Shoot-and-Trap: Use of specific x-ray damage to study structural protein dynamics by temperature-controlled cryo-crystallography

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Communicated by David Eisenberg, University of California, Los Angeles, CA, May 20, 2008 (received for review December 11, 2007)

Although x-ray crystallography is the most widely used method for macromolecular structure determination, it does not provide dynamical information, and either experimental tricks or complementary experiments must be used to overcome the inherently static nature of crystallographic structures. Here we used specific x-ray damage during temperature-controlled cryo-crystallographic experiments at a third-generation synchrotron source to trigger and monitor (Shoot-and-Trap) structural changes putatively involved in an enzymatic reaction. In particular, a nonhydrolyzable substrate analogue of acetylcholinesterase, the “off-switch” at cholinergic synapses, was radiocleaved within the buried enzymatic active site. Subsequent product clearance, observed at 150 K but not at 100 K, indicated exit from the active site possibly via a “backdoor.” The simple strategy described here is, in principle, applicable to any enzyme whose structure in complex with a substrate analogue is available and, therefore, could serve as a standard procedure in kinetic crystallography studies.

acetylcholinesterase | kinetic crystallography | structure–dynamics–function relationships | energy landscape | synchrotron radiation

Protein function depends critically on the synergy of structure and dynamics. Structural dynamics, stemming from the interconversion of conformational states in the complex energy landscape of a protein (1, 2), are not readily accessible to conventional x-ray crystallography. The advent of third-generation synchrotron sources, producing highly brilliant x-ray beams, has opened up exciting possibilities for studying macromolecular structural dynamics by using an ensemble of techniques known as kinetic crystallography (3). Although specific radiation damage in the course of data collection is often an issue at highly intense insertion-device synchrotron beamlines (4–7), it has been used for certain aspects of macromolecular x-ray structure determination, such as for phasing purposes (8), to structurally follow the catalytic pathway of redox enzymes (9), and for monitoring the dynamical behavior of crystalline proteins at cryogenic temperatures (10, 11). Amino acid residues directly involved in protein function, including those at the active sites of enzymes, are among the most radiation-sensitive entities (4–6, 10, 12–16). This observation has been linked to the strain of residue conformations within active sites that is released upon specific radiation damage (17). In this study, temperature-controlled x-ray cryo-crystallography was shown to result in radiolytic cleavage of a substrate analogue bound at the active site of the enzyme Torpedo californica acetylcholinesterase (TcAChE), and permitted monitoring of subsequent clearance of a radiolysis product from the active site.

AChE terminates transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (ACh) (Fig. 1) to choline (Ch) and acetate (18). The reaction proceeds in two steps. First, the enzyme is acetylated and the Ch-product expelled. Then, a water molecule regenerates the free enzyme with concomitant release of acetic acid. AChE is one of fastest enzymes in nature, with a turnover of 102–103 s⁻¹, and is the target of most currently approved anti-Alzheimer drugs (19), of insecticides (20), and of chemical warfare agents (21). The crystal structure of TcAChE revealed that its active site is buried at the bottom of a deep and narrow gorge (Fig. 2), an unexpected architecture in view of its high catalytic efficiency. Significant molecular breathing motions are essential for traffic of substrate and products to occur along this gorge, and it has been suggested that products may exit the active site via a putative, transiently opened backdoor (23, 24). Structural snapshots of substrate and products bound within the gorge have been obtained recently (25, 26). Binding of a nonhydrolyzable substrate analogue [4-oxo-N,N,N-trimethylpentanaminium iodide (OTMA)] (Fig. 1) in the active site uncovered the structural features of the tetrahedral intermediate state, while the structure of the enzyme in the action of expelling a product analogue at room temperature provided evidence for a transient opening of the backdoor (27). Here, we report the radiolytic breakdown of OTMA in the OTMA/TcAChE complex during collection of a series of crystallographic data sets at 100 and 150 K. Structural changes at 150 K indicate that the enzyme is flexible enough at that temperature to allow the exit of an analogue of the natural hydrolysis product, Ch, from the deeply buried active site. Radiolytic breakdown of substrate analogues, combined with temperature-controlled cryo-crystallography, is suggested as a methodology for studying product traffic and related structural dynamics in other crystalline enzymes.

