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Relation between dynamics, activity and thermal stability within the cholinesterase family

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ABSTRACT

Incoherent neutron scattering is one of the most powerful tools for studying dynamics in biological matter. Using the cold neutron backscattering spectrometer IN16 at the Institut Laue Langevin (ILL, Grenoble, France), temperature dependence of cholinesterases' dynamics (human butyrylcholinesterase from plasma: hBChE; recombinant human acetylcholinesterase: hAChE and recombinant mouse acetylcholinesterase: mAChE) was examined using elastic incoherent neutron scattering (EINS). The dynamics was characterized by the averaged atomic mean square displacement (MSD), associated with the sample flexibility at a given temperature. We found MSD values of hAChE above the dynamical transition temperature (around 200 K) larger than for mAChE and hBChE, implying that hAChE is more flexible than the other ChEs. Activation energies for thermodynamical transition were extracted through the frequency window model (FWM) (Becker et al. 2004) [1] and turned out to increase from hBChE to mAChE and finally to hAChE, inversely to the MSDs relations. Between 280 and 316 K, catalytic studies of these enzymes were carried out using thiocholine esters: at the same temperature, the hAChE activity was systematically higher than the mAChE or hBChE ones. Our results thus suggest a strong correlation between dynamics and activity within the ChE family. We also studied and compared the ChEs thermal inactivation kinetics. Here, no direct correlation with the dynamics was observed, thus suggesting that relations between enzyme dynamics and catalytic stability are more complex. Finally, the possible relation between flexibility and protein ability to grow in crystals is discussed.

1. Introduction

It is now well accepted that proteins are dynamic objects and that there are strong correlations between their structure, dynamics and function. The achievement of a good understanding of macromolecular activity and stability is therefore intimately correlated to the understanding of dynamics. EINS spectroscopy is a technique uniquely suited for the measurement of atomic fluctuations in the ps–ns time scale, on samples that need not to be crystalline or even monodisperse [2]. Moreover, neutrons essentially see hydrogen atoms, as their incoherent scattering cross section is higher than for all other atoms in biomolecules, and as hydrogen atoms are almost uniformly distributed in biological matter, this technique probes the average proteins dynamics [3]. Despite all these advantages, EINS is not often employed, mainly because of the large amount of biological matter needed to perform an experiment (about 100 mg of highly purified sample) and also because the access to neutron sources is not easy.

To better characterize dynamics-activity and dynamics-stability relationships in proteins, it is necessary to study the molecular flexibility of as many different protein systems as possible. We previously studied activity and molecular dynamics of hBChE from plasma and recombinant hAChE and we suggested possible correlations between their dynamical behavior and their activity [4]. To even better understand the influence of the dynamics, we included now the recombinant *mouse* acetylcholinesterase (mAChE) from the same ChE's family having essentially the same structure. Catalytic activities and stabilities of these three ChEs were analyzed and relationships between dynamics, enzyme activity, thermal stability and ability to form crystals are presented.

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2. Materials and methods

2.1. Enzymes production and purification

hBChE from plasma and recombinant hAChE were purified as previously described in [4,5]. mAChE was expressed in chinese hamster ovary cells (CHO-K1 cells) and was purified as hAChE. ChEs activity measurements were carried out at 25 °C according to the Ellman method [6] using 1 mM butyrylthiocholine (BTC) for BChE and acetylthiocholine (ATC) for AChEs as substrates and 0.5 mM 5–5′-dithio-bis (2-nitrobenzoic acid) (DTNB) in 0.1 M phosphate buffer pH 7.0.

2.2. Sample preparation for neutron scattering

The mAChE sample was prepared following exactly the same protocol as previously described for hBChE and hAChE [4]. Briefly, mAChE was first dialyzed against ammonium acetate dissolved in D_2O (25 mM, pD 7.0) and then freeze dried (12 h) at 220 K under vacuum. The salt free protein powder, placed in an appropriated aluminum sample container, was dried for 12 h at atmospheric pressure over P_2O_5 and weighed. This measured weight was the sample dry weight ($h = 0$ g D_2O/g dry powder, denoted by g/g). For neutron experiments, the sample was hydrated by vapor exchange over D_2O at ambient temperature in a dessicator, until final water content of about 0.4 g/g was achieved, corresponding to at least one full hydration layer at the protein surface. To verify that no loss of material had occurred and that the hydration state was the same, sample was weighed before and after the neutron scattering experiments and no loss was detected.

2.3. Elastic incoherent neutron scattering

All neutron experiments were performed on the IN16 cold neutron backscattering spectrometer [7] at the Institut Laue Langevin (Grenoble/France), but in different years. It is important to note that no essential modifications were undertaken on the instrument since Gabel's first experiment [5]. Thus, mAChE dynamics could be compared to those previously obtained for hAChE and hBChE [4]. We recall that IN16 has an energy resolution of $\Delta E \sim 0.9$ μeV, corresponding to a time window up to ~1 ns.

