Enzyme Dynamics and Activity: Time-Scale Dependence of Dynamical Transitions in Glutamate Dehydrogenase Solution

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ABSTRACT We have examined the temperature dependence of motions in a cryosolution of the enzyme glutamate dehydrogenase (GDH) and compared these with activity. Dynamic neutron scattering was performed with two instruments of different energy resolution, permitting the separate determination of the average dynamical mean square displacements on the sub-~100 ps and sub-~5 ns time scales. The results demonstrate a marked dependence on the time scale of the temperature profile of the mean square displacement. The lowest temperature at which anharmonic motion is observed is heavily dependent on the time window of the instrument used to observe the dynamics. Several dynamical transitions (inflexions of the mean squared displacement) are observed in the slower dynamics. Comparison with the temperature profile of the activity of the enzyme in the same solvent reveals dynamical transitions that have no effect on GDH function.

INTRODUCTION

It is generally accepted that enzymes require internal flexibility for catalytic activity (e.g., Frauenfelder, 1989). However, which motions are required is not yet well understood. Of particular interest is the role in activity of the fast (e.g., picosecond and nanosecond time scale) structural fluctuations that are probed by molecular dynamics simulations. An important question is whether these fast motions are coupled to the structural changes associated with the catalytic rate-limiting step, which itself may take place on time scales several orders of magnitude slower. One important question that can be and has been addressed is whether the fast motions need to be anharmonic for protein function. This might be the case if, for example, picosecond-nanosecond motions involve rearrangements of the protein that are required to permit slow dynamics across the highest-energy reaction barrier.

In this context it is of interest that a variety of techniques have shown a temperature-dependent transition in the dynamic behavior of hydrated proteins at ~200–220K, involving cessation of anharmonic motion below this temperature. Much of this work has been done on myoglobin with Mossbauer spectroscopy (Keller and Debrunner, 1980; Parak et al., 1982; Knapp et al., 1982), neutron scattering (Doster et al., 1989; Cusack and Doster, 1990), or x-ray crystallography (Frauenfelder et al., 1979) of hydrated crystals, powders, or frozen solutions, but similar results have been found in x-ray crystallographic studies of ribonuclease A (Tilton et al., 1992) and in Mossbauer (Parak et al., 1980) and neutron scattering (Ferrand et al., 1993) studies of membrane proteins. Some protein functions have been observed to cease with the loss of equilibrium anharmonic dynamics as the protein is cooled through the dynamic transition. Among these are electron tunneling in *Rhodospirullum rubrum* chromatophores (Parak et al., 1980), some elements of the photocycle of bacteriorhodopsin in hydrated membranes of *Halobacterium salinarum* (Ferrand et al., 1993), and ligand binding/release in ribonuclease A crystals (Rassmussen et al., 1992).

Motions in proteins are known to exist over a range of time scales. An important remaining issue then is the time scale dependence of the dynamical transition. Whether there is a time scale dependence depends on the nature of the underlying potential surface. The first parallel studies of enzyme activity and dynamics, using glutamate dehydrogenase (GDH) in a cryosolution, probed picosecond time scale motions. The results showed no deviation from Arrhenius behavior through the dynamical transition (Daniel et al., 1998). These results indicate that there is a range of temperatures (190–220K) at which the enzyme rate-limiting step does not require, and is not affected by, anharmonic motions taking place on the picosecond time scale.

To further address the time scale problem we have performed here a study comparing the temperature dependence of the activity of GDH with the dynamics of the enzyme solution on two different time scales, determined by neutron scattering. The experiments make use of the different energy resolutions of two neutron scattering spectrometers: IN16 and IN6 at the Institut Laue-Langevin reactor in Grenoble. Whereas IN6 probes motions on time scales faster than ~100 ps, those detected by IN16 extend to ~5 ns. The experiments were performed over a temperature range extending through the previously observed dynamic transition, in a 70% v/v methanol/water cryosolvent in which the enzyme is active and stable.

