3D Structural Studies on Nervous System Proteins & Their Complexes with Inhibitors

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Acetylcholinesterase

By rapid hydrolysis of the neurotransmitter, acetylcholine (ACh), acetylcholinesterase (AChE) terminates neurotransmission at cholinergic synapses. AChE is a very fast enzyme, especially for a serine hydrolase, functioning at a rate approaching that of a diffusion-controlled reaction. The powerful toxicity of organophosphorus (OP) poisons is attributed primarily to their being potent AChE inhibitors. AChE inhibitors are utilized in the treatment of various neurological diseases, and provide the first generation of drugs approved by the FDA for management of Alzheimer's disease (AD). Many carbamates and OPs act as potent insecticides by selectively inhibiting insect AChE.

We determined the 3D structure of AChE from *Torpedo californica* (*Tc*AChE), permitting visualization, for the first time, at atomic resolution, of a binding pocket for ACh. It also allowed identification of the active site of AChE, which, unexpectedly, is located at the bottom of a deep gorge lined with aromatic residues. This unusual structure permitted us to work out structure-function relationships for AChE. The so-called 'anionic' binding site for the quaternary moiety of ACh does not contain several negative charges, as earlier postulated. Moreover, AChE shows a remarkable asymmetric charge distribution resulting in an unusually large dipole moment (~1,700 Debye) aligned along the active-site gorge. Modeling studies suggested that the quaternary group of ACh interacts primarily with the indole ring of the conserved Trp residue, W84, via cation-π interaction, as well as with F330. Crystallographic studies on several AChE-ligand complexes confirmed this, in agreement with labeling studies in solution and with theoretical studies on the π-cation interaction. From the various inhibitor/AChE complexes we have studied, including all currently available cholinesterase-inhibitor (ChEI) drugs for treatment of AD (viz. Cognex, Exelon, Aricept, Reminyl, and huperzine), we see that although many interact very tightly with AChE (binding constants ~10^{-10}), interaction is mediated mostly via waters and van der Waals contacts, with few direct interactions with the protein. We are also studying complexes of various potential AD drugs, e.g. huprine-X, a hybrid of huperzine and tacrine (Cognex), and bivalent analogs of huperzine.

One of our most surprising findings was the breaking of the catalytic triad (E327-H440-S200) by modification with the nerve agent VX ('pro-aged' conjugate), followed by its reformation upon 'aging'. On carbamylation with the Novartis AD drug, Exelon (rivastigmine), H440 also moved away from its hydrogen-bonded partner in the triad, E327, resulting in similar disruption. This movement may provide an explanation for the unusually slow kinetics of reactivation of the carbamyl enzyme.

The only drugs approved for use against Alzheimer's disease are those that provide symptomatic relief for the patients, with most being AChEIs. Nonamethylene-bis-(–) normeptazinol (nMEP) is a novel nanomolar AChEI which inhibits aggregation of the Aβ peptide to amyloid fibrils. We recently determined the 3D structure of its complex with AChE (Figure 1). One normeptazinol moiety binds at the active site, whereas the second binds at the peripheral anionic site, at the mouth of the gorge, with the linker spanning them. The normeptazinol bound at the active site disrupts the catalytic triad by binding to the side chain of His440, and causes a 100º rotation about $\chi 1$ of Ser200, increasing the distance between Ser200Oγ and His440Nε^2 from ~3Å in the native structure to ~4.2Å. These findings may facilitate the rational design of improved normeptazinol-based bifunctional ChEIs.

