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Correlation of the dynamics of native *human* acetylcholinesterase and its inhibited huperzine A counterpart from sub-picoseconds to nanoseconds

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It is a long debated question whether catalytic activities of enzymes, which lie on the millisecond timescale, are possibly already reflected in variations in atomic thermal fluctuations on the pico- to nanosecond timescale. To shed light on this puzzle, the enzyme *human* acetylcholinesterase in its wild-type form and complexed with the inhibitor huperzine A were investigated by various neutron scattering techniques and molecular dynamics simulations. Previous results on elastic neutron scattering at various timescales and simulations suggest that dynamical processes are not affected on average by the presence of the ligand within the considered time ranges between 10 ps and 1 ns. In the work presented here, the focus was laid on quasi-elastic (QENS) and inelastic neutron scattering (INS). These techniques give access to different kinds of individual diffusive motions and to the density of states of collective motions at the sub-picoseconds timescale. Hence, they permit going beyond the first approach of looking at mean square displacements. For both samples, the autocorrelation function was well described by a stretched-exponential function indicating a linkage between the timescales of fast and slow functional relaxation dynamics. The findings of the QENS and INS investigation are discussed in relation to the results of our earlier elastic incoherent neutron scattering and molecular dynamics simulations.

1. Introduction

Cholinesterases (ChEs) play a fundamental role in the cholinergic and non-cholinergic functioning of the nervous system [1,2]. Acetylcholinesterase (AChE), hydrolyses the neurotransmitter acetylcholine (ACh), thereby terminating the action of the transmitter at the cholinergic synapses and neuromuscular junctions. Hence, a strong or even complete blockade of ACh-mediated neurotransmission has severe implications on the nervous system and can be lethal. Clinically, moderate inhibition of *human* AChE (*hAChE*) is effective in the treatment of certain neurological and neuromuscular diseases to prolong the action of ACh on the receptor. Such a treatment is desirable either if there are not enough ACh-receptors, as in the case of myasthenia gravis, or if there is reduced production of ACh, as in the case of Alzheimer's disease.

Huperzine A (HupA), a naturally occurring alkaloid isolated from the Chinese medicinal herb *Huperzia serrata* [3], is a powerful reversible inhibitor of *hAChE*. It is envisaged for the palliative treatment of Alzheimer's disease

and memory impairments and found to display neuro-protectant properties of interest in mitigation of nerve agent poisoning [4].

hAChE is one of the fastest enzymes known [5], operating nearly at the diffusion limit. Its three-dimensional structure is similar to that of other ChEs [6]. The first crystallographic structure of *Torpedo californica* AChE (*TcAChE*) [7] revealed a surprising feature, which is at odds with the speed of the enzyme: the active site is located at the bottom of a deep and narrow gorge lined by 14 conserved aromatic residues. Owing to the restrictive dimensions of the active site gorge (20 Å depth, $\varnothing \approx 5$ Å), substrate hydrolysis takes place in a closed space virtually isolated from the bulk solvent. In parts, the gorge appears to be so narrow that only water molecules could fit through, and substrates or inhibitors would have no access to the active site if the enzyme structure was rigid. Thus, large amplitude fluctuations are necessary to explain the entry of molecules such as substrates, ligands and inhibitors or activators. Studies of the enzyme's dynamics, using molecular dynamics (MD) simulations [8,9] and quantum mechanical–molecular mechanical (QM/MM) simulations [10], indicate the presence of such motions.

MD simulations are a powerful computing tool to analyse the dynamics in more detail and such efforts have revealed a whole hierarchy of motions acting together at different timescales [11,12]. The groups of McCammon (1 ns) and Xu [13] (20 ns) have undertaken simulations of *mouse* AChE (*mAChE*), unliganded and in complex with HupA [14–17]. The authors analysed the dynamics of the breathing mode of the gorge and the possible opening of a so-called backdoor and other possible side channels in detail.

Recently, we performed 10 ns MD simulations of native *hAChE* and its HupA-inhibited counterpart [18] starting from the crystal structure of *hAChE* published by Dvir *et al.* in 2010 [19]. Meanwhile, the crystal structure of a recombinant truncated *hAChE* monomer in complex with HupA was released under PDB entry 4EY5 [6]. However, no significant difference between our starting model and the experimental structure was found. We compared the simulation results with results from elastic incoherent neutron scattering (EINS) experiments and concluded that dynamical processes were not affected on average by the presence of the ligand within the considered time ranges between 10 ps and 1 ns.

