

Enzyme activity and dynamics: xylanase activity in the absence of fast anharmonic dynamics

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The activity and dynamics of a simple, single subunit enzyme, the xylanase from *Thermotoga maritima* strain Fj SS3B.1 have been measured under similar conditions, from -70 to $+10$ °C. The internal motions of the enzyme, as evidenced by neutron scattering, undergo a sharp transition within this temperature range; they show no evidence for picosecond-timescale anharmonic behaviour (e.g. local diffusive motions or jumps between alternative conformations) at temperatures below

-50 °C, whereas these motions are strongly activated at higher temperatures. The activity follows Arrhenius behaviour over the whole of the temperature range investigated, -70 to $+10$ °C. The results indicate that a temperature range exists over which the enzyme rate-limiting step is independent of fast anharmonic dynamics.

Key words: cryoenzymology, neutron scattering, thermophile.

INTRODUCTION

There is good evidence that most, if not all, enzymes require molecular flexibility for function, as is implicit in the concept of induced fit [1–5]. The most direct evidence is the large conformational changes which crystallographic studies show accompany substrate binding and/or product release in some enzymes [6,7], and on a smaller scale the changes in covalent bond lengths that occur during catalysis [8]. More general evidence arises from a variety of studies of enzymes from extreme thermophiles that show them to be, at any given temperature, less flexible and less active than enzymes from mesophiles [9–13].

Proteins exhibit a variety of internal motions, that cover a wide range of amplitudes (0.01–100 Å) and timescales (10^{-15} to > 1 s) [1,2]. Which of these are essential for enzymatic activity, and how these motions couple to each other is not yet known. While it is evident that many of the motions involved in enzyme flexibility must occur over shorter timescales than those of the enzyme rate-limiting step, the nature of the dynamic changes required for catalysis is unclear: for example, whether they are local or global, fast or slow. One possibility is that fast, small-amplitude fluctuations of single atoms and amino-acid side-chains, on the picosecond (10^{-12} s) and sub-Angstrom time and length scales, are essential to other motions that occur on longer length scales, such as relative motions of domains, and on physiological timescales. The shorter motions may therefore be coupled to the longer ones, serving as the ‘lubricant’ that makes the latter possible. This idea is consistent with results that indicate that the temperature dependence of the fast hydrogen mean-square displacements in hydrated myoglobin, as obtained by neutron scattering, resembles that derived by Mossbauer spectroscopy, which detects motions of the iron atom in the haem group on slower timescales down to 10^{-7} s. Both techniques provided evidence for a dynamical transition in myoglobin, at about -50 to -70 °C [14–16]. Below this, the protein is relatively solid-like, exhibiting only vibrational, harmonic

motion, and above this more liquid-like, anharmonic motion is seen, indicating increased flexibility of the protein. The anharmonic motion involves the activation of non-vibrational random motion, such as jumps between closely-related conformations or diffusive motion of groups of atoms in effective cages [17]. The similarity in the temperature dependence obtained with the two techniques lead to the suggestion that the picosecond fluctuations observed with neutron scattering could couple to the haem group, and were likely to be an important factor in the slower motions resolved in the Mossbauer spectrum [14].

The dynamical transition has now been observed with a number of biophysical techniques [14–16,18–22], and some protein functions including ligand binding have been shown to cease at temperatures below the transition [21,23,24]. The implication of this is that flexibility at the active site is required for efficient substrate binding and/or protein function. This, and the interdependence of the fast and slow motions, is consistent with the idea that the fast anharmonic motions are required for enzyme activity. If this were the case we might expect that an enzyme would not exhibit catalytic activity below the transition temperature, where these motions are absent. However, it has recently been shown that glutamate dehydrogenase, a large multisubunit enzyme, catalysing a complex reaction, is active below the dynamical transition (as evidenced by the cessation of picosecond timescale anharmonic motions) at -50 °C [22]. However, it is not clear whether this applies to all enzymes, and in particular whether it applies to simple, single-subunit enzymes, for which intersubunit motions play no role. In the present work this question is addressed. We report here the result of the concomitant determination of the activity and dynamics of a single-subunit enzyme, a xylanase, catalysing a relatively simple hydrolytic reaction. The activity determinations were carried out over the temperature range of $+10$ to -70 °C, in a 70% (v/v) methanol cryosolvent. The dynamics were determined in the same solvent by elastic neutron scattering, giving the average dynamical mean square displacement in the solution over time-

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scales of up to approx. 100 ps. The results thus enable the direct correlation of xylanase activity with fast dynamics over a wide temperature range. It is found that a temperature range exists in which the enzyme rate-limiting step is independent of the fast anharmonic dynamics.