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Glutamate dehydrogenase is a key enzyme of central metabolism. It catalyzes the reversible conversion of 2-ketoglutarate, ammonia, and reduced nicotinamide adenine dinucleotide phosphate (NADP) to glutamate and oxidized NADP. The reaction is complex and has multiple steps. Kinetic analysis of this enzyme (Hudson and Daniel, 1995) suggests the reaction proceeds by a sequential, steady-state, random mechanism. At the growth temperature of the source organism, *Thermococcus zilligii* strain AN1, ~350K, the turnover number of the enzyme is ~1500 s^{-1}. In fully deuterated cryosolvent at 220K the turnover number is ~0.01 s^{-1}. For comparison, the turnover number of lysozyme at room temperature is 0.5 s^{-1}. The turnover number of course applies to the rate-limiting step, and given the complexity of the reaction and the necessary rapid equilibration of intermediates, some reaction steps will be very much faster.

The results demonstrate a marked dependence on time scale of the temperature profile of the mean square displacements. The dynamical transitions occurring within the temperature range for which enzyme activity was measured have no significant effect on function.

MATERIALS AND METHODS

The glutamate dehydrogenase is from *Thermococcus* strain AN1 (now known as *T. zilligii* strain AN1) (DSM 2770) and was purified, assayed, and prepared for neutron scattering as described elsewhere (Hudson et al., 1993; More et al., 1996; Daniel et al., 1998). Temperature-activity determinations were carried out in hydrogenated cryosolvent: isolated determinations carried out in the perdeuterated (completely deuterated) cryosolvent used for the neutron scattering experiments, at various temperatures, showed that Arrhenius behavior (apparent activation energy) was unaffected. At the completion of each neutron scattering experiment the activity was assayed to ensure that no denaturation had occurred.

The dynamic neutron scattering measurements were performed on the IN6 time-of-flight spectrometer and on the IN16 backscattering spectrometer at the Institut Laue-Langevin, Grenoble. The incident neutron wavelengths were 5.12 Å on IN6 and 6.28 Å on IN16. All data were collected with the sample holder oriented at 135° relative to the incident beam. The samples were contained in aluminum flat-plate cells, of 0.3-mm and 0.5-mm path lengths on IN6 and IN16 respectively.

Two samples were run: 1) on IN6, 100 mg ml^{-1} of enzyme in 70% v/v CD_3OD/D_2O solvent, and 2) on IN16, 56 mg ml^{-1} of enzyme in 70% v/v CD_3OD/D_2O solvent. The samples were cooled to 80K then heated progressively to 320K over 16–24 h. The measured transmissions were 0.92 for the IN6 sample and 0.85 for the IN16 sample. Raw data on the two instruments were corrected in identical fashion. The elastic intensity was determined 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, 80K, and to the lowest wavevector, q.

The elastic incoherent scattering intensity $S_{inc}(q, \omega = 0)$ (where q is the magnitude of the scattering wave vector and ω is the energy transfer) was used to obtain $\langle u^2 \rangle$ by using the relationship $\ln S_{inc}(q, \omega = 0) = -\langle u^2 \rangle q^2/3$, 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 semilog plot of $S(q, \omega = 0)$ versus q^2 in the linear regime, which was found at $0.12\ Å^{-2} < q^2 < 1.07\ Å^{-2}$ and $0.10\ Å^{-2} < q^2 < 1.13\ Å^{-2}$ in the IN6 and IN16 experiments, respectively. The linear regime was found to be well separated from the Bragg scattering of the solution, which was found at $1.4\ Å^{-1} < q < 2.0\ Å^{-1}$, and no evidence was found for a low-q protein-protein interaction peak. As the scattering was normalized with respect to the 80K intensities, 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. In practice, the measured $\langle u^2 \rangle$ corresponds to the H atoms, the scattering cross section of which is strongly dominant. For the samples used on IN16 and IN6, 70% and 80% of the incoherent signal are due to the enzyme, respectively. The $\langle u^2 \rangle$ values obtained for these samples are therefore dominated by the enzyme motions. The energy resolution of IN16 is 1 μeV, whereas that of IN6 is 50 μeV. The inverses of these energy resolutions correspond to times of 5 ns and 100 ps, respectively.

The quasielastic scattering experiment was run on IN16, using 60 mg ml^{-1} of enzyme in 70% v/v CD_3OD/D_2O solvent. Data were collected during heating from 80K, using the same proceedure as for the elastic scans, and during cooling from 320K over 16–24 h. No statistically significant differences were observed between the two sets of data. Data were corrected by normalization of the detectors with vanadium and subtraction of the empty cell scattering.