The above-mentioned studies revealed the dynamic complexity of the fluctuations. The latter fact seems to be a general characteristic of macromolecules due to their elaborate energy landscapes [20]. Within complex dynamic systems, such as proteins, diffusive motions are often confined in space. Therefore, they can be described as diffusion processes in the presence of systematic forces. The corresponding auto-correlation functions exhibit long-term memory effects and lead to a non-exponential decay in time. This effect is the signature of a linking of the timescales of fast and slow functional relaxation dynamics [21]. Occurring on different levels of interactions (van der Waals, hydrogen bonding, hydrophobic, etc.) all of these motions are important for the proper functioning of the macromolecule but can also compete with each other. The analysis of the variation of the gorge radius over 10 ns showed clearly that the corresponding autocorrelation function complies with a non-exponential relaxation [16]. For instance, the local motions of side chains seem to contribute significantly to the opening of the gorge, but they are strongly correlated with larger domains of collective dynamics. At least half the

protein was found to be involved in large-scale fluctuations. The authors of these publications are speaking about a hierarchy of relaxations divided into tiers of faster and slower motions, where the relaxations in the slower tiers only become accessible when the fastest tiers have moved into the required conformations. A similar interpretation was given by Henzler-Wildman & Kern [20], stating that transitions are slow if they are improbable, arising from many individual attempts by local groups to overcome the energy barrier separating the conformational states. The low success rate results in the collective nature of such large-scale motions.

More recently, a combined study of MD simulations and experimental data has shown that picosecond mean square displacements (MSDs), as measured by EINS, satisfy a universal scaling law with respect to viscosity measured over much longer timescales (up to seconds or more) [22,23]. Such universal correlation was observed to be valid for a wide range of simple liquids, supercooled liquids and glass formers, and thus confers on picosecond MSDs a more general and intriguing role of fast predictor for slower dynamical processes governed by viscosity and relaxations.

Members of the ChE family differ from each other in catalytic activity, oligomerization state and glycosylation [24]. The question of whether there exists a correlation between these characteristics and dynamics is still an unresolved matter [25]. Catalytic activities lie on the millisecond timescale, which are comparable to those required for protein folding. Are differences in catalytic activities reflected in variations in atomic thermal fluctuations on the pico- to nanosecond timescale? The first evidence supporting this hypothesis comes from comparative studies on *hAChE*, *human* butyrylcholinesterase (*hBChE*) [26] and *mAChE* [27]. In a next step, we wanted to block enzymatic activity with an inhibitor to probe its effects on the dynamics. The inhibitor HupA was chosen for the experiments because it binds tightly to the enzyme. Owing to its low hydrogen content, the molecule itself will be almost invisible in the neutron dynamics studies, but when bound to *hAChE*, it switches off the enzyme activity.

Elastic, quasi-elastic (QENS) and inelastic neutron scattering (INS) was used to probe experimentally and quantitatively the MD of *hAChE*, with and without HupA inhibition, in order to investigate the relationships between MD, activity and inhibitor binding. The elastic results obtained on the three different neutron spectrometers IN6, IN13 and IN16 (<http://www.ill.eu/instruments-support/instruments-groups/instruments/in6/description/instrument-layout/>; <http://www.ill.eu/instruments-support/instruments-groups/instruments/in16/>; <http://www.ill.eu/instruments-support/instruments-groups/instruments/in13/description/instrument-layout/>) at the Institut Laue Langevin (ILL) in Grenoble, France, were presented in a separate publication and compared to a 10 ns MD simulation [18]. Elastic measurements mainly probe local vibrational motions. The Gaussian approximation was used [28] to extract atomic MSDs. This approximation supposes a Gaussian distribution of atoms around their equilibrium positions and is only valid within a small domain of momentum transfers Q . Within this framework, no difference was detectable between the wild-type enzyme and its inhibited counterpart.

QENS permits distinguishing and quantifying the motions of atoms and molecules on a microscopic scale and on characteristic times of the order of 10^{-9} – 10^{-12} s, corresponding to energy transfers of the order of 1 μ eV to 1 meV [29]. In contrast to elastic neutron measurements, this technique helps to

disentangle different kinds of individual motions, examples of which include: long-range diffusion of intercalated atoms in a host matrix, consecutive short-range jumps and motions of molecules in liquids, rotations of chemical groups or of whole molecules in solid disordered phases, which occur at slightly different timescales. QENS was performed on IN6 and IN16, but only the dynamical range of IN6 permitted INS measurements.

