K V T M T C
caa att gtt ctc acc cag tct cca gca atc atg tct gca tct cca ggg gag aag gtc acc atg acc tg
                                         I S
AJ231209 Musmus IGHV4-61*01 F -----a t-----

----->----- CDR1 - IMGT -----<-----
      25      30      35      40      45
S A S S S V      S Y M Y W Y H Q K
c agt gcc agc tca agt gta ... .. agt tac atg tac tgg tac cat cag aag
                                         Q
AJ231209 Musmus IGHV4-61*01 F -----g-----

FR2 - IMGT ----->----- CDR2 - IMGT -----<-----
      50      55      60      65
P G S S P K P W I Y R T      S N L A
cca gga tcc tca ccc aaa ccc tgg att tat cgc aca ... .. tcc aac ctg gc
AJ231209 Musmus IGHV4-61*01 F -----c-----

----- FR3 - IMGT -----
      70      75      80      85      90
S G V P      A R F S G S G      S G T S Y S L S
t tct gga gtc cct ... gct cgc ttc agt ggc agt ggg ... .. tct ggg acc tct tac tct ctc tca
                                         T
AJ231209 Musmus IGHV4-61*01 F -----a-----

----->----- CDR3 - IMGT -----
      95      100      104
V S S V E A E D A A T Y Y C Q Q Y N S H P M T
gtc agc agc gtg gag gcc gaa gat gct gcc act tat tac tgc cag cag tac aat agt cac ccc atg ac
I      M      H      Y      P
AJ231209 Musmus IGHV4-61*01 F a-- --a-- --t-- --t c-- --t-- --a ccc --

-
F G G G T K L E I K R
g ttc ggt gga ggc acc aag ttg gaa atc aaa cgg
AJ231209 Musmus IGHV4-61*01 F

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VH 410

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<----- FR1 - IMGT -----
1      5      10      15      20
E V Q L V E S G G      G L V Q P K G S L K L S C
gag gtg cag ctt gtt gag tct ggt gga ... gga ttg gtg cag cct aaa gga tca ttg aaa ctc tca tg
AJ073561 Musmus IGHV10-3*01 F -----

----->----- CDR1 - IMGT -----<-----
      25      30      35      40      45
A A S G F T F      N T Y A M H W V R Q A
t gcc gcc tct ggt ttc acc ttc ... .. aat acc tat gcc atg cac tgg gtc cgc cag gct
AJ073561 Musmus IGHV10-3*01 F -----

FR2 - IMGT ----->----- CDR2 - IMGT -----<-----
      50      55      60      65
P G K G L E W V A R I R S K S N K Y A T H Y A
cca gga aag ggt ttg gaa tgg gtt gct cgc ata aga agt aaa agt aat aaa tat gca aca cat tat gc
S      N      Y
AJ073561 Musmus IGHV10-3*01 F -----g- --t-- --t--

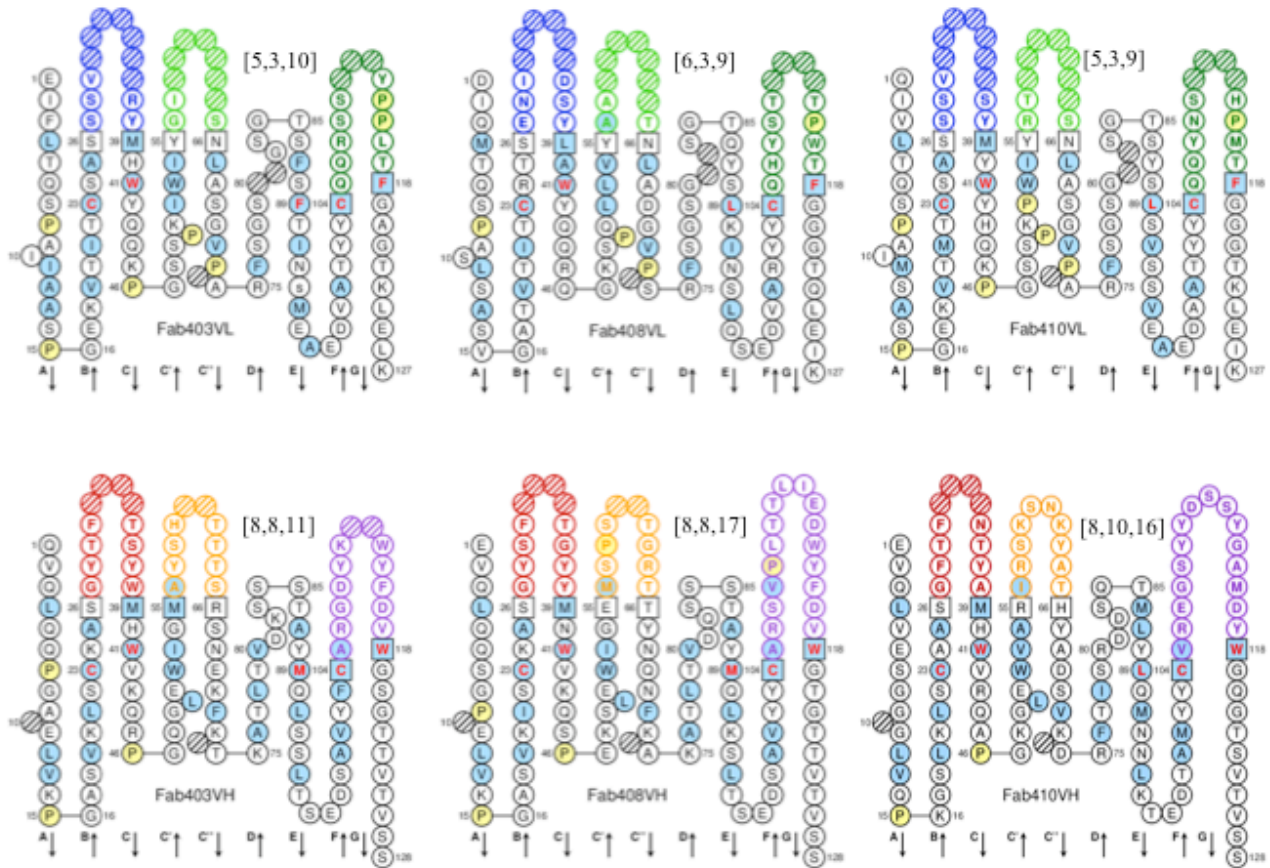
----- FR3 - IMGT -----
      70      75      80      85      90
D S V K      D R F T I S R D D S Q T M L Y L Q
c gat tca gtg aaa ... gac aga ttc acc atc tcc aga gat gat tca caa acc atg ctc tat ctg caa
S
AJ073561 Musmus IGHV10-3*01 F -----g-----