EXPERIMENTAL

Materials

The xylanase was obtained from an *Escherichia coli* clone containing the gene from the extremely thermophilic bacterium, *Thermotoga maritima* strain FjSS3-B.1 [25,26]. The *E. coli* clone was grown on Luria broth media, with 50 mg/l ampicillin for the initial inoculum, at pH 7.0. It was grown as a batch-fed culture, in a 10 l fermenter at 28 °C. Xylanase production was induced at the end of the logarithmic growth phase by raising the temperature to 38 °C. Further nutrients were also added at the time of induction. The cells were then harvested at the end of the second logarithmic growth phase (approx. 12–18 h after induction). The xylanase was purified as described by Bergquist et al. [26]. The cells were concentrated by hollow fibre filtration, collected by centrifugation at 15000 g and washed, re-centrifuged, and re-suspended in Bis-Tris buffer at pH 7.0. The solution was then taken through two freeze-thaw cycles to help lyse the cells, followed by sonication for 15 min, on ice. The cell debris was removed by centrifugation at 15000 g for 30 min. DNase is added to the supernatant, and the solution stirred for 3 h at 37 °C. The supernatant was heat treated at 75 °C for 30 min, and allowed to stand at 4 °C for 2–3 days, to ensure complete precipitation of the denatured protein. The resulting precipitate was removed by centrifugation at 15000 g for 60 min. Ammonium sulphate was then added to a concentration of 1 M. The protein solution was loaded onto Phenyl Sepharose, and the xylanase eluted by a single-step gradient to water. The ammonium sulphate was removed from the eluted xylanase by filtration on an Amicon ultrafiltration cell, with a YM3 membrane and equilibrated with Bis-Tris buffer at pH 6.7 to a salt concentration of approx. 20 mM in the same ultrafiltration cell. The protein solution was then loaded on to Fast Flow Q-Sepharose, and eluted with 150 mM NaCl in Bis-Tris buffer at pH 6.7. The eluted xylanase was then dialysed against 10 mM Mes buffer at pH 5.3, loaded on to high load S-Sepharose column, and eluted with a linear gradient of 1–100 mM NaCl. From a 10 l fermenter culture, approx. 100 mg of purified xylanase was obtained.

Assays

Xylanase activity was routinely assayed by measuring the release of nitrophenol from *o*-nitrophenyl- β -D-xyloside at 80 °C, for 10 min. The reaction mixture contained 10 mM *o*-nitrophenyl- β -D-xyloside, 0.1 M sodium acetate/acetic acid buffer, pH₈₀ 5.5, and 0.14–5 μ g of enzyme, in a total assay volume of 100 μ l. The reaction was stopped by the addition of 500 μ l of ice-cold 1 M sodium carbonate, and the absorbance at 420 nm read.

In the cryosolvent, 70% (v/v) methanol, assays were carried out at temperatures of 10 °C and below. Sodium acetate/acetic acid buffers, giving a final pK_{aH} of approx. 5.4 at the assay temperature, were used [27]. The total assay volume was 100 μ l, with the enzyme solution and 0.1 M sodium acetate/acetic acid buffer making up 30% of this. The substrate, *o*-nitrophenyl- β -D-xyloside, was dissolved in the miscible organic solvent. The assays were started by the addition of the enzyme, cooled to the assay temperature. For assay temperatures below 0 °C, the enzyme was dissolved in the cryosolvent to prevent freezing upon addition to the cooled assay solution.

The reaction was stopped by the addition of 500 μ l of precooled ethanolamine/DMSO/water (3:5:2; by vol.). At temperatures between 0 °C and –50 °C, the assays were cooled to at least 10 °C below the assay temperature before the addition of the stopping reagent. For assays at –50 °C and below, the assay tubes were immersed in liquid nitrogen to ensure efficient stopping of activity. The assay tubes were allowed to gradually warm, after thorough mixing with the stopping reagent below the assay temperature. The absorbance at 420 nm was read once the assays had warmed to room temperature. Control measurements were made in the absence of enzyme to account for any background absorbance of the substrate and solvents.