The scattered elastic intensity can be described within the Gaussian approximation by

$$I_{el}(Q, \omega = 0 \pm \Delta E) \approx I_0 \exp(-\frac{1}{3}\langle u^2 \rangle Q^2) \quad (1)$$

where $\langle u^2 \rangle$ is the average atomic mean square displacement. For $Q \to 0$, the approximation is strictly valid, and it holds up to $\langle u^2 \rangle Q^2 \approx 1$. At elevated temperatures deviation from the linear behavior occurs for higher Q. Therefore the Q-range used in the fitting procedure was restricted to 0.43 $Å^{-1} < Q < 1.06$ $Å^{-1}$ (see Fig. 1).

The MSD can thus be obtained for each temperature by the slope of the semi-logarithmic plot of the incoherent scattering function through

$$\langle u^2 \rangle \approx -3\frac{d \ln I_{el}(Q, \omega = 0 \pm \Delta E)}{dQ^2}. \quad (2)$$

The data treatment, including subtraction of the scattering from the empty sample holder, normalization to the lowest temperature (20 K) and absorption correction based on the correction formula of Paalman-Pings coefficients [8] was performed using the ILL program LAMP [9] as described in [4].

Using the frequency window model of Becker et al. [1], which takes the instrumental resolution explicitly into account, it was possible to fit the MSD according to

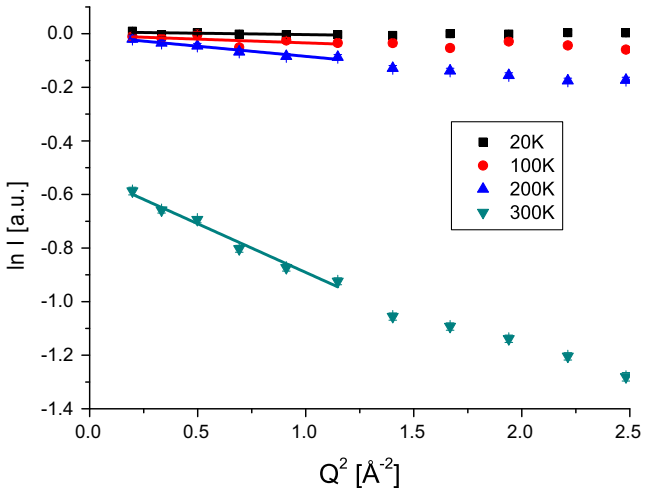

Fig. 1. Logarithm of the normalized intensities as a function of Q^2 for mAChE at IN16 for four representative temperatures (20 K: black squares, 100 K, red circles, 200 K blue triangles, 300 K green diamonds). The full lines correspond to linear fits used to extract the MSD. The data show a deviation from Gaussian behavior at higher temperatures and higher Q values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$$\langle u^2 \rangle_{FWM} = \langle u^2 \rangle_{fast} + \langle u^2 \rangle_{slow}(1 - \frac{2}{\pi}\arctan\frac{\Delta\omega}{\kappa_{FWM}}) \quad (3)$$

where $\langle u^2 \rangle_{fast}$ and $\langle u^2 \rangle_{slow}$ correspond to fast and slow contributions to the mean square displacement, respectively. $\Delta\omega = 7.595 \times 10^8$ s^{-1} is the half-width at half maximum of the elastic instrumental resolution function of IN16 and κ_{FWM} represents the long-time relaxation frequency corresponding to the characteristic time scale of the underlying process. $\langle u^2 \rangle_{fast}$ is assumed to depend linearly on temperature as $\langle u^2 \rangle_{fast} = \alpha T$ and can be obtained by fitting the data at low temperatures with a straight line. $\langle u^2 \rangle_{slow}$ is a fit parameter taking into account slow diffusive processes. Assuming an Arrhenius behavior for κ_{FWM}, it can be expressed as:

$$\kappa_{FWM}(T) = ae^{-E_a/RT} \quad (4)$$

where a is a prefactor, E_a the activation energy, R the ideal gas constant and T the absolute temperature.

2.4. Temperature dependence of substrate hydrolysis

The temperature dependence of hBChE, hAChE and mAChE-catalyzed hydrolysis of BTC or ATC were determined at various temperatures from 7 to 38 °C. Reaction rates (k) were measured at intervals of 1–2 °C, at saturating substrate concentration (1 mM in 0.1 M phosphate buffer pH 7.0 containing 0.1% bovine serum albumin) and 0.17 nM hBChE, 0.17 nM hAChE or 0.10 nM mAChE. All observed reaction rates were corrected for spontaneous substrate hydrolysis. Activation energies ($E_a^{\ddagger}$) and thermodynamic parameters ($\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta G^{\ddagger}$) for substrate hydrolysis were calculated from lnk and lnk/T *vs.* 1/T plots (where T is the absolute temperature).

2.5. Thermal inactivation

Kinetics of ChEs thermal inactivations were studied at different temperatures between 40 and 60 °C. Samples of enzyme (between 1 and 4 nM) were incubated in phosphate buffer 0.1 M pH 7.0 containing 0.1% bovine serum albumin. As ChEs thermal stability depends on protein concentration, bovine serum albumin was added to achieve approximately the same protein concentration as previously used in neutron experiment. Aliquots were with-

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