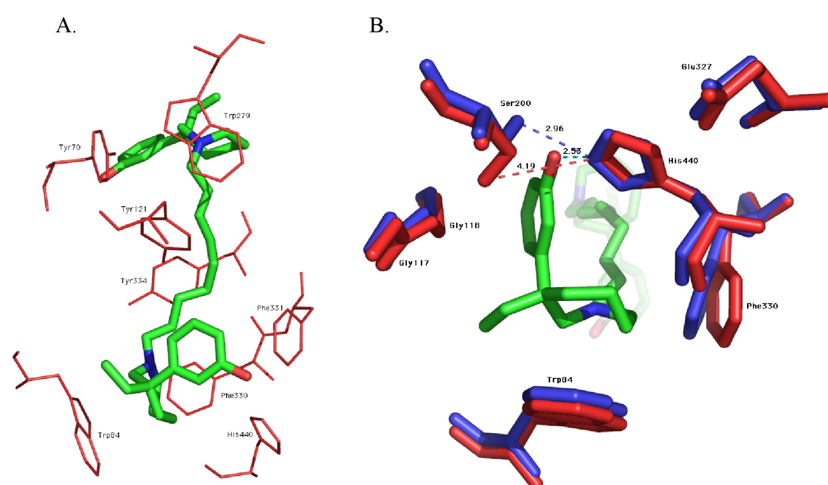

Fig. 1 *Views of refined structure of the complex between nMEP and AChE.. A. nMEP spans the gorge of AChE. nMEP represented in green with side-chains of AChE gorge residues displayed as red lines. B. View from the bottom of the catalytic active site of nMEP, and superposition of side chains of the nMEP complex (red sticks), and the native enzyme (blue sticks). The catalytic activity of AChE is disrupted by a dual mechanism: The side chain of His440 hydrogen bonds to the oxygen of the MEP phenol ring (cyan dashed line), and Ser200 is rotated away from His440, widening the distance between the residues to 4.19Å (red dashed line) in comparison to the native distance of 2.96Å (blue dashed line). In addition, Phe330 is tilted away from nMEP.*

Radiation damage is an inherent problem in X-ray crystallography.

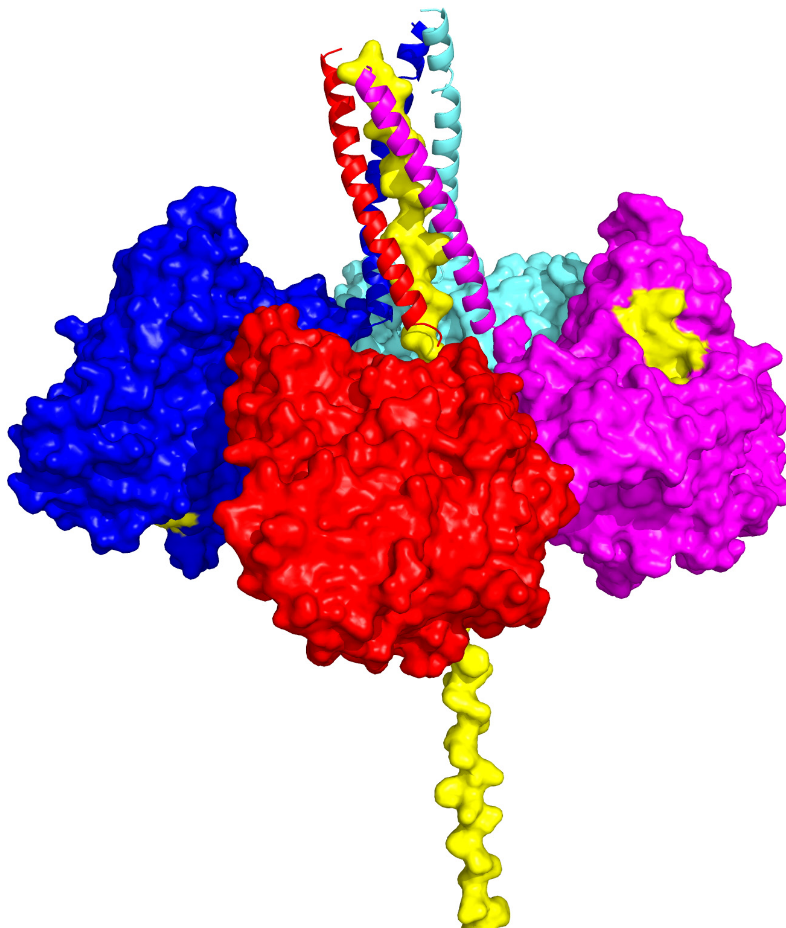

Fig. 2 *Model of the physiological ColQ-linked AChE tetramer with the ColQ polypeptide shown vertically. The WAT polypeptides are displayed as ribbons, and the PRAD as a yellow surface model.*

(a)

(b)

Fig. 3 *Homology models of Nlg 3 and Nlg4 bearing autism-causing mutations, based on the crystal structure of AChE. (a) A highly conserved Arg residue is converted into a Cys (R451C) in Nlg 3. The mutated residue is displayed as spheres, and the rest of the residues in ribbon format. (b) A stop codon at position 396 of Nlg4 leads to premature termination of the polypeptide chain. Residues in green are before the premature termination, those in gray after it.*