INS gives access to collective modes or vibrational excitations (phonons) and to the density of states (DOS), which corresponds to the phonon dispersion curve. These methods were employed to investigate the same samples (hAChE and hAChE + HupA) on the spectrometer IN6 at the ILL and are presented here.

2. Material and methods

Production, purification and sample preparation of recombinant hAChE with or without two molar equivalents of (–)-HupA (<http://www.sigmaaldrich.com/sigma-aldrich/home.html>, H5902(–)) are described in more detail by Peters *et al.* [26]. Briefly, after a lyophilization step, the protein powders were placed in aluminium sample containers, dried over P₂O₅ and re-hydrated from D₂O vapour exchange to a final water content of 0.4 g water/g protein. This hydration corresponds to at least a first full hydration layer and ensures that the protein is in its active state [30]. Moreover, to verify that no loss of material had occurred, both samples were weighed before and after neutron scattering experiments. No losses were detected for any sample.

2.1. Neutron scattering experiments

Experiments were performed on the cold time-of-flight spectrometer IN6 and on the cold neutron backscattering spectrometer IN16 at the ILL, Grenoble, France. QENS spectra were recorded at physiological temperature (300 K). Recorded data are corrected for the scattering coming from the sample holder, normalized to the 20 K data or vanadium and binned. On IN6, two incident wavelengths λ of 5.1 and 5.9 Å, corresponding to two different elastic energy resolution of $\Delta E = 90 \mu\text{eV}$ and $\Delta E = 50 \mu\text{eV}$, respectively, were investigated. The covered reciprocal space was $0.44 < Q < 1.9 \text{ Å}^{-1}$ in the case of the $90 \mu\text{eV}$ setting and $0.38 < Q < 1.6 \text{ Å}^{-1}$ for $\Delta E = 50 \mu\text{eV}$. Owing to the two different resolutions used in the experiments on IN6, the accessible Q -ranges differ slightly for the two resolutions. In the case of $\Delta E = 50 \mu\text{eV}$ and an energy transfer of $\pm 0.75 \text{ meV}$, the Q -range is limited to $0.5\text{--}1.6 \text{ Å}^{-1}$. Choosing a ΔQ of 0.1 Å^{-1} these result in 12 data points. For $\Delta E = 90 \mu\text{eV}$, the energy range can be extended to an energy transfer of $\pm 1 \text{ meV}$ and a Q -value of 1.9 Å^{-1} . The same binning of $\Delta Q = 0.1 \text{ Å}^{-1}$ was chosen, resulting in 15 groups. On IN16, data were collected with an energy resolution of $1 \mu\text{eV}$ and an energy transfer of $\pm 15 \mu\text{eV}$. Data were analysed in a scattering vector range of $0.54 \text{ Å}^{-1} < Q < 1.06 \text{ Å}^{-1}$, corresponding to a space–time measurement window of 1 Å in 1 ns .

INS spectra were taken on IN6 at 80 K, where the motions are supposed to be strictly harmonic. Data collection time was between 4 and 8 h for both samples. All measured spectra were corrected for empty can scattering, normalized to the spectra measured at 20 K or to vanadium in order to correct for detector efficiency, grouped and transformed into energy transfer using the standard ILL LAMP routines (http://www.ill.eu/data_treat/lamp/the-lamp-book/) [31]. The transmission of all samples was more than 94%; therefore, multiple scattering effects were estimated to be negligible.

Analysing quasi-elastic scattering gives a more detailed picture about the observed dynamics. The theoretical scattering

function $S_{\text{theo}}(Q, \omega)$ reads [29]

$$S_{\text{theo}}(Q, \omega) = e^{-\langle x^2 \rangle Q^2} [A_0(Q) \delta(\omega) + \sum_n A_n(Q) L_n(Q, \omega)], \quad (2.1)$$

where $e^{-\langle x^2 \rangle Q^2}$ is the Debye–Waller factor, representing vibrations, and $\hbar\omega$ is defined as the energy transfer. The delta function δ accounts for dynamics that lie within the instruments' resolution. The amplitude $A_0(Q)$ is the elastic incoherent structure factor (EISF) and contains information about the geometry of the motion. The quasi-elastic contributions are mimicked by a sum of Lorentzian functions $L_n(Q, \omega)$ of half width half maximum (HWHM) $\Gamma(Q)$ and the corresponding quasi-elastic incoherent structure factors $A_n(Q)$ with

$$L(Q, \omega) = \frac{1}{\pi} \frac{\Gamma(Q)}{\omega^2 + \Gamma(Q)^2}. \quad (2.2)$$