For the corresponding deuterated solvent assays, deuterium oxide replaced water, and CD₃OD replaced CH₃OH, throughout. The pD of the buffer solution was checked with a conventional pH meter, calibrated with aqueous buffers, using the relationship pD = pH_(meter) + 0.4 units [28].

For assay temperatures above –60 °C, a methanol bath cooled by a Flexi Cool Immersion Cooler (FTS Systems) was used. For –70 °C an ultra deep freeze was used to incubate the assays.

Neutron scattering

The dynamic neutron scattering measurements were carried out on the IN6 time-of-flight spectrometer at the Institut Laue-Langevin, Grenoble. The energy resolution of IN6 is 50 μ eV, which allows determination of the average dynamical mean-square displacements of the enzyme protons over timescales of up to approx. 100 ps. To minimize the contribution of the solvent to the scattering, fully deuterated solvents were used. Exchange of labile enzyme protons was carried out by twice dissolving the xylanase in warm D₂O and freeze-drying to ensure no exchange took place during neutron scattering. The samples were contained in aluminium flat plate cells of 0.7 mm thickness, and were oriented at 135° with respect to the incident neutron beam. The incident neutron wavelength was 5.12 Å. The sample consisted of 68 mg/ml xylanase in 70% (v/v) CD₃OD/D₂O cryosolvent.

The sample was cooled to –163 °C and then heated progressively, with elastic scan data measurements taken at temperature intervals of 10° or 20° up to 30 °C. The measured transmission was 0.891. Raw data were corrected to determine the elastic intensity by integrating the detector counts over the energy range of the instrumental resolution. The detectors were calibrated by normalizing with respect to a standard vanadium sample. The cell scattering was subtracted, taking into account the attenuation of the singly scattered beam. Finally, the scattering was normalized with respect to the scattering at the lowest measured temperature, –163 °C.

The measured scattering contains a contribution from the protein hydrogen atoms, and also a contribution of similar magnitude from self-coherent scattering from the solvent. The mean-square displacement derived is therefore approximately equivalent to averaging over the elastic scattering from both the solvent and protein. The incoherent scattering from hydrogen atoms, due to self-correlations in their dynamics, is a significant component of the neutron scattering from proteins. As the hydrogen atoms are uniformly distributed over the macromolecule, the technique gives a global view of protein dynamics [29]. The elastic scattering intensity $S(q, \omega = 0)$ (where q is the magnitude of the scattering wave vector, and ω is the energy transfer) is then used to obtain the average mean square displacement $\langle u^2 \rangle$, using the relationship $\ln S(q, \omega = 0) = -\langle u^2 \rangle q^2/3$, which is valid in the regime $q^2 \langle u^2 \rangle / 3 < 1$. $\langle u^2 \rangle$ was thus obtained by fitting a straight line to a semi-log plot of $S(q, \omega = 0)$

versus q^2 in the linear regime which was found at $0.12 < q^2 < 1.06$. As the scattering is normalised with respect to the -163 °C intensities, the $\langle u^2 \rangle$ determined is equal to $(\langle u^2 \rangle_T - \langle u^2 \rangle_{-163})$, where $\langle u^2 \rangle_T$ is the absolute mean-square displacement at temperature T . The $\langle u^2 \rangle$ obtained by dynamic neutron scattering is purely dynamical and, unlike crystallographic atomic displacements, does not contain a static disorder contribution.

RESULTS AND DISCUSSION

To ensure valid measurement of activity at low temperatures in cryosolvent, a number of preliminary experiments were carried out. The stability of the xylanase in the 70% methanol cryosolvent was determined by incubation in the cryosolvent at 0 °C over a 12-h period, with aliquots removed and assayed at various time intervals. The remaining activity, assayed at 80 °C in an aqueous buffer, showed no significant decrease over the 12-h period, and was the same as that obtained for xylanase not exposed to the cryosolvent.