It has commonly been assumed to be non-specific, and manifested as gradual decay in the data quality as data collection proceeds, even at cryo temperatures. In preparation for time-resolved crystallography experiments, we collected nine successive complete data sets on the same *Tc*AChE crystal, at 100K, on a powerful undulator beamline at the ESRF. We found that radiation damage can be highly specific. Thus one disulfide was cleaved completely, while another was resistant. Highly exposed COOH groups, and those in the active site, are especially susceptible, and active-site H440 much more than others. Our findings have practical implications for routine data collection at high-energy synchrotrons and for interpretation of data so obtained. They also offer a direct approach to studying the radiation chemistry of proteins and nucleic acids, and may yield information concerning flexible regions or putative weak links in a given macromolecule.

CPT-11, which has recently been approved as a drug for treatment of colon cancer, is really a prodrug, meaning that it must undergo a chemical change in the body to become active. Thus the active drug molecule is packaged in a larger molecule that helps to keep it stable when injected. Inside the body, a naturally occurring enzyme hydrolyzes the prodrug to release the potent anti-cancer drug. The side effects, which include nausea, vomiting and diarrhea, are similar to those experienced by some AD patients. This led us to suspect that a second enzyme, with a similar structure to that targeted by the AD drug, might be involved. We soaked crystals of *Tc*AChE with a solution of CPT-11, obtaining crystals of the CPT-11/*Tc*AChE complex, and determined its 3D structure, thus obtaining a detailed image of how the two interact. Comparing the structure of the CPT-11/AChE complex with computer-generated models of other enzymes that convert the prodrug to the drug, we found the cause of the problem to be a physical misfit. In the converting enzymes, too, the CPT-11 molecule enters a narrow cavity like that in AChE in order to be converted. In AChE, however, it is trapped in such a

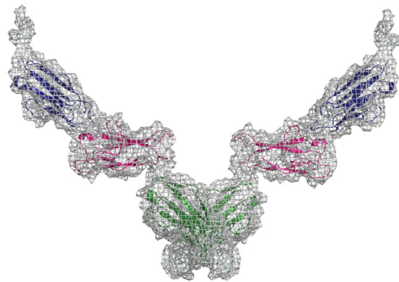

Fig. 4 *Overall structure of Ama based on SAXS data*

way that the active site of AChE cannot cleave it. Instead, the prodrug blocks the enzyme like a cork, rendering it inactive. This is a rare, atomic-level view, of the molecular basis of the side effects of a drug, which is now being used to assist the design of new drugs so as to tweak the shape of the AChE-blocking segment of the prodrug molecule (which is discarded in any case), so that it will bind less tightly to AChE. The study may also have implications for the design of new AD drugs.

Functional localization of AChE in vertebrate muscle and brain depends on interaction of the tryptophan amphiphilic tetramerization (WAT) sequence, at the C-terminus of its major splice variant (T), with a proline-rich attachment domain (PRAD) of the anchoring collagenous polypeptide (ColQ). The crystal structure of the WAT/PRAD complex reveals a novel supercoil structure in which four parallel WAT chains form a left-handed superhelix around an antiparallel left-handed PRAD helix resembling polyproline II. The WAT coiled coils possess a *WWW* motif that makes repetitive hydrophobic stacking and hydrogen-bond interactions with the PRAD. The WAT chains are related by a ~4-fold screw axis around the PRAD. Each WAT makes similar but unique interactions, consistent with an asymmetric pattern of disulfide linkages between the AChE tetramer subunits and ColQ. The P59Q mutation in ColQ, which causes the neuromuscular disease, congenital endplate AChE deficiency, and is located within the PRAD sequence, disrupts crucial WAT–WAT and WAT–PRAD interactions. A model is proposed for the synaptic AChE tetramer (Figure 2).

Cholinesterase-Like Adhesion Molecules (CLAMs)

In addition to its 'classical' role in terminating transmission at cholinergic synapses, AChE is also believed to play 'non-classical' roles, e.g. as an adhesion protein. Furthermore, it accelerates assembly of the Aβ peptide into amyloid fibrils which are the major components of the plaques characteristic of AD.