For data analysis the theoretical scattering law S_{theo} has to be convoluted with the instrumental energy resolution, which can be mimicked by e.g. vanadium:

$$S_{\text{meas}}(Q, \omega) = S_{\text{theo}}(Q, \omega) \otimes S_{\text{res}}(Q, \omega). \quad (2.3)$$

For the description of diffusion in confined space, such as hydrogen atoms in proteins, Volino and Dianoux developed the model of diffusion in a sphere. Here, diffusive motions are allowed only inside a sphere with radius r and impervious walls [32]. The corresponding scattering law can be written as follows:

$$S(Q, \omega) = A_0^l(Q) \delta(\omega) + \sum_{(l,n) \neq (0,0)} (2l+1) A_n^l(Q) \frac{1}{\pi} \frac{\lambda_n^l D}{\omega^2 + (\lambda_n^l D)^2}. \quad (2.4)$$

where λ_n^l are numerical coefficients of the series and D a diffusion parameter.

Volino and Dianoux found an analytical expression for the EISF, which reads

$$A_0(Q) = \left[\frac{3 j_1(Qr)}{Qr} \right]^2, \quad (2.5)$$

where $j_1(x) = \sin x/x^2 - \cos x/x$ is the first-order spherical Bessel function and r the radius of the sphere, which can be extracted from fitting equation (2.5) to the obtained data. Bellissent-Funel and co-workers [33] expanded the model for the EISF by an immobile fraction p , where p denotes strongly bound protons. Equation (2.5) then reads

$$A_0(Q) = p + (1-p) \left[\frac{3 j_1(Qr)}{Qr} \right]^2. \quad (2.6)$$

For continuous diffusion, a linear dependence of the HWHM Γ of the quasi-elastic component as function $(rQ)^2$ is expected [29]. However, for small Q -values ($Q < \pi/r$) Γ tends towards a constant value Γ_0 , which is a signature of motion in confinement. Γ is then related to the diffusion parameter D and the radius of the sphere via $\Gamma_0 = 4.33 D/r^2$.

Hall & Ross [34] extended the Volino–Dianoux model to random jump diffusion within the restricted geometry of a sphere. For small Q -values, it shows the same behaviour as the diffusion in a sphere, but for large values of Q it converges towards the jump diffusion model introduced by Singwi & Sjölander [35]. The HWHM can then be described by

$$\Gamma = \frac{DQ^2}{1 + DQ^2\tau}, \quad (2.7)$$

where D is again a diffusion parameter and τ the residence time between two jumps. τ can be described in the limit of large Q as $\Gamma_\infty = 1/\tau$.

From INS data, the vibrational DOS $g(\omega)$ can be extracted. It corresponds to the frequency distribution of the collective vibrational modes. The DOS can be obtained from the scattering

Figure 1. Semi-logarithmic plot of $S(Q, \omega)$ for $hAChE$ taken on IN6 ($\Delta E = 90 \mu\text{eV}$ energy resolution, at 300 K) with corresponding fits. Data are shown as black points with corresponding error bars. The elastic contribution is shown in green, in blue is a single Lorentzian curve and in cyan a linear background is drawn. (Online version in colour.)

function $S(Q, \omega)$ in the limit $Q \Rightarrow 0$ [36,37] through

$$g_{\text{exp}}(\omega) = \lim_{Q \rightarrow 0} \frac{6\omega}{\hbar Q^2} (e^{\hbar\omega/k_B T} - 1) S(Q, \omega), \quad (2.8)$$

where $\hbar$ is Planck's constant divided by 2π , k_B is the Boltzmann constant and T the absolute temperature. The integral of $g(\omega)$ over the whole energy range should be normalized to 1. Experimentally, one has no access to the complete energy range needed for the normalization. Thus, the DOS was normalized by a multiplicative factor assuring that

$$\int_{\omega_{\min}}^{\omega_{\max}} g_{hAChE}(\omega) d\omega = \int_{\omega_{\min}}^{\omega_{\max}} g_{hAChE+HupA}(\omega) d\omega, \quad (2.9)$$

where $\omega_{\min}$ and $\omega_{\max}$ were fixed such that at the lower limit the elastic peak contributions were excluded and at the higher energy transfer limit the very noisy and small contributions. Concretely, $\omega_{\min}$ was obtained by determining the minimum of the function $g(\omega)/\omega^2$.