Results

The substrate analogue OTMA differs from the natural substrate, ACh, only in the replacement of the ester oxygen by a carbon, thus being composed of acetyl (Ac) and pseudo-Ch (PsCh) moieties (Fig. 1); PsCh (n-propyltrimethylammonium) is

Author contributions: J.P.C. and M.W. designed research; J.P.C., B.S., and M.W. performed research; D.F. contributed new reagents/analytic tools; J.P.C., D.B., D.F., J.L.S., I.S., and M.W. analyzed data; and J.P.C., I.S., and M.W. wrote the paper.

The authors declare no conflict of interest.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2vja, 2vjb, 2vjc, 2vjd, 2vt6, and 2vt7).

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This article contains supporting information online at www.pnas.org/cgi/content/full/0804828105/DCSupplemental.

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an analogue of the enzymatic product Ch. OTMA binds, in
orthorhombic crystals of TcACHoE, at the catalytic anionic
subsite (CAS) of the active site and at the peripheral anionic
site (PAS), in exactly the same manner as reported for trigonal
TcACHoE crystals (25) (PDB ID code 2CSF). In the active site, its
quaternary nitrogen makes cation–π interactions with Trp-84
and Phe-330, and an electrostatic interaction with the acidic side
chain of Glu-199 (Fig. 3A). Its carbonyl oxygen hydrogen bonds
to Gly-118N, Gly-119N, and Ala-201N in the oxyanion hole, and
its carbonyl carbon is modeled at a covalent-bonding distance
from the catalytic Ser-200 Oy. At the PAS, a second OTMA
molecule is bound, with its CH3CO moiety pointing toward the
active site. Its quaternary group makes cation–π interactions with
the aromatic rings of Trp-279 (see Fig. 4A) and Tyr-70, whereas
its carbonyl oxygen is weakly H-bonded to Tyr-121 Oy.

Four consecutive data sets (I–IV) were collected from the
same volume of a single crystal of an OTMA/TcACHoE complex
at 100 K. The crystal was then translated, and a second series of
four data sets was collected at another spot on the crystal at 150
K [see supporting information (SI) Table S1]. Thus, the two
series reveal the x-ray dose-dependent evolution of the OTMA/ TcACHoE complex at these two temperatures. From the first
to the last data set, the resolution deteriorated from 2.3 to 2.4 Å at
both temperatures. The cumulative doses absorbed after collec-
tion of data sets IV at 100 K and III at 150 K are nearly identical,
0.92 and 0.95 x 107 Gy, respectively. They correspond to
approximately one-third of the experimentally determined Gar-
man limit (3 x 107 Gy), above which the biological information
extracted from a macromolecular structure is likely to be com-
promised (28).

**Radiolytic Breakdown of OTMA in the Active Site of TcAChE at 100 K.**
The OTMA in the CAS of the structure corresponding to data
set I at 100 K (PDB ID code 2VJA) is well defined, as judged
from 2Fo – Fc composite-omit electron density maps (Fig. S1A),
reproducing the structural features reported previously (25).
Electron density observed around OTMA is still continuous in
data set IV at 100 K (PDB ID code 2VJB) while noticeably
thinner (Fig. S1B). A strong negative peak is observed on OTMA
in the Fourier difference map (Fig. 3A) computed with observed
structure factor amplitudes of data sets I and IV and with
calculated phases from model I [(Fo100K-IV – Fo100K-I)re-100K,I].
Inspection of B factors revealed a higher loss of definition of the
PsCh moiety of OTMA, but not of the Ac group on Ser-200,
compared with the loss of definition averaged over all protein
and solvent atoms (see Materials and Methods). Radiation-
induced cleavage of OTMA into PsCh and an Ac group is thus
the most likely scenario. The strong positive peak (1 in Fig. 3A)
below Phe-330 indicates that repositioning of the radio-released
PsCh occurs within the active site. As a consequence, a water
molecule interacting with the PsCh moiety of OTMA changes its
position concomitantly, as suggested by a pair of positive and
negative peaks (2 and 3 in Fig. 3A). The repositioning of the
PsCh moiety occurs just below Phe-330, which, together with
Tyr-121 (residue not shown in Fig. 3), forms a bottleneck in the
middle of the gorge (22). A negative peak is seen on the Phe-330
phenyl ring in the Fourier difference map (Fig. 3A), as well as on
the adjacent Tyr-334 (not shown), suggesting that both residues
become partially disordered upon repositioning of the radiation-
freed PsCh. The binding locus indicated for PsCh by the positive
peak in the Fourier difference map is different from that
reported earlier for the steady-state complex of TcACHoE with
thiocholine (TCh) (25). The positive peak (4 in Fig. 3A) observed
behind the acetate moiety of the OTMA molecule in the Fourier
difference map had been attributed earlier to a radiation-
induced movement of the catalytic His-440 (10).