X-ray scattering experiments on the solvent and solvent plus enzyme were carried out on line 9.1 at the Daresbury synchrotron radiation source, using a curved image-plate detector system. Enzyme concentrations and cooling and heating rates were chosen to match those used in the neutron scattering experiments. Differential scanning calorimetry measurements were made on samples of the same concentrations during both cooling and heating, but with higher rates of temperature change (10°/min).

RESULTS AND DISCUSSION

The elastic incoherent scattering intensities obtained on IN6 and IN16 for a range of temperatures are shown in Fig. 1. The fitted straight lines were used to plot $\langle u^2 \rangle$ versus temperature in Fig. 2. The upward inflexion of the $\langle u^2 \rangle$ from $\langle u^2 \rangle \approx 0$ in Fig. 2 indicates the onset of anharmonic dynamics as the temperature is increased. There is a clear indication from Fig. 2 of a time scale dependence of this dynamical transition. The onset of anharmonic motion occurs at ~140K on IN16 (motions <~5 ns) and at ~220K on IN6 (<~100 ps). In addition to the dynamical transition at ~140K, there are inflexions in the IN16 $\langle u^2 \rangle$ at ~185K, ~210K, and ~280K. If one defines a "dynamical transition" as an inflexion in $\langle u^2 \rangle$, then the IN16 profile demonstrates the presence of four dynamical transitions in the sample. The three highest-temperature transitions do not correspond to transitions from anharmonic to harmonic behavior, but rather to modification of the anharmonic behavior itself.

To investigate whether any of the various transitions in Fig. 2 are coupled with changes in solvent behavior, a number of supplementary experiments were performed. Differential scanning calorimetry (DSC) indicated the presence of one thermodynamic phase transition, at 180K in the pure cryosolvent and 170K in the enzyme/cryosolvent solution. No qualitative differences in behavior were seen when the data were gathered during heating rather than cooling. The associated exothermic change is 2.5 times smaller for the enzyme/cryosolvent system. Synchrotron x-ray diffraction experiments indicate that the 180K transition in the pure cryosolvent is associated with the melting of one crystalline phase. However, the x-ray diffraction patterns obtained from the enzyme/cryosolvent system showed no detectable crystallization at any temperature down to