3. Results

3.1. Quasi-elastic neutron scattering results

The obtained data were fitted using a convolution of the measured instrumental resolution function with a delta function for the elastic contribution, a single Lorentzian to mimic the quasi-elastic broadening and a slope background. The resulting fits for the native $hAChE$ sample at $\Delta E = 90 \mu\text{eV}$ are shown exemplary for $Q = 0.5 \text{ \AA}^{-1}$ in figure 1.

Fitting Bellissent-Funel's model to the Q -dependence of the EISF (figure 2) enabled values for the radius of the sphere and the percentage of immobile protons to be obtained; corresponding values are given in table 1. For both resolutions, the obtained values for the EISF of the two samples agree within experimental errors. As expected, the derived radius is larger for $\Delta E = 50 \mu\text{eV}$ than for $\Delta E = 90 \mu\text{eV}$ due to the longer observation time. For the second parameter extracted from the fits, the fraction of immobile water molecules, the errors are much larger. However, for both resolutions this fraction tends to be slightly reduced in the presence of the inhibitor.

The HWHM of the Lorentzians used in the fitting procedure are given in figure 3 for the 90 and 50 μeV set-ups as a function of Q^2 . For small Q -values, i.e. large distances,

Figure 2. EISF for both resolutions ((a) 90 μeV , (b) 50 μeV) measured on IN6. Data and corresponding fits for pure $hAChE$ are drawn as black squares and line, for $hAChE$ –HupA data and fits are depicted as red circles. (Online version in colour.)

a confinement effect is visible as a levelling off towards a constant value. According to the diffusion in a sphere model [32] and the modification proposed by Bellissent-Funel *et al.* [33], a diffusion parameter D can be estimated from the limit $\Gamma_0 = 4.33 D/r^2$ for $Q \rightarrow 0$ for the 90 μeV resolution. For the 50 μeV energy resolution, the generalized model of Hall & Ross [34] was used to extract diffusion parameters. Note that for each of the resolutions the obtained D values of the two samples match within their reliability parameters (cf. table 2). However, they differ by more than a factor of two between the different resolution settings. This effect highlights the approximate nature of the diffusion models applied in this work. In the presence of an appreciable distribution of relaxation modes, the exploitation of a limited spectral range for the fitting of these models results in apparent diffusion parameters. Their values are subject to the limiting conditions and may have only a useful meaning within these conditions. This does not necessarily apply to properties and quantities such as the activation energies derived from the apparent observables as we will show and discuss hereafter.

For large Q -values, the line width does not follow a free diffusion in which case Γ can be written as $\Gamma = DQ^2$. In this investigated case, the line width shows a jump diffusion behaviour. High Q -values correspond to short length scales, thus local motions such as jumps of the observed protons become dominant. The residence time τ between two jumps is given by $\tau = 1/\Gamma_\infty$, where Γ_∞ can be extracted from the

Table 1. Results obtained from fitting the EISF with the diffusion in a sphere model. Values are given for both samples and both investigated energy resolutions.

		native <i>hAChE</i>	<i>hAChE</i> –HupA
$\Delta E = 90 \mu\text{eV}$	radius a (Å)	2.69 ± 0.07	2.71 ± 0.07
	immobile fraction p (%)	71.19 ± 3.71	69.81 ± 4.31
$\Delta E = 50 \mu\text{eV}$	radius a (Å)	2.93 ± 0.09	2.96 ± 0.06
	immobile fraction p (%)	69.37 ± 6.00	68.19 ± 4.23

Figure 3. (a) Lorentzian width for an elastic energy resolution of $\Delta E = 90 \mu\text{eV}$. (b) Lorentzian width for an elastic energy resolution of $\Delta E = 50 \mu\text{eV}$. Fit curves correspond to the jump diffusion model. The data and fits for pure *hAChE* are as black squares and for *hAChE*–HupA as red circles. (Online version in colour.)

asymptotic behaviour of Γ for high Q , where a constant value Γ_∞ is reached. However, for both resolutions studied here the line width is still increasing at high Q -values and has not yet reached a plateau. Therefore, the values have to be extracted from fitting equation (2.7) to the data. The characteristic jump distance can be calculated using: $l = \sqrt{D\tau}$. As for all other parameters obtained, the jump lengths for native *hAChE* and *hAChE*–HupA also agree within experimental errors. All the obtained fitting parameters are given in table 2.