To ensure that substrate did not become limiting, K_m and V_{max} determinations were carried out in cryosolvent at temperatures down to -30 °C. As found for other enzymes [30] the K_m decreased with temperature (results not shown).

Figure 1 shows the effect of temperature on xylanase activity in both hydrogenated and fully deuterated cryosolvent, over the temperature range of $+10$ to -70 °C. From the similarity of the data, it does not appear that the catalytic activity of xylanase is significantly affected by deuterated solvents. The Arrhenius plots are essentially linear for both the hydrogenated and deuterated cryosolvents. This indicates that the rate-limiting step remains the same, and suggests that the catalytic mechanism is unchanged, as the temperature is lowered. As the temperature is reduced from 0 °C to -70 °C the activity declines by about 5 orders of magnitude. In comparison, the viscosity of the cryosolvent declines by less than 2 orders of magnitude. As the diffusion coefficient of a solute is inversely proportional to the viscosity of the solution, this implies that the activity of the xylanase is not likely to be diffusion limited at any temperature studied here.

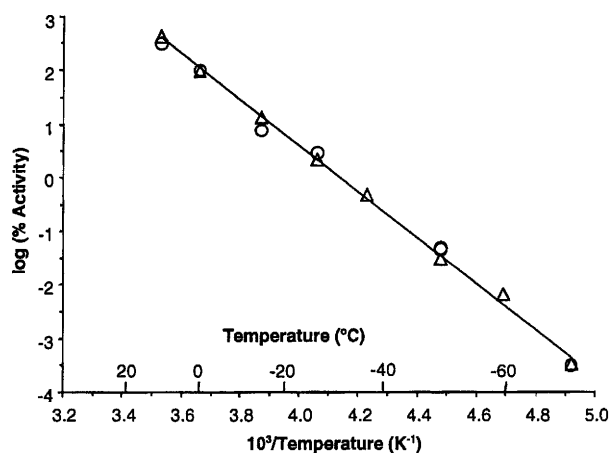


Figure 1 Effect of temperature on the activity of a xylanase enzyme in 70% aqueous methanol

The activity is expressed as the percentage residual activity corresponding to the activity in 70% methanol at 0 °C in hydrogenated (Δ) and fully deuterated (\circ) solvents. 100% activity corresponds to 0.57 and 0.62 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in the hydrogenated and deuterated solvent, respectively.

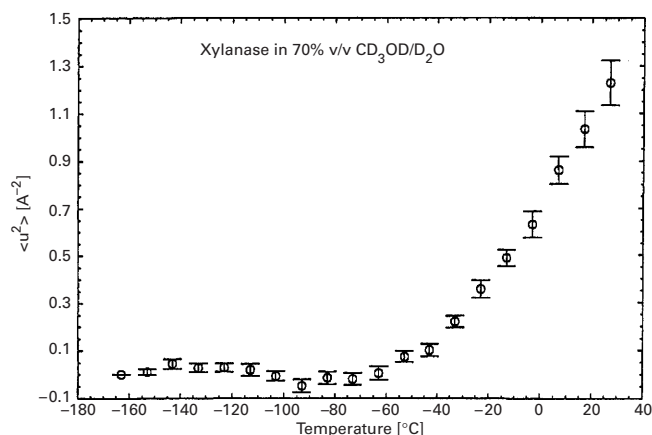


Figure 2 Effect of temperature on the dynamics of a xylanase enzyme in fully deuterated 70% aqueous methanol, as measured by neutron scattering

The dynamic results from the neutron scattering study of xylanase are shown in Figure 2, expressed as $\langle u^2 \rangle$ as a function of temperature. The $\langle u^2 \rangle$ values are scaled to zero at $T = -163$ °C, the lowest temperature used in the experiment. For $T \leq -70$ °C, $\langle u^2 \rangle$ remains at zero within experimental error: any small increase in $\langle u^2 \rangle$ over this range due to the increase with temperature of the amplitude of the harmonic vibrations is likely to be below the experimental error. At approx. -50 °C there is a dynamical transition, at which $\langle u^2 \rangle$ begins to rise rapidly, indicating the activation of anharmonic dynamics. In comparison, in the activity data there is no corresponding transition, the Arrhenius plot remaining linear down to at least $T = -70$ °C.