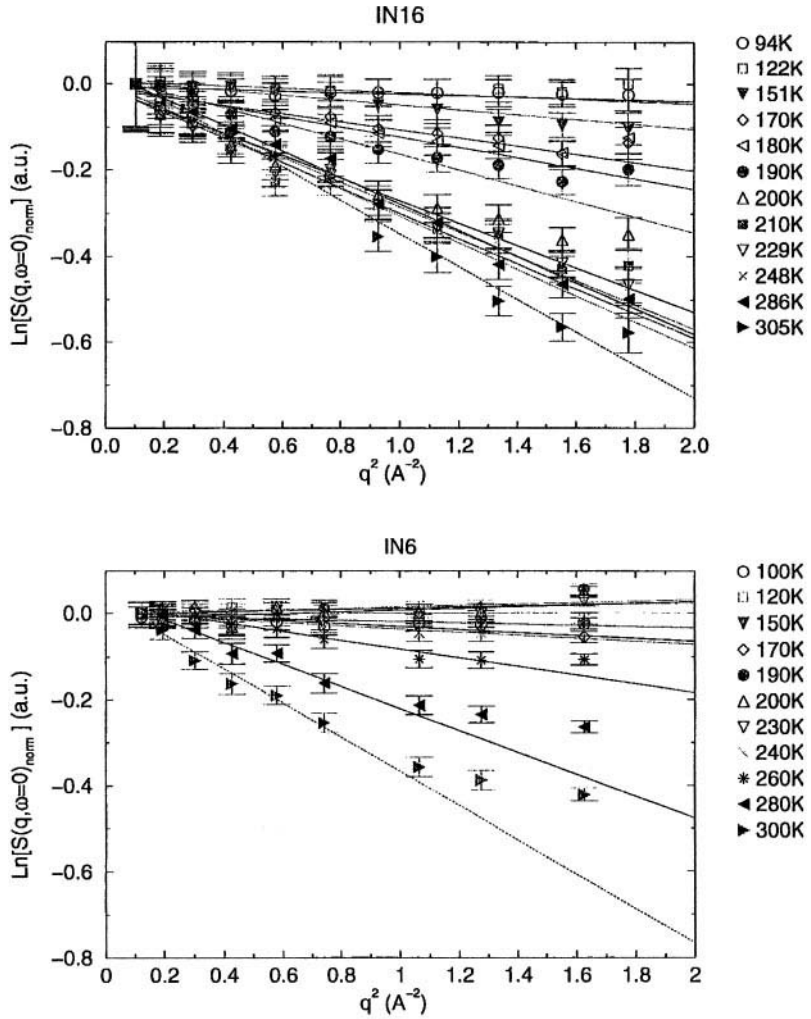

FIGURE 1 Variation of the logarithm of the elastic incoherent scattering of glutamate dehydrogenase in CD_3OD/D_2O (70:30), $S(q,\omega = 0)$, normalized to 80K, as a function of q^2. The IN6 spectrometer has an energy resolution corresponding approximately to motions faster than 10^{-10} s (Daniel et al., 1998), and the IN16 spectrometer an energy resolution corresponding to motions faster than 5×10^{-9}s .

110K. In contrast, crystallization was visible in neutron scattering in the enzyme/solvent system at 170K, as evidenced by the appearance of Bragg diffraction at $Q > 1.4$ $Å^{-1}$. Moreover, the neutron peaks were found at the same d-spacing as those appearing in the 70% methanol/water x-ray diffraction pattern at 180K. The differences between the neutron and x-ray diffraction results may be related to differences in the sample sizes, which are a few microliters for the x-ray experiments, ~50 μl for the DSC, and a few milliliters for the neutron scattering. It is therefore possible that the DSC phase transition at 170K and the discontinuity observed at 185K in the temperature profile of the IN16 $\langle u^2 \rangle$ are associated with the melting of one component of the cryosolvent. However, no features are found in either the x-ray or DSC results that accompany either of the dynamical transitions at 140K or at 210K. We therefore conclude that these two transitions are not correlated with detectable structural or thermodynamic changes in the cryosolvent.

To verify the dynamic origin of the observed transitions, additional experiments were performed to detect associated changes in quasielastic neutron scattering on IN16. Quasielastic scattering, which is visible as a broadening under the elastic peak, arises from the presence of nonvibrational motion in the sample, such as jump or continuous diffusion (Bee, 1988). In favorable cases quasielastic scattering can be analyzed to give details on the geometries and time scales of the contributing motions. Here, however, the counting statistics were found to be insufficient for such an analysis. Rather, the presence of a qualitative change in the quasielastic scattering was used to confirm that the origin of the IN16 scattering changes in Fig. 2 are indeed dynamic. Measured spectra are presented in Fig. 3. At 130K the form of the spectrum is essentially that of the vanadium control. As vanadium is a pure elastic scatterer, this indicates that no quasielastic scattering and hence no diffusive dynamics are present. Quasielastic scattering appears between 130K and 170, and intensifies between 170K and 190K and again between 190K and 210K. These results are consistent with the positions of the dynamical transitions in the IN16 $\langle u^2 \rangle$ data in Fig. 2. From 225K to 290K little change is seen in the quasielastic spectrum, again consistent with the behavior of $\langle u^2 \rangle$ in Fig. 2. The quasielastic scattering data confirm the IN16 $\langle u^2 \rangle$ transitions at ~140K, ~210K, and ~280K as arising from changes in the anharmonic motion in the enzyme.

In previous work on a number of proteins (Keller and Debrunner, 1980; Parak et al., 1982; Knapp et al., 1982;