In order to perform a jump, protons have to overcome an energy barrier imposed by the surrounding energy landscape. The height of this energy barrier is linked to the residence time via the Arrhenius relation:

$$\tau \sim e^{E_a/k_B T}, \quad (3.1)$$

where τ_0 is a pre-exponential factor and E_a the activation energy of the process. When τ_0 is assumed to be the same for native *hAChE* and its inhibited counterpart ΔE_a can be written as

$$\Delta E_a = k_B T \ln \frac{\tau_{\text{AChE}}}{\tau_{\text{HupA}}}. \quad (3.2)$$

The activation energy difference between the two investigated systems is thus found to be $\Delta E_{a,90 \mu\text{eV}} = 10.47 \pm 0.30 \text{ cal mol}^{-1}$ and $\Delta E_{a,50 \mu\text{eV}} = 11.90 \pm 0.43 \text{ cal mol}^{-1}$, respectively. Both values correspond to approximately $0.02 k_B T$, e.g. 2% of the thermal energy, and therefore only to very small energy differences.

3.2. Density of states

The INS measurements were performed on IN6 at 80 K to ensure that the sample dynamics was purely harmonic. Both resolutions were investigated, but as the results are very similar, we present them only for $90 \mu\text{eV}$.

Figure 4 shows the measured spectra of the samples integrated over the whole range of scattering angles. The origin of the maximum around $-(3-4) \text{ meV}$, called Boson peak, is not yet completely understood, but there is some evidence that it is related to collective harmonic vibrations [38] within the protein. The main difference between both samples appears in the Boson peak region, where $S(Q, \omega)$ seems higher for the complexed enzyme. The DOS (figure 4b) permits extraction of the vibrational contribution to the experimental structure factor and thus localized motions. After normalization according to equation (2.9), again no difference is detectable for the DOS in the presence of the inhibitor when compared with the pure enzyme, confirming that the vibrational motions are maintained. Balog *et al.* [39] found that the binding of methotrexate to dihydrofolate reductase does result in a more flexible form than the uncomplexed protein. The comparison between the two studies illustrates the effects of substrate binding to the entropy does strongly depend on the system under investigation.

3.3. Intermediate scattering function

The second question addressed in this paper was the question of the existence of a correlation of motions and possible linking of timescales. QENS data allow extraction of the autocorrelation function $I(Q, t)$ by Fourier transformation of $S(Q, \omega)$ using the Fourier transform toolkit included in the DAVE software package [40]. We applied it to IN6 data, deconvoluted with the resolution function, and selected Q -values at room temperature (figure 5). Comparing both graphs of figure 5, the difference in accessible time ranges for the two energy resolutions and a clear decay of $I(Q, t)$ for longer times indicating the presence of slow relaxation processes can be seen. This proves the predicted existence of a whole hierarchy of motions present in the

Table 2. Results for fitting equation (3.2) to the 90 and 50 μeV data.

	90 μeV		50 μeV	
	native AChE	AChE + HupA	native AChE	AChE + HupA
D ($10^{-5} \text{ cm}^2 \text{ s}^{-1}$)	1.58 ± 0.07	1.51 ± 0.06	3.53 ± 0.33	3.45 ± 0.31
τ (ps)	3.30 ± 0.29	3.36 ± 0.31	9.11 ± 0.23	8.93 ± 0.22
l ($\text{\AA}$)	0.72 ± 0.04	0.71 ± 0.04	1.79 ± 0.17	1.76 ± 0.16
ΔE_a (cal/mol)	10.47 ± 0.30		11.90 ± 0.43

Figure 4. (a) Dynamic structure factor at 90 μeV measured on IN6 at 80 K. Data for pure *hAChE* are drawn as black squares, for *hAChE*–HupA data are depicted as red circles. Data from all scattering angles are summed. The vertical line shows ω_{min} determined for the normalization. (b) DOS $g(\omega)$ as function of energy transfer ω . Data are normalized as described in the text. (Online version in colour.)

enzymes, the Debye–Waller factor accounting for all fast dynamics, comprising vibrational and fast diffusive motions.