The results in Figures 1 and 2 throw light on the relationship between fast, picosecond motions, and enzyme activity. The slowest step in the catalytic cycle of the xylanase is the rate-limiting step, and can be expressed as the reciprocal of the turnover number. For the xylanase, the timescale of the rate-limiting step is approx. 1 s at 0 °C and this increases by at least 3 orders of magnitude at -50 °C. The mechanism of xylanase action involves multiple steps [31], including rapid equilibration of intermediates, and the crossing of each associated energy barrier may require one or more dynamical modes on the seconds/milliseconds timescale, at -50 °C. This is several orders of magnitude slower than the 100 ps dynamic motions being observed by neutron scattering. The absence of a transition in the activity data corresponding to the observed transition in the dynamic data at -50 °C shows that over the temperature range -50 to -70 °C picosecond anharmonic dynamics are not required for activity (whether this applies at higher temperatures is an open question). Therefore, anharmonic fast motions are not necessarily coupled to the conformational transitions involved in the enzyme reaction, i.e. to the much slower motions required for barrier-crossing transitions along the enzyme reaction coordinate. However, this conclusion should be tempered by consideration of the experimental error in $\langle u^2 \rangle$ in Figure 2, which is of the order of 0.05 Å². As $\langle u^2 \rangle$ obtained by the neutron technique is averaged over the sample, it is conceivable that functionally important fast anharmonic motions may be activated locally at the active site, at levels below the noise in Figure 2.

The xylanase used in this work is a very stable enzyme from an extreme thermophile [26,32], chosen to ensure stability in the

cryosolvent. There is no indication that deuterated or non-deuterated cryosolvent has a significant effect on the xylanase activity or stability. While it is not possible to compare the dynamics of the enzyme in cryosolvent with that in aqueous buffer below 0 °C, its dynamic behaviour as evidenced by neutron scattering is similar in a variety of different cryosolvents below 0 °C, including 40% methanol and 40 and 80% DMSO (R. V. Dunn, V. Réat, J. Finney, M. Ferrand, J. C. Smith and R. M. Daniel, unpublished work). The enzyme itself is not atypical [26], and there is already evidence that thermophilic and mesophilic enzymes behave similarly at their respective optimum temperatures in terms of their global dynamics and cryoenzymology [22,30]. Similar results to those obtained for xylanase have been observed for the multi-subunit protein glutamate dehydrogenase. That is, Arrhenius plots showed no break through the temperature of the picosecond dynamic transition [22,30]. The xylanase is a single subunit enzyme that catalyses a simple hydrolytic reaction, whereas the glutamate dehydrogenase is a multi-subunit enzyme that catalyses a complex multi-substrate reaction. The observation that these two dissimilar enzymes both show an independence of anharmonic picosecond dynamics for activity suggests that this finding may be applicable to enzymes generally. This would be consistent with the finding that the activity of two other multi-subunit enzymes, calf intestinal alkaline phosphatase, and beef liver catalase, have been measured down to approx. -100 °C (J. Bragger, R. V. Dunn and R. M. Daniel, unpublished work), without any Arrhenius plot break, although the corresponding dynamical measurements for these enzymes have not yet been performed.

Recent dynamic measurements on glutamate dehydrogenase over slower, nanosecond, timescales have shown dynamical transitions occurring at significantly lower temperatures than observed here, leading to the suggestion that the temperature of the dynamic transition is dependant on the timescale of the experimental technique [33]. As a corollary, it is confirmation of the idea that the anharmonic behaviour of an enzyme is timescale-dependent, and that anharmonic motions on timescales slower than picoseconds (e.g. functionally important dynamics) can occur in the absence of anharmonic picosecond motions. This latest finding has not yet been probed with other enzymes, but suggests that a way forward to understanding the relationship between enzyme dynamics and activity is to measure dynamical transition behaviour on a variety of timescales, and, as in the present work, to compare the results with activity determinations made under similar conditions. A variety of techniques exist for probing longer-timescale protein dynamics [1], including NMR, Mossbauer and fluorescence spectroscopies, neutron spin-echo [34] and molecular dynamics simulation, the latter technique now being feasible up to the microsecond timescale [35]. Although each of these techniques presents particular difficulties, overall prospects for examining enzyme motions on longer timescales are encouraging.

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