A family of four proteins with substantial sequence similarity to ChEs has been identified, the ChE-like adhesion molecules (CLAMs). These proteins are assumed to be catalytically inactive since they lack the active-site serine. They all play roles in nervous system development and patterning, and in formation and differentiation of synaptic junctions, and appear to make both intracellular and extracellular functional protein complexes. The neuroligin (Nlg) family consists of five members; all isoforms are highly expressed in excitatory or inhibitory synapses in the CNS, and have been shown to act as trans-neuronal signals for induction of presynaptic differentiation at neuron-neuron connections. Recently, mutations in human Nlgs 3 and 4, that cause defective neuroligin trafficking, have been identified in a number of autism-spectrum families and in families displaying mental retardation (Figure 3).

The *Drosophila* CLAM, gliotactin (Gli), is expressed in peripheral glia and in epithelial cells. It contributes to the formation of two cellular junctions: the blood–nerve barrier, and septate junctions between epithelial cells, during embryonic development. Another *Drosophila* CLAM, neurotactin (Nrt), is expressed in neuronal and epithelial tissues during embryonic and larval development. Nrt promotes heterophilic cell aggregation through interaction with the protein amalgam (Ama).

Currently, we are focusing attention on characterization of these three CLAMs, (which are all single-pass transmembrane proteins, of their known binding partners), and of the corresponding CLAM-partner complexes, using molecular biology, biochemical and biophysical techniques, as well as X-ray crystallography, small-angle X-ray scattering (SAXS) and NMR. In addition, we are attempting to identify novel interactions between the CLAMs and other proteins.

Recently we have shown that the intracellular domains of both Gli and Nlg are intrinsically unordered in solution. Intrinsically unordered proteins (IUPs) are flexible, noncompact (extended), and have little or no ordered secondary structure under physiological conditions. These proteins question the known structure-function relationship in proteins; despite their lack of structure, they are indeed functional. A combination of low sequence complexity, low overall hydrophobicity and relatively high net charge are important prerequisites for a protein to be intrinsically devoid of compact structure. Functionally, IUPs are involved in protein-protein interaction networks and protein-DNA complexes, and play roles in such processes as signal transduction, transcriptional activation, nucleic acid recognition and cell cycle regulation. IUPs are thought to have high specificities together with low affinities, which enables them to have multiple binding partners and function as hub proteins in interactomes.

In collaboration with Prof. Yossi Shaul, we have shown that 20S proteasomes digest IUP sequences, under conditions in which native proteins, and even partially unfolded molten globule states, are resistant. Furthermore, we demonstrated that protein–protein interactions with their partners can protect IUPs against 20S proteasomal action. Taken together, our results thus suggest that the 20S proteasome degradation assay provides a powerful system for operational definition of IUPs.

We have developed an algorithm to predict which regions of proteins are intrinsically unordered, i.e. FoldIndex©, http://bioportal.weizmann.ac.il/fldbin/findex

Ama is a secreted neuronal

adhesion protein belonging to the immunoglobulin superfamily, which has been identified as a ligand for Nrt. It has both homophilic and heterophilic cell adhesion properties, and is required for axon guidance and fasciculation during early stages of *Drosophila* development. Its sequence predicts an N-terminal signal sequence, three immunoglobulin domains, and a short C-terminal segment. We recently determined its structure in solution by use of SAXS (Figure 4). We found that Ama has a V-shaped dimer structure, in which the two chains are parallel to the dimer dyad (see figure). The dimerization interface is formed by domain 1, and domains 2 and 3 protrude. The V-shaped structure of Ama, as determined, provides a structural basis for explaining its homophilic adhesion properties. Furthermore, it suggests an adhesive mechanism for interaction with Nrt in which each 'arm' of Ama binds to Nrt, and thus promotes its clustering on the outer face of the plasma membrane.

Selected publications

Zeev-Ben-Mordehai, T., Rydberg, E.H., Solomon, A., Toker, L., Botti, S., Auld, V.J., Silman, I. and Sussman, J.L. (2003) The intracellular domain of the Drosophila cholinesterase-like neural adhesion protein, gliotactin, is natively unfolded. Proteins, 53, 758-767.

Greenblatt, H.M., Dvir, H., Silman, I. and Sussman, J.L. (2003) Acetylcholinesterase: a multifaceted target for structure-based drug design of anticholinesterase agents for the treatment of Alzheimer's disease. J. Mol. Neurosci., 20, 369-384.

Dvir, H., Harel, M., Bon, S., Liu, W.-Q., Vidal, M., Garbay, C., Sussman, J.L., Massoulié, J. and Silman, I. (2004) The synaptic acetylcholinesterase tetramer assembles around a polyproline-II helix. EMBO J., 23, 4394-4405.