We can still go further in our analysis by estimating the width of the distribution of activation energies involved. For this, we are modelling the autocorrelation by a stretched-exponential $A\exp(-[t/\tau]^\beta)$, known as the Kohlrausch–Williams–Watt (KWW) function, which has been used extensively to model dielectric relaxation processes [41]. A is a pre-exponential factor, τ represents the relaxation time and β is a fit factor varying between 0 and 1. In the limit $\beta \rightarrow 1$, a single exponentially decaying correlation function is retrieved.

Figure 5. Autocorrelation function $I(Q, t)$ from data measured at IN6 at 300 K for *hAChE* and *hAChE*–HupA for different Q -values. For one and the same instrument resolution, the two samples give very similar results. Here, we show as an example *hAChE* at 50 μeV instrumental resolutions and *hAChE*–HupA at 90 μeV instrumental resolution. (Online version in colour.)

4. Discussion

The main objective of our investigation was the question of whether a ligand (e.g. an inhibitor) bound to an enzyme would influence its MD or not. Therefore, *hAChE* in the presence or the absence of the non-covalent inhibitor HupA was investigated by EINS on different spectrometers corresponding to various time windows and compared to a 10 ns MD simulation [18]. Within the precision of the measurements and the Gaussian approximation to extract MSD values, no differences were detectable on all instruments for such local vibrational motions for the protein under investigation.

QENS is a technique that helps to probe diffusive motions and contains information about the geometry of the movement. Using the generalized diffusion in a sphere model of Bellissent-Funel *et al.*, we obtained radii for both instrumental resolutions, which were close to identical for the two samples and typical for confined motions in proteins [42,43]. The immobile fractions correspond to atoms that do not contribute to motions within the time window of the instrument. Although, they also agree within the error bars, they are by trend smaller for the inhibited enzyme, which gives a little hint about a higher flexibility in this case.

The quantities which can be obtained from the fits of the HWHM of the QENS curves, i.e. the diffusion parameter D , the residence time τ and the jump distance l are almost identical for both samples, so that it can be stated as a general conclusion about the QENS measurements that no differences are found with respect to the diffusive motions.

The situation appears very similar for the DOS, extracted from INS measurements and translating collective harmonic vibrations on a timescale of a few tens of femto-seconds according to Heisenberg's uncertainty principle. The main difference in the two samples consists of a blockade of the active site, which is located at the bottom of a gorge, but the dynamics inside of this gorge does not seem much influenced by such a non-covalent inhibitor. A biological system can indeed appear softer when the sampling of conformational substates is increased due to entropic effects. The following structural changes were found from the experimental structure [6] and the MD simulations presented in [18] for the active site structure of *hAChE* in complex with HupA: the carbonyl oxygen of HupA was H-bonded to Tyr133, and the NH group formed an H-bond with a structural water molecule H-bonded to Glu202. The amino group was H-bonded to water molecules to these residues, very similarly to what was observed for huprines in *mAChE* [44]. Other interactions were hydrophobic or van der Waals interactions and involved Trp86, Tyr337, Phe338, His447, Tyr449 and Tyr124. Over the course of the MD simulations, the network of hydrogen bonds prone to proton shuttling from Ser203 to Glu202, through His447, Glu334, Ser229 and Glu450, remained stable. Also the 180° flip of Gly121 observed in the *hAChE*–HupA crystal structure [6] and during the MD simulation of the *mAChE*–HupA complex [14,15] occurred early in the *hAChE*–HupA simulation at 100 ps. It seemed to be driven by the orbital repulsion of the carbonyl of HupA and the main chain carbonyl of Gly120. Besides, new H bonds were formed between Gly120-CO and Ala204-NH, Ser125-CO and Gly121-NH, and the methylene of Gly121 fitted perfectly in the groove formed by the two cycles of HupA. These interactions lead to an increased hydrophobicity within the gorge, in which both entropic terms (through solvent water effects) and enthalpic terms (through increased van der Waals interaction of apolar groups) contribute, but the effects are hardly visible on the timescales investigated here.

