Enzyme Activity below the Dynamical Transition at 220 K

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ABSTRACT Enzyme activity requires the activation of anharmonic motions, such as jumps between potential energy wells. However, in general, the forms and time scales of the functionally important anharmonic dynamics coupled to motion along the reaction coordinate remain to be determined. In particular, the question arises whether the temperature-dependent dynamical transition from harmonic to anharmonic motion in proteins, which has been observed experimentally and using molecular dynamics simulation, involves the activation of motions required for enzyme function. Here we present parallel measurements of the activity and dynamics of a cryosolution of glutamate dehydrogenase as a function of temperature. The dynamical atomic fluctuations faster than ~100 ps were determined using neutron scattering. The results show that the enzyme remains active below the dynamical transition observed at ~220 K, i.e., at temperatures where no anharmonic motion is detected. Furthermore, the activity shows no significant deviation from Arrhenius behavior down to 190 K. The results indicate that the observed transition in the enzyme's dynamics is decoupled from the rate-limiting step along the reaction coordinate.

INTRODUCTION

It is widely accepted that enzymes require internal flexibility for catalytic activity (e.g.,; Frauenfelder et al., 1979; Artymiuk et al., 1979; Karplus and Petsko, 1990; Gerstein et al., 1994); the concept of induced fit, for example, is based on this idea (Yankeelov and Koshland, 1965). However, the time scales and forms of the functionally important atomic motions remain poorly understood. Measurements using a number of methods have revealed a temperature-dependent transition in equilibrium dynamical fluctuations of several proteins that is observed at ~200–220 K (Cohen et al., 1981; Knapp et al., 1982; Doster et al., 1989; Rasmussen et al., 1992; Daniel et al., 1996). At temperatures below the transition, the motions are mostly vibrational, whereas above the transition anharmonic dynamics are observed, involving continuous or jump diffusion (Elber and Karplus, 1989; Kneller and Smith, 1994). Correlations have been observed between protein function, such as ligand binding or proton pumping, and the presence of equilibrium anharmonic motion (Frauenfelder et al., 1991; Rasmussen et al., 1992; Ferrand et al., 1993). The increased flexibility accompanying anharmonic dynamics may allow proteins to rearrange their structures to achieve functional configurations.

Enzyme activity involves barrier crossing and therefore anharmonic motion along the reaction coordinate. However, to fully understand enzyme activity it is necessary to determine which other motions in the protein and surrounding solvent are coupled to or required for progression along the reaction coordinate. One way of addressing this problem is to specifically compare enzyme catalytic activity with direct measurements of dynamical amplitudes and time scales. The existence of the temperature-dependent dynamical transition, below which fluctuations in proteins are much reduced (Cohen et al., 1981; Knapp et al., 1982; Doster et al., 1989; Rasmussen et al., 1992), offers the possibility of probing the relationship between enzyme activity and dynamics. However, the low temperature of the dynamical transition requires the use of enzymes capable of functioning in cryosolvents over a wide temperature range (Jaenicke and Zavodsky, 1990; Daniel et al., 1996). We have therefore performed parallel dynamic neutron scattering and activity measurements on a glutamate dehydrogenase (GDH) enzyme in a cryosolvent consisting of 70% methanol and water. The use of this cryosolvent permits activity measurements to be made at temperatures below those at which the dynamical transition has previously been observed in proteins (Douzou et al., 1979; Hudson and Daniel, 1995).

Dynamic neutron scattering affords a means of directly probing atomic fluctuations in protein systems (Lovesey, 1984; Cusack et al., 1988; Smith, 1991). By measuring the exchange of energy and momentum between the neutrons and the sample, information can be obtained simultaneously on the time and length scales of atomic motions. In the present work the dynamical information was obtained via the elastic scattering, which can be used to derive the average dynamical mean square displacement, $\langle u^2 \rangle$, in the sample (see Materials and Methods). $\langle u^2 \rangle$ contains contributions from all motions resolvable by the instrument used. In the present work measurements were made using the IN6 spectrometer at the Institut Laue-Langevin reactor in Grenoble. The energy resolution of IN6 is 50 μeV. Consequently, only motions faster than ~100 ps contribute to the $\langle u^2 \rangle$ determined using the instrument. The neutron experi-

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ment therefore permits determination of the dynamical transition temperature for motions on the sub-~100-ps time scale. When combined with the activity measurements, the results of the neutron experiment allow information to be obtained on the role of fast motions in protein function. The work presented here is the first concomitant determination of activity and dynamics of an enzyme through a dynamical transition.