Haviv, H., Wong, D.M., Greenblatt, H.M., Carlier, P.R., Pang, Y.P., Silman, I. and Sussman, J.L. (2005) Crystal packing mediates enantioselective ligand recognition at the peripheral site of acetylcholinesterase. J. Am. Chem. Soc., 127, 11029-11036.

Prilusky, J., Felder, C.E., Zeev-Ben-Mordehai, T., Rydberg, E., Man, O., Beckmann, J.S., Silman, I. and Sussman, J.L. (2005) FoldIndex©: a simple tool to predict whether a given protein sequence is intrinsically unfolded. Bioinformatics, 21, 3435-3438.

Harel, M., Hyatt, J.L., Brumshtein, B., Morton, C.L., Yoon, K.J., Wadkins, R.M., Silman, I., Sussman, J.L. and Potter, P.M. (2005) The crystal structure of the complex of the anticancer prodrug 7-Ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin (CPT-11) with Torpedo californica acetylcholinesterase provides a molecular explanation for its cholinergic action. Mol. Pharmacol., 67, 1874-1881.

Silman, I. and Sussman, J.L. (2005) Acetylcholinesterase: 'Classical' and 'Non-Classical' Functions and Pharmacology. Curr. Opin. Pharmacol., 5, 293-302.

Brumshtein, B., Greenblatt, H. M., Butters, T. D., Shaaltiel, Y., Aviezer, D., Silman, I., Futerman, A. H. & Sussman, J.L. (2007) Crystal structures of complexes of N-butyl- and N-nonyl-deoxynojirimycin bound to acid-β-glucosidase: Insights into the mechanism of chemical chaperone action in gaucher disease. J. Biol. Chem. 282, 29052-29058.

Hodis, E., Schreiber, G., Rother, K. & Sussman, J.L. (2007) eMovie: a storyboard-based tool for making molecular movies. Trends Biochem Sci. 32, 199-204.

Tsvetkov, P., Asher, G., Paz, A., Reuven, N., Sussman, J. L., Silman, I., and Shaul, Y. (2008) Operational definition of intrinsically unstructured protein sequences based on susceptibility to the 20S proteasome. Proteins 70, 1357-1366.

Manicka, S., Peleg, Y., Unger, T., Albeck, S., Dym, O., Greenblatt, H., Bourenkov, G., Lamzin, V., Krishnaswamy, S. & Sussman, J.L. (2008) Crystal structure of YagE, a putative DHDPS like protein from Escherichia coli K12. Proteins (in press).

Paz, A., Zeev-Ben-Mordehai, T., Lundqvist, M., Sherman, E., Mylonas, E., Weiner, L., Haran, G., Svergun, D. I., Mulder, F. A. A., Sussman, J. L., and Silman, I. (2008) Biophysical Characterization of The Unstructured Cytoplasmic Domain of The Human Neuronal Adhesion Protein Neuroligin 3. Biophysical Journal (in press).

Xu, Y., Colletier, J.P., Jiang, H., Silman, I., Sussman, J.L. & Weik, M. (2008) Induced-fit or pre-existing equilibrium dynamics? Lessons from protein crystallography and MD simulations on acetylcholinesterase Prot. Sci. (in press).

Xu, Y., Colletier, J.P., Weik, M., Jiang, H., Moult, J., Silman, I. & Sussman, J.L. (2008) The Side-chain Flexibility of 14 Aromatic Residues Lining the Active-site Gorge of Acetylcholinesterase Biophys J (in press).

Colletier, J.-P., Bourgeois, D., Fournier, D., Silman, I., Sussman, J.L. & Weik, M. (2008) "Shoot to Snap - X-ray damage as a method to study structural dynamics in protein crystals" Proc Natl Acad Sci USA (in press).

Acknowledgements

JLS is The Morton and Gladys Pickman Professor of Structural Biology

Granting agencies: European Commission, The Israel Ministry of Science, Culture and Sport, Autism Speaks, Israel Science Foundation and Israel Ministry of Trade & Industry

Internal support

The Divadol Foundation, The Neuman Foundation, Jean and Jula Goldwurm Memorial Foundation, Helen & Milton A. Kimmelman Center for Biomolecular Structure and Assembly, Benoziyo Center for Neuroscience, Minerva Foundation, Bruce and Rosalie N. Rosen Family Foundation, Kalman and Ida Wolens Foundation, Nalvyco Foundation, Research grant from Mr. Erwin Pearl, Yeda