Negative and positive peaks indicative of well documented
damage to acidic residues and disulfide bonds (4–7) are also seen
in the difference Fourier map (data not shown).

**Radiolytic Breakdown of OTMA in the Active Site of TcAChE at 150 K.**
The 2Fo – Fc composite-omit electron density for the OTMA in
the CAS of the structure corresponding to data set I at 150 K
(PDB ID code 2VJC) is as well defined (Fig. S1C) as the one
corresponding to data set I at 100 K (Fig. S1A). However, as the
x-ray dose increases, much greater structural changes occur
in the active site at 150 K than at 100 K. At a cumulative absorbed
dose similar to that for data set IV at 100 K, the 2Fo – Fc
composite-omit map of data set III at 150 K (PDB ID code
2VJD) shows a hole corresponding to data set IV at 100 K (PDB ID code
2VJA) is well defined, as judged
from 2Fo – Fc composite-omit electron density maps (Fig. S1A),
reproducing the structural features reported previously (25).
Electron density observed around OTMA is still continuous in
data set IV at 100 K (PDB ID code 2VJB) while noticeably
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Negative and positive peaks indicative of well documented
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in the difference Fourier map (data not shown).

**Radiolytic Breakdown of OTMA in the Active Site of TcAChE at 150 K.**
The 2Fo – Fc composite-omit electron density for the OTMA in
the CAS of the structure corresponding to data set I at 150 K
(PDB ID code 2VJC) is as well defined (Fig. S1C) as the one
corresponding to data set I at 100 K (Fig. S1A). However, as the
x-ray dose increases, much greater structural changes occur
in the active site at 150 K than at 100 K. At a cumulative absorbed
dose similar to that for data set IV at 100 K, the 2Fo – Fc
composite-omit map of data set III at 150 K (PDB ID code
2VJD) shows a hole corresponding to data set IV at 100 K (PDB ID code
2VJA) is well defined, as judged
from 2Fo – Fc composite-omit electron density maps (Fig. S1A),
reproducing the structural features reported previously (25).
Electron density observed around OTMA is still continuous in
data set IV at 100 K (PDB ID code 2VJB) while noticeably
thinner (Fig. S1B). A strong negative peak is observed on OTMA
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Negative and positive peaks indicative of well documented
damage to acidic residues and disulfide bonds (4–7) are also seen
in the difference Fourier map (data not shown).
2VJD) shows poorly defined and discontinuous electron density for the OTMA molecule bound at the CAS (Fig. S1a); only residual density is observed for the PsCh moiety next to Trp-84. The absence of electron-density around the Phe-330 phenyl group in the $2F_o - F_c$ composite-omit maps (Fig. S1c and d) indicates that it is more disordered in data sets I and III at 150 K than at 100 K. Analogously, the water molecule interacting with the PsCh moiety of the OTMA molecule in the CAS at 100 K (Fig. S1a) has no discernable electron density in the $2F_o - F_c$ composite-omit maps calculated for data sets I and III at 150 K (Fig. S1c and d). Thus, the crystalline enzyme is more flexible at 150 K than at 100 K. The Fourier difference map, calculated with the structure factor amplitudes of data sets I and III at 150 K, and with the calculated phases from model I ($|F_{o150K-III} - F_{c150K-III}|e^{i\phi_{150K-III}}$), reveals an elongated negative peak on OTMA in the CAS (Fig. 3B), indicating radiolysis of the substrate analogue. No strong positive peak is seen adjacent to this negative peak, suggesting that the radiolytically generated PsCh does not reposition below Phe-330 at 150 K, as it does at 100 K. In the putative backdoor region, strong positive and negative peaks are observed on the side chains of Met-83, Trp-84, Val-129 (Fig. 3B), and on both the main and side chains of Tyr-442, Ser-79, and Gly-81 (data not shown). Again, the positive peak attributed earlier to a radiation-induced movement of the catalytic His-440 (10) is visible in the CAS (4 in Fig. 3B).