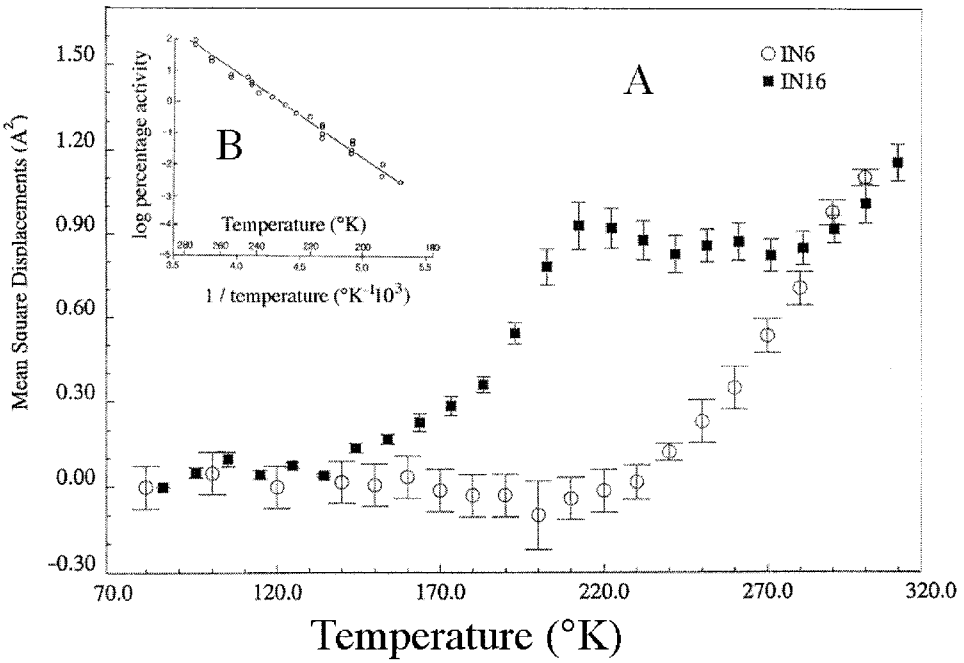

FIGURE 2 Effect of temperature on the enzyme activity (*inset, B*), and the dynamics as measured by neutron scattering, of glutamate dehydrogenase in CD_3OD/D_2O (70:30). Neutron scattering data are from Fig. 1. Enzyme activity data are from More et al. (1996).

Doster et al., 1989; Cusack and Doster, 1990; Frauenfelder et al., 1979; Tilton et al., 1992; Parak et al., 1980; Ferrand et al., 1993), a dynamical transition was observed at various temperatures between 170 and 230K. Moreover, although the different techniques used have sometimes covered different time scales, comparison between them, and with activity, has been hampered by the use of different proteins in a variety of physical states. In the experiments presented here, the use of a consistent method on a single preparation reveals a shift of the lowest transition temperature to lower temperatures as the time scale probed increases from approximately picoseconds to approximately nanoseconds. This effect was also seen on the same preparation in preliminary measurements using the IRIS spectrometer at the ISIS spallation neutron source in Oxford, which has an energy resolution intermediate between that of IN16 and IN6, and resolves motions on the <500-ps time scale. The lowest IRIS transition was found at an intermediate temperature, between 150K and 180K (Finney and Daniel, 1998).

Given the direct nature of neutron scattering as a determinant of global protein motions (Smith, 1991) (as compared with Mossbauer spectroscopy, which examines Fe dynamics, and x-ray crystallography, which gives time-averaged displacements and includes static disorder), the conclusion that the temperature dependence of the transition in average protein motions is time scale dependent can be considered to be relatively robust. The lowest dynamical transition temperature for the present system can therefore be seen to be heavily dependent upon the instrument time scale, rather than occurring at a fixed temperature. As the temperature is lowered, the anharmonic motions slow down, moving from fast to slow time windows. This qualitative effect is consistent with a description of the dynamics associated with the lowest transition as essentially activated, i.e., as involving energy-barrier crossing. A remaining question is therefore whether at time scales longer than nanoseconds the lowest dynamical transition will be found below 140K, and correspondingly at time scales significantly shorter than 10^{-11} s it will be found above 220K. Within the framework of an activated process, the recent simulation evidence for the lowering of the dynamical transition temperature in a viral capsid protein upon ligand binding (Phelps et al., 1998) could be interpreted as the ligand leading to a decrease in the effective associated energy barrier.