----->----- CDR3 -
      95      100      104
M N N L K T E D T A M Y Y C V R E G S Y Y D S
atg aac aac ctg aaa act gag gac aca gcc atg tat tac tgt gtg aga gaa ggg agt tac tac gat ag
AJ073561 Musmus IGHV10-3*01 F -----

IMGT -----
S Y G A M D Y W G Q G T S V T V S S
t agc tac ggt gct atg gac tac tgg ggt caa gga acc tca gtc acc gtc tcc tca
AJ073561 Musmus IGHV10-3*01 F

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(Figure S1 A, part 3)



(Figure S1 B)

Figure S1. The variable regions of Elec403, Elec408 and Elec410. (A, parts 1-3) Alignments of the rearranged nucleotide sequences of the VL and VH regions with the IMGT/V-QUEST reference directory sets of germline V-REGION alleles (only this part of the variable region, not the entire variable region, is shown). **Bold red letters** denote the modified amino acid residues and nucleotides in the antibody compared to the germline. **Dashes** indicate identical nucleotides. **Dots** indicate gaps according to the IMGT unique numbering. **(B)** IMGT canonical representation of the Fab403, Fab 408 and Fab410 variable domains. CDR-L1, CDR-L2, CDR-L3 are displayed in blue, light green, dark green, and CDR-H1, CDR-H2, CDR-H3 in red, orange, purple, respectively. The five conserved residues/positions of the VL and VH domains are displayed with red bold letters. Anchor positions are squared. Gaps in the IMGT numbering are hatched. Pro residues are shown on a yellow background. Arrows indicate the theoretical main β -strands and their direction. The greater lengths of CDRs H3 in Fab408 and Fab410, and of CDR-H2 in Fab410, compared with their counterparts in the other Fabs, is evident. Theoretical pI values for the Fab403 CDRs are: 8.46 (L1), 5.52 (L2), 8.22 (L3) and 5.24 (H1), 8.52 (H2), 5.95 (H3). For the Fab408 CDRs they are: 3.67 (L1), 5.52 (L2), 6.73 (L3) and 5.25 (H1), 6.10 (H2), 4.03 (H3). And for the Fab410 CDRs they are 5.24 (L1), 8.75 (L2), 6.73 (L3) and 5.24 (H1), 11.10 (H2), 4.03 (H3). (Figure made with the *IMGT/Collier-de-Perles* tool using the 50% hydrophobic position option.)