To address the effect of x-ray irradiation on native orthorhombic $Tc$AChE crystals at 150 K, a control experiment was performed, in which a similar cumulative x-ray dose was administered (i.e., $0.94 \times 10^7$ Gy, distributed over five consecutive data sets; see SI Materials and Methods). A Fourier difference map was then calculated between the first (control-I) and last (control-V) data set (Fig. S4 and Table S2). Peaks on Trp-84, Met-83, Glu-199 and His-440 are observed (Fig. S4) that are qualitatively similar to those in Fig. 3B.

**Radiation-Induced Changes at the Peripheral Substrate-Binding Site (PAS).** At 100 K, two positive peaks are observed in the Fourier difference map next to a negative one on the carboxyl group of Glu-278, the residue adjacent to Trp279 at the PAS (Fig. 4A). No significant negative peak is observed on the OTMA molecule bound at the PAS (Fig. 4A and B), indicating that it is not affected by x-ray irradiation at 100 and 150 K, and that it does not move from its original position. Unlike at 100 K, no positive peaks are observed at 150 K next to the negative peak on the carboxyl group of Glu-278 (Fig. 4B).

**Discussion**

**Build-Up of an Intermediate at 100 K and Exit of PsCh from the Active Site at 150 K.** Two series of structures were collected, at 100 K and at 150 K, on two separate regions of a single crystal of a complex of $Tc$AChE with the nonhydrolyzable substrate analogue OTMA. At both temperatures, difference Fourier maps indicate loss of definition of the PsCh moiety of OTMA at increased absorbed x-ray doses, which we ascribe to radiolytic cleavage of the substrate analogue into an Ac group on the catalytic Ser-200 and PsCh, an analogue of the enzymatic product, Ch. The series of structures determined at 100 K is interpreted as illustrating the cleavage of the tetrahedral intermediate that forms subse-
quent to binding of ACh in the CAS (Fig. 3A). PsCh repositions below Phe-330, a residue that is part of a bottleneck at midway down the active-site gorge. Phe-330 is a very mobile residue, and it has been suggested that it is involved in gating of traffic of substrate and products within the gorge (18, 25). The repositioning of PsCh, evidenced by the strong positive peak observed in the Fourier difference map calculated at 100 K, does not overlap with the position of TCh in complex with TcAChE (PDB ID code 2CSG) (25), nor with that observed for TCh or Ch in complex with mouse AChE (PDB ID codes 2HA2 and 2HA3, respectively) (26). This difference might be ascribed either to the more hydrophobic character of PsCh, relative to TCh or Ch, leading to a different binding mode, or to the fact that the structures presented in refs. 25 and 26, although collected at cryo-temperatures, reflect equilibrium states obtained by soaking at room temperature that were trapped upon cryo-cooling.

At 150 K, no signs of reorientation of PsCh are observed after OTMA radiolysis. However, strong negative and positive features in the Fourier difference map (Fig. 3B) indicate a small shift in the equilibrium position of the indole ring of Trp-84 that suggests an exit trajectory (indicated by green arrows in Figs. 2 and 3B) for PsCh and, by analogy, for the natural enzymatic hydrolysis product, Ch, via the putative backdoor (23, 24, 27). A similar, but less pronounced displacement of the indole ring of Trp-84 was observed in a radiation-damage control on native orthorhombic TcAChE crystals at 150 K (Fig. S4 and SI Materials and Methods). Thus, the Trp-84 movement shown in Fig. 3B is partially due to a relaxation at 150 K that might be a local response to radiation-induced changes elsewhere in the enzyme. In any case, the backdoor region is intrinsically more flexible at 150 than at 100 K. Together with the fact that the Fourier difference map is almost featureless at the top of the gorge (Fig. 4B), the observed Trp-84 movement argues that at 150 K PsCh exits predominantly via the backdoor. In a second control experiment, the absence of peaks on Trp-84 in a difference Fourier map computed between data set 100 K and a data set of the native enzyme collected at 100 K (Rmerge of structure factor amplitudes, 18%; data not shown) excluded the possibility that the movement of Trp-84 observed at 150 K (Fig. 3B) is due to unbinding of PsCh from Trp-84.