Figure 6 shows fits to $I(Q,t)$ with the KWW function. They are all compatible with a value of $\beta \approx 0.30$ for *hAChE*, with and without inhibitor. The values of the relaxation time τ are almost identical for both samples, but strongly dependent on Q . For $Q = 1 \text{ \AA}^{-1}$, it is around 300 ps at the 90 μeV resolution and around 400 ps at the 50 μeV resolution. Once more the dynamics of both samples does not seem to be influenced by the presence or the absence of HupA, but they are distributed over a large domain in time. The relaxation times are

Figure 6. Fits to $I(Q,t)$ by a KWW function at $Q = 0.5 \text{ \AA}^{-1}$ for *hAChE* at 50 μeV (a) and for *hAChE*–HupA at 90 μeV (b). (Online version in colour.)

indeed much longer than the instrumental resolution times. Typical values of β found in the literature are between ≈ 0.6 and 0.8 for polymers [45], 0.3 and 0.5 for proteins [46] and 0.4 and 0.7 for glasses [47], and Kneller found values around 0.5 analysing measured and simulated data of lysozyme and myoglobin [21]. A physical interpretation of this value is, however, not straightforward.

As remarked by Kneller [21] the KWW function has no associated memory function, as it should be for dynamics in proteins, and thus does not belong to the class of 'admissible' models. Together with Hinsin [48], he proposed a scattering function $S(Q,\omega)$ containing an elastic part similar to equation (2.9) and a part composed by generalized Lorentzians accounting for the quasi-elastic scattering. It is the Fourier transform of a Mittag-Leffler function [49], which exhibits long-time memory effects. Fitting our data in the (Q,ω) -space with such a generalized Lorentzian resulted in very similar results for β and τ for both instrumental resolutions, which means that the approximation with a KWW function seemed to be very reasonable.

To better understand the effect of the instrumental resolution on the measured curves, we modelled the autocorrelation function by an analytical KWW function with the value of $\beta = 0.35$ and $\tau = 300$ ps at the 90 μeV resolution and $\tau = 400$ ps at the 50 μeV resolution. We Fourier transformed these functions into the (Q,ω) -space and cut them in ω according to the energy ranges (-15 to $+15 \text{ \mu eV}$), which are also accessible on IN16. Finally, we Fourier transformed them back into the (Q,t) space. We combined the autocorrelation function obtained from the QENS data collected on IN6 and IN16 (figure 7). It permits coverage of a broad range of time

Figure 7. Autocorrelation function $I(Q,t)$ extracted from data obtained on IN16 and on IN6 at three different resolutions and $Q = 0.5 \text{ \AA}^{-1}$. (Online version in colour.)

from below the picosecond to a few nanoseconds. Beside little shifts between the curves, probably due to normalization, the data can be superposed on the different time ranges and clearly show a common stretched-exponential function corresponding to fractional Brownian dynamics in the proteins [48] and indicating the linking of timescales over more than four orders of magnitude. Fitting the combined data with a KWW function results in an even smaller β than simply fitting data from one energy resolution.

5. Conclusion

Results of QENS and INS measurements of free and HupA-inhibited *hAChE* performed on the spectrometers IN6 and IN16 of the ILL in Grenoble, France, were presented. Various parameters were extracted from the QENS fits at the energy

resolutions of 50 and 90 μeV , including the radius of confinement and the fraction of immobile protons, a diffusion parameter, the residence time, a characteristic jump distance and the activation energy difference between the two investigated systems. All values are very similar for both systems at both energy resolutions, indicating the similarity of the two systems on average. Only the radius of confinement is slightly larger and the fraction of immobile protons slightly reduced for the complexed form of the enzyme, which could be a hint for a higher flexibility of this sample. The high dynamical similarities between free *hAChE* and the *hAChE*–HupA complex are also confirmed by the results for the DOS. One should, however, keep in mind that this conclusion is specific to the enzyme studied here and not necessarily transferable to other proteins.

The autocorrelation function was extracted from the scattering function by Fourier transform to investigate the question of correlation of motions within many timescales. A comparison of the experimental curves with a KWW and a generalized Lorentzian function show the non-exponential behaviour of this function. The autocorrelation functions thus exhibit long-term memory effects, which is the signature of a linking of the timescales of fast and slow functional relaxation dynamics [21], resulting from different scales of interactions that each have important but competitive contributions.

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