MATERIALS AND METHODS

The glutamate dehydrogenase was from the thermophilic *Thermococcus* strain AN1 and was purified and assayed as described elsewhere (Hudson et al., 1993; More et al., 1995). Activity measurements on this enzyme gave results similar to those obtained for other members of the glutamate dehydrogenase family (Hudson et al., 1993; Hudson and Daniel, 1993, 1995; More et al., 1995). To minimize the contribution to the measured scattering from the solvent, the neutron experiments were performed with perdeuterated solvent and, to exchange the labile enzyme protons, the enzyme was twice dissolved in D_2O and freeze-dried.

The dynamic neutron scattering measurements were performed on the IN6 time-of-flight spectrometer at the Institut Laue-Langevin, Grenoble. The incident neutron wavelength was 5.12 Å. All data were collected with the sample holder oriented at 135° relative to the incident beam. The sample was contained in aluminum flat-plate cells of 0.3 mm thickness. The sample consisted of 100 mg ml^{-1} of enzyme in 70% MeOD/D_2O solvent. It was cooled to 80 K and then heated progressively to 320 K over 16–24 h. The measured transmission was 0.917. 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 attenuation of the singly scattered beam. Finally, the scattering was normalized with respect to the scattering at the lowest measured temperature, 80 K.

The measured signal contains a contribution from incoherent scattering from the protein hydrogen atoms, due to self-correlations in their dynamics. As the hydrogens are approximately uniformly distributed, the technique gives a global view of equilibrium protein dynamics (Smith, 1991). The scattering also contains contribution of a similar magnitude from self-coherent scattering from the solvent. The mean square displacement derived is approximately equivalent to that obtained from averaging over the elastic scattering from the solvent and protein. 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 $\langle u^2 \rangle$, using the relationship $\ln S(q, \omega = 0) = -\langle u^2 \rangle q^2/3$, which is valid in the regime $q^2\langle u^2 \rangle/3 < 1$ (19). $\langle u^2 \rangle$ was thus obtained by fitting a straight line to a semilog plot of $S(q, \omega = 0)$ versus q^2 in the linear regime. The linear regime was found at $0.12\ Å^{-2} < q^2 < 1.07\ Å^{-2}$ and was well separated from the Bragg scattering which was found at $1.4\ Å^{-1} < q < 2.0\ Å^{-1}$. No evidence was found for a low-q protein-protein interaction coherent scattering peak. As the scattering was normalized with respect to the intensities measured at 80 K, the $\langle u^2 \rangle$ determined is equal to $(\langle u^2 \rangle_T - \langle u^2 \rangle_{80})$, 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 displacement parameters, does not contain a static disorder contribution. The $\langle u^2 \rangle$ can be compared with crystallographic heavy-atom atomic displacement parameters. The present experiments are performed in solution and thus contain a significant component of rigid-molecule translational and rotational diffusion that will be significantly reduced in the crystalline state. Moreover, the hydrogen and deuterium atoms probed here are lighter than the heavy atoms seen by x-ray crystallography, leading to larger average displacements. The internal flexibility of parts of a protein in a crystal may also be reduced relative to that found in solution.

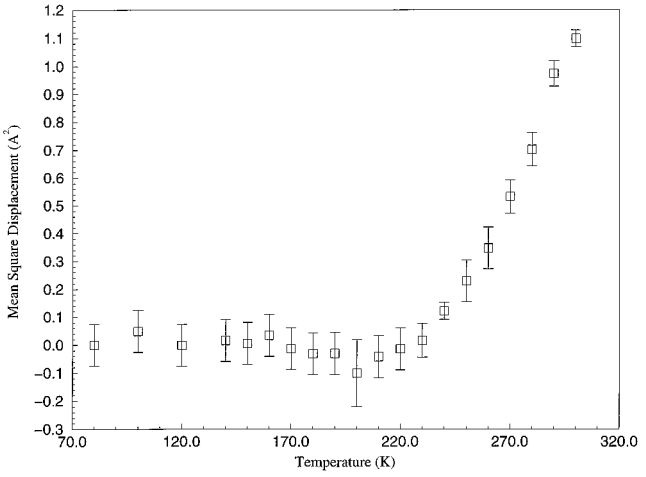

FIGURE 1 Effect of temperature on the dynamics of a glutamate dehydrogenase enzyme in 70% aqueous methanol, as measured by neutron scattering.