Our experiments do not allow to determine conclusively the radiochemical mechanism responsible for the cleavage of OTMA (Fig. 1). Secondary electrons or electron holes (7), mobile at both 100 and 150 K, most likely trigger the event by targeting the C–C bond between the Ac group and the PsCh moiety. In the natural substrate, ACh, the equivalent C–O bond between the Ac group and the Ch moiety is weakened by partial electron withdrawal in the tetrahedral intermediate and subsequently cleaved during substrate hydrolysis. OTMA binding in the active site mimics the tetrahedral intermediate that forms during enzymatic hydrolysis of ACh. Consequently, electrons might also be partially withdrawn from the C–C bond between the Ac group and the PsCh moiety in OTMA when it binds to the catalytic Ser-200, thus rendering the Ac carbon electrophilic. An increased radiation-sensitivity of the C–C bond might be the consequence, resulting in the observed OTMA cleavage. Indeed, x-ray irradiation has no significant effect on OTMA bound at the PAS, where ACh binds only transiently en route to the active site, and does not adopt a conformation resembling the transition state. This observation is in agreement with the early proposed “rack-and-strain” theory for enzymatic action, in which strain on the reactive bond is a consequence of the binding of the substrate within the active site of the enzyme (29).

Putative Trapping of Radio-Generated CO₂ Molecules at 100 K

Apart from the structural changes described above that are directly related to the radiolytic breakdown of the substrate analogue OTMA within the active site, most of the radiation damage features observed in the Fourier difference maps have already been reported for the native protein, namely the breakage of disulfide bridges and loss of definition of the carboxyl groups of acidic residues (4, 5, 10). The latter has been interpreted as resulting from the release of a CO₂ molecule by decarboxylation (5, 30). However, a previously undescribed feature was observed here for Glu-278, the residue adjacent to Trp-279 whose indole ring is the principal component of the PAS. For Glu-278 at 100 K, not only is a negative peak seen, but also two strong positive peaks on either side of it (Fig. 4A). A partially occupied CO₂ molecule could be fitted by real space refinement into each of the two positive peaks (Fig. 4C), and occupancies were estimated to be 5% and 13% (see Materials and Methods for details). Likewise, the occupancy of the Glu-278 carboxyl group was estimated to have been reduced to 79%. We thus suggest that either one or both of these positive peaks might reflect partial occupancy by a CO₂ molecule generated by decarboxylation. We conjecture that the Trp-279 indole ring, which blocks access to the active-site gorge, allows trapping of CO₂ at 100 K. Indeed, none of the other decarboxylated residues of the enzyme displays such clear and strong positive peaks next to the negative one. The fact that these peaks are absent from the Fourier map at 150 K (Fig. 4B) suggests that, at this temperature, the active-site gorge residues as a whole display sufficient flexibility for the CO₂ molecule to escape. Based on IR spectroscopic experiments, it has been reported that migration of photogenerated CO₂ in GFP occurs in a similar temperature range (31).

Conclusion and Perspectives

A close interdependency exists between the dynamics of a protein and of its immediate environment (32). Therefore, it is likely that dynamical changes in the hydration-water at the protein surface are involved in the observed change in protein flexibility with temperature (11, 33). Similar results were observed (at both 100 and 150 K) when analogous experimental protocols were conducted on a complex of TcAChE (crystallized in the same space group under the same conditions) with a photolabile precursor of the enzymatic product analogue, arsenecholine, namely “caged arsenecholine” (27) (data not shown), and on a “pro-aged” complex of human butyrylcholinesterase (34) with the organophosphate nerve agent soman (J.-P.C., F. Nachon, B.S., P. Masson, and M.W., unpublished results). These findings indicate that the results described here are neither protein- nor ligand-dependent, and that the Shoot-and-Trap strategy is applicable to other crystalline protein systems. Synchrotron-radiation-induced cleavage at 100 K of a N–S bond in an inhibitor complexed with a liver X receptor has indeed been reported (35).