The amplitude of the slower motions (IN16) remains essentially unchanged from 210K to 280K. This surprising result is consistent with an effective square-well potential over this temperature range. Also of interest is the apparent coincidence (to within experimental error) of the IN6 and IN16 data points above 280K. This coincidence between the $\langle u^2 \rangle$ values above 280K would indicate that the contribution to the overall motion at these higher temperatures from motions on the time scales intermediate between IN6 and IN16 is very small; i.e., these intermediate time scale motions have risen to a peak at ~210K (observable by subtracting IN6 $\langle u^2 \rangle$ values from IN16 $\langle u^2 \rangle$ values) and decline steadily to a low value at ~280K. This is consistent with the conclusion (above) that the dynamic transition is heavily dependent upon the time scale window of the instrument used to observe dynamics, with motion over a given time scale progressively replaced by slower motions as the temperature is lowered. However, it is possible that the IN16 values are affected by systematic error at high values of $\langle u^2 \rangle$, possibly associated with the relatively low corresponding elastic counting intensities. Therefore, the approximately constant $\langle u^2 \rangle$ between 220K and 280K and the coincidence of the IN6 and IN16 data above 280K merit

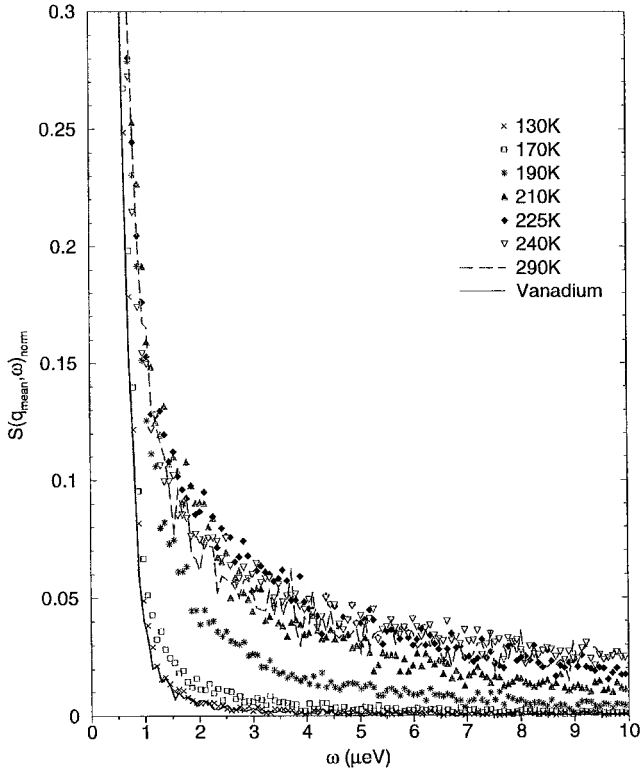

FIGURE 3 IN16 dynamic structure factor of glutamate dehydrogenase in CD_3OD/D_2O (70:30) over a range of temperatures. Data averaged over angles corresponding to the q range over which the data fit in Fig. 2 were linear, i.e., $0.10 < q < 1.13$ Å^{-1}. Spectra were normalized to 1 for zero energy transfer to visualize the variation in shape of the quasielastic intensity.

further confirmation. Experimental errors are also probably responsible for the absence of a detectable $\langle u^2 \rangle$ at temperatures below the lowest dynamical transition on both instruments: small-amplitude vibrational motions are active in the protein at these temperatures. In parallel measurements on low hydration samples of GDH, the better statistics of the data possible in the absence of excess solvent showed a clear, steady rise in $\langle u^2 \rangle$ from the lowest temperature measured.

The inset to Fig. 2 shows that enzyme activity in the cryosolvent used in the neutron scattering experiments exhibits no deviation in Arrhenius behavior down to the lowest temperature at which activity could be measured, 185K. Three of the dynamical transitions visible in Fig. 2 occur at temperatures significantly above 185K: those at 210K and 280K on IN16 and at 220K on IN6. Therefore, these apparent changes in dynamic behavior have no visible affect on activity. Whether the remaining transitions at lower temperatures (140K and 185K on IN16) involve the activation of dynamics required for function is unknown.