RESULTS AND DISCUSSION

Fig. 1 shows $\langle u^2 \rangle$ as a function of the temperature, T, determined using the neutron spectrometer. The $\langle u^2 \rangle$ values are scaled to zero at $T = 80$ K, the lowest temperature in our experiments. For $T \leq 200$ K, $\langle u^2 \rangle$ remains at zero within experimental error; the very small amplitude vibrational motion ($<0.05\ Å^2$) is not detectable in the data. At ~220 K there is a dynamical transition, at which $\langle u^2 \rangle$ begins to rise rapidly, indicating the activation of anharmonic dynamics. The dynamical origin of this transition in the scattering was confirmed by the observation of the simultaneous activation of quasielastic scattering from the sample. GDH activity data in the same system are presented in Fig. 2 (More et al., 1995). There is no apparent deviation from Arrhenius behavior over the whole temperature range (190–350 K) for which activity was determined. In particular, no change in slope is seen in the activity plot that could correlate with the

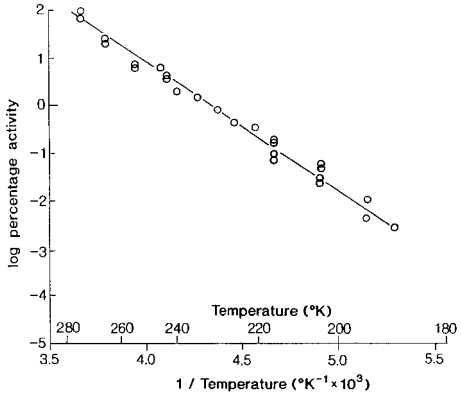

FIGURE 2 Effect of temperature on the activity of a glutamate dehydrogenase enzyme in 70% aqueous methanol. Activities are presented as a percentage of the activity at 273 K (from More et al., 1995). Measurements at selected temperatures in perdeuterated cryosolvent showed no significant deviation from the results in Fig. 2.

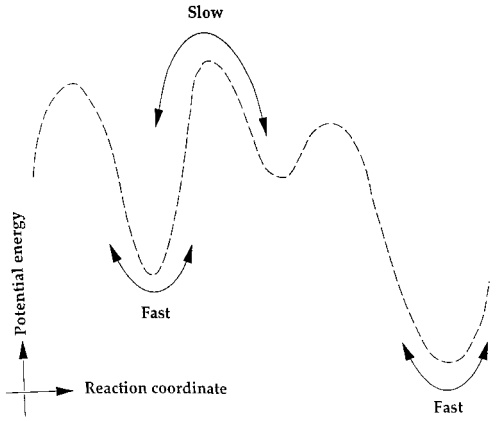

FIGURE 3 Schematic representation of the potential energy profile along an enzyme reaction coordinate.

dynamical transition shown in Fig. 1. These results therefore assert that the dynamical transition observed over the present time scale (<100 ps) is not directly related to the onset of enzyme activity.

The results in Figs. 1 and 2 shed light on the relationship between enzyme activity and equilibrium fluctuations on the measured time scale. The activity of GDH is not diffusion controlled at physiological temperatures (Hudson and Daniel, 1995), and the viscosity increase of the cryosolvent down to 190 K is small compared with the activity decrease (Douzou et al., 1976; More et al., 1995), i.e., at all temperatures examined in this work it is barrier-crossing events inside the enzyme that are rate limiting. Fig. 3 shows a schematic representation of the potential energy profile along a reaction coordinate in an enzyme. The profile consists of a series of wells and barriers. The highest barrier-crossing event, which corresponds to the rate-limiting step, takes place on a time scale that is the reciprocal of the turnover number. For the GDH sample the turnover number varies between 0.1 s^{-1} at 180 K and 10^4 s^{-1} at 300 K. The present experiments explore equilibrium fluctuations on much faster time scales, <100 ps. These fluctuations contain components along the reaction coordinate that are mostly confined to regions associated with the bottoms of the wells. We address here the question of whether these fast fluctuations need to be anharmonic for the slower, barrier-crossing catalytic events to exist.

There is measurable enzyme activity down to 190 K, i.e., significantly below the 220 K dynamical transition in Fig. 1. This demonstrates that, at least in the case of GDH between 190 K and 220 K, the existence of the slower, rate-limiting barrier-crossing dynamics that determine enzyme activity does not require the anharmonic motion activated in the protein solution on the <100-ps time scale in Fig. 1. Moreover, the absence of an observed deviation of the activity from Arrhenius behavior in response to the qualitative change in the dynamics of the enzyme at ~220 K indicates that the rate-limiting barrier-crossing kinetics are effectively decoupled from the observed dynamical transition.

Anharmonic motions must be activated to permit GDH activity down to 190 K. These motions are likely to be slower than those detected here. However, we cannot exclude the possibility that a small proportion of the measured scattering below the dynamical transition may originate from anharmonic, functionally important picosecond motions, but these would be below the noise level of the data in Fig. 1. Our measurements do show that, averaged over the molecule, picosecond time scale motions below 220 K need not be anharmonic for activity. Further experiments are required to determine whether this result is a general property of enzymes and to construct a general picture of the relationships between enzyme activity and dynamical transitions. In particular, there is a need to further characterize the forms, amplitudes, and time evolution of motions in active enzymes if we are to fully unravel the dynamic component of their biological activity. This is currently being pursued with a range of spectroscopic, diffraction, and simulation techniques.

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