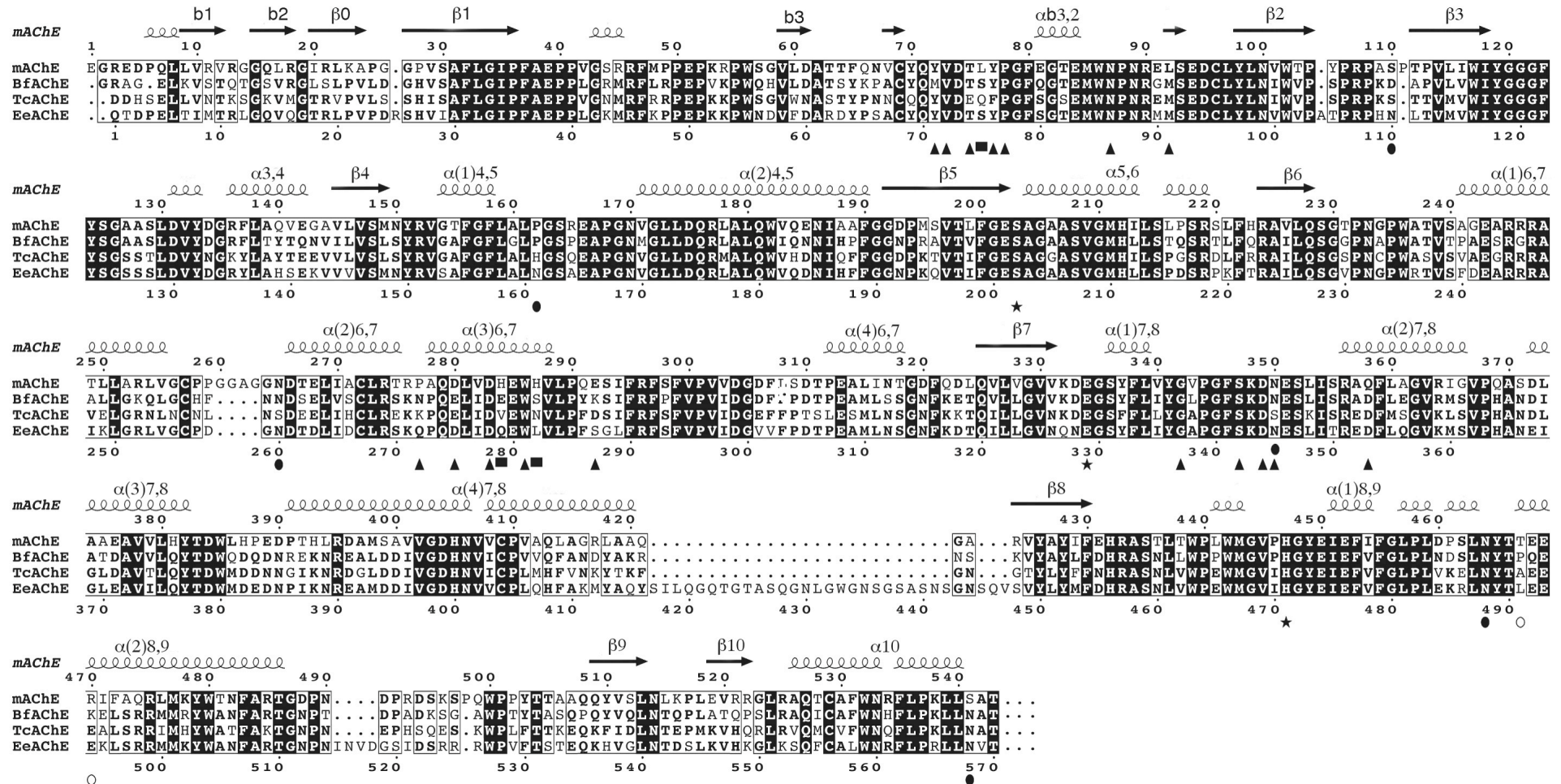


Figure S2. Sequence alignment of the AChE species cited in this study. The sequences of the EeAChE, TcAChE, BfAChE and mAChE subunits are displayed. The residue numbering displayed below the alignment is that of EeAChE, while the residue numbering and secondary structure elements displayed above the alignment are those of mAChE. Conserved residues are shown on a *black* background and non-conserved residues on a *white* background. The symbols below the alignment point to: the catalytic triad residues (stars); EeAChE Asn residues that belong to consensus N-glycosylation sequences (black dots) [48]; EeAChE residues whose substitution by rat AChE residues abolished Elec403 binding (Ser75, Gln279, Leu282; black squares), Elec410 binding (Ser75) or Elec408 binding (Leu491, Glu494; white dots) [33]; and EeAChE residues found to be buried at the interface of the theoretical Fab403-EeAChE complex (triangles).

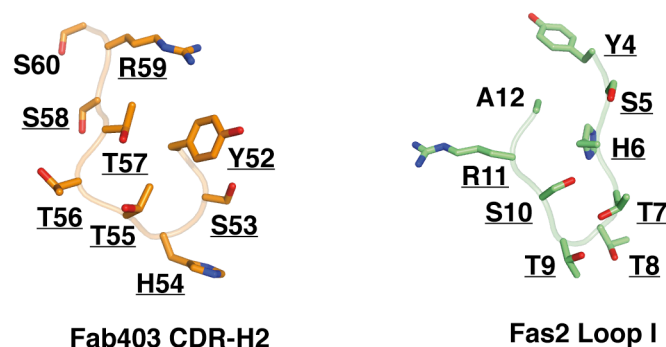


Figure S3. Structural comparison of Fab403 CDR-H2 and Fas2 loop I. Side by side views of Fab403 CDR-H2 (AYSHTTTSRS) and Fas2 loop I (YSHTTTSRAILTN) showing the distinctive $\text{C}\alpha$ conformations and side chain repartitions despite their high sequence homology. The respective loop conformations are stabilized by intra-loop interactions. Fas2 residue Arg11 protrudes into the solvent and is well positioned at the loop I edge for interaction with AChE, whereas Fab403 residue Arg59 lies at the CDR base with limited accessibility for partnering.