The use of specific radiation damage, in combination with temperature-controlled cryo-crystallography, is a simple experimental approach capable of providing valuable insight into functional aspects of the structural dynamics of enzymes. Whereas most kinetic crystallography techniques are highly time consuming, and require the use of dedicated equipment and materials (3, 27), the Shoot-and-Trap strategy has the advantages of being fast and accessible, in principle, to any synchrotron user. The experiments described here were indeed performed in less than 2 h, including the temperature ramping from 100 to 150 K, at a typical third-generation insertion-device beamline. Moreover, using only a single crystal throughout the experiment permitted conservation of very high isomorphism between the data sets, which is critical for the calculation of accurate Fourier difference maps (3, 27, 36). Although this method obviously requires predetermination of the radiation damage characteristics of a given native enzyme, it is, nevertheless, theoretically applicable to any crystalline enzyme for which a substrate analogue is available. We propose the Shoot-and-Trap strategy...
as a simple technique that could allow the standardization of kinetic crystallography studies.

Materials and Methods

Chemicals. The nonhydrolyzable substrate analogue, OTMA, (Fig. 1B), was synthesized according to Thanei-Wyss and Waser (37).

Crystallization of TaChE and Soaking Procedure. TaChE was purified and crystallized as described (22, 38). To obtain the TaChE/OTMA complex, a large orthorhombic (P2₁2₁2₁) crystal (300 × 300 × 50 μm) was soaked for 2 h at 4°C in a mother liquor solution [30% polyethylene glycol (PEG)200/150 mM morpholineethanesulfonic acid (Mes), pH 6.0] containing 500 μM OTMA.  

Data Collection. Because of the cryoprotective capacity of PEG200, the crystal was directly flash-cooled in the cryostream of a cooling device (700 series, Oxford Cryosystems) at 100 K. X-ray diffraction data were collected on beamline ID14-EH4 at the European Synchrotron Radiation Facility (ESRF) by using the unattenuated beam at a wavelength of 0.932 Å. A first series of four data sets (I–IV) was collected at 100 K on one area of the crystal by using an x-ray beam of dimensions 100 × 100 μm. Subsequently, the crystal was translated by 200 μm along the spindle axis, and the temperature of the cooling device was increased to 150 K at 360 K/m. A second series of four data sets (I–IV) was then collected at 150 K without changing the beam size. For each of the eight data sets, 180 frames were collected, with an oscillation range of 1° and an exposure time of 1 s per frame. The collection time of each series of four data sets was on average 12 h with 10%–15% of the beam time between 100 and 150 K was unused. Data sets were indexed, merged, and scaled by using XDS/XSCALE, and structure-factor amplitudes were generated by using XDSCONV (39). The resolution data collection are shown in Table S1. 

Structure Determination and Refinement. The native structure of TaChE in the orthorhombic space group (PDB ID code 1W75) (41) served as the starting model for the rigid body refinement of data set 100K in the resolution range 50–4 Å. Subsequently, the structure underwent simulated annealing to 2,000 K with cooling steps of 10 K, and their occupancies were refined. The occupancies of the two putative CO₂ molecules and of the Glu-278 carboxyl group were refined to 0.32 and 1.00, respectively.  

ACKNOWLEDGMENTS. We thank Lilly Toker and Michal Cohen for purification of TaChE; Raimond Ravelli, Joanne McCarthy, and Elspeth Garman for continuous discussions and help during data collection; Ian Carmichael, Chan- tal Houëve-Levin, and David Milstein for fruitful discussions; David Eisenberg, Richard Dickerson, Duiilo Cascio, Michael Sawaya, and Arthur Lagana for critically reading the manuscript; and the European Synchrotron Radiation Facility for their light-time under long-term protocol ( MX51 and MX66 (radiation-damage BAG) and MX498 (IIS BAG). This work was supported by the Commissariat à l’Énergie Atomique, the Centre National de la Recherche Scientifique, the Université Joseph Fourier, Agence Nationale de la Recherche Grant (Sécurité et Santé en vie), the Institutes of Health CounterACT Program (the U.S. Army Defense Threat Reduction Agency; the Nalvyco Foundation, the Kimmelman Center for Biomolecular Structure and Assembly (Rehovot, Israel); the Minerva Foundation (J.L.S.); the Benoziyo Center for Neuroscience (J.L.S.); the Israel Ministry of Science, Culture, and Sport (the Israel Structural Proteomics Center); the Divacol Foundation; and the European Commission 7th Framework “SPIINE-COMPLEXES” Project (LSHG-CT-2006-031232). J-P.C. was supported by a Université Joseph Fourier grant and European Molecular Biology Organization Short-Term Fellowship ASTF230-2006. J.L.S. is the Pickman Professor of Structural Biology.