In considering the significance of the above results it is important to assess whether enzyme structure/activity in the cryosolvent is likely to be similar to that in a physiological solvent. Many studies of protein dynamics have been carried out on hydrated crystals or powders, and there is evidence that, given a hydration of >0.2 g H_2O/g protein, many protein properties are close to those in solution (Rupley and Careri, 1991). The present measurements were carried out under conditions where enzyme activity can be accurately measured. Given the relatively small effect of cryosolvent on the activity of the enzyme and the low water activity in the interior of a cell, the physical and functional properties of the enzyme in the 70% methanol cryosolvent are likely to be at least as close to those of the in vivo enzyme as are those dissolved in dilute buffer or as hydrated powders. The solvent has little influence on activity or stability under the experimental conditions used here. The reaction rate in cryosolvent is about half the rate in buffer. K_m values are somewhat lower, but these effects are relatively small and with appropriate precautions do not affect activity determinations (More et al., 1996). The viscosity of the cryosolvent rises by ~200-fold between 273K and 188K (Weast, 1974), but the decrease in reaction velocity is very much greater than this, so that activity does not approach diffusion limitation. Full activity was found to be recoverable after the neutron beam experiments. However, solvent properties will be expected to influence the dynamical properties examined here. An example of this is given in the recent work on myoglobin in trehalose, where the protein was found to remain approximately harmonic up to room temperature (Cordone et al., 1999). The dynamical transitions examined here might be expected to change with the properties of the solvent. Work is in progress to examine this point.

The present results may not be representative of all proteins. Neutron experiments performed on bacteriorhodopsin (an insoluble, integral membrane protein) in hydrated membranes of *Halobacterium salinarum* have not detected a time dependence of the dynamical transitions (Réat et al., 1997). Whether the present findings will be generalized to other (water) soluble enzyme classes under the same environmental conditions remains to be seen. The GDH used here is a large, hexameric enzyme from an archeal extreme thermophile. It is a good subject for work of this type, being stable and active in the cryosolvent over a very wide temperature range, as well as being very soluble. There is no evidence that such enzymes in general have systematic differences of structure or function from mesophilic or non-archeal organisms; i.e., the differences between mesophilic and thermophilic enzymes, and between archeal and bacterial enzymes are no greater than the differences found within each group (Daniel et al., 1997). Specific studies on this GDH, including those on Arrhenius behavior, have shown that it is no more different from other GDHs than these are from one another (Hudson and Daniel, 1993, 1995; Hudson et al., 1993; More et al., 1996), and GDHs are not atypical of soluble multisubunit enzymes. We currently have no reason to believe that the results reported here will not apply to this class of enzymes and possibly also to simpler soluble enzymes. A variety of enzymes, including xylanase, catalase, alkaline phosphatase (Dunn, Bragger, and Daniel, unpublished observations), β-glucosidase, and β-galactosidase (More et al., 1996) do exhibit straight Ar-

rhenius plots to below 200K, well below the picosecond dynamical transition temperature for GDH.

The work described here is the first determination of the global dynamics of an active enzyme over a range of time scales, measured under similar conditions. It seems likely from the present and earlier work (Daniel et al., 1998) that anharmonic fast (ps) time scale motions are not required at all temperatures for the enzyme rate-limiting step. Therefore, anharmonic fast motions are not necessarily coupled to the much slower motions describing transitions along the enzyme reaction coordinate. However, the neutron technique used here reveals average dynamics, and it is conceivable that functionally important fast motions may occur locally in the protein at the active site, at levels below the noise in Fig. 2.

Glutamate dehydrogenase is a multisubunit enzyme catalyzing the interconversion of five reactants by a rather complex mechanism (Hudson and Daniel, 1993, 1995). Therefore, it is not feasible to relate the global dynamics we have described here to elements in the structure critical to function or to the rate-limiting step. Clearly more needs to be done in combining techniques with which the dynamical transitions can be studied. Further work is required to examine the intramolecular localization of functionally important motions. It is encouraging in this respect to see that site-specific labeling studies have recently been shown to be successful in dynamic neutron scattering studies on proteins (Lehnert et al., 1998; Réat et al., 1998). Moreover, techniques sensitive to local dynamics, such as optical spectroscopy, can also provide useful information (Di Pace et al., 1992; Melchers et al., 1996). The availability of the crystal structure of GDH may also help future work in this regard. It is of interest to note that the crystallographic study of a related GDH provided evidence for a large relative movement of the two domains between which the active site is located, during the catalytic cycle (Baker et al., 1997).

The results of further investigations can be expected to improve our understanding of the relationships between protein activity, flexibility, and stability. For example, there is a commonly held view that stability is inversely related to activity because molecular flexibility is required for activity, but excessive flexibility tends to be destabilizing (see Daniel et al., 1997). The recent engineering of subtilisin to withstand 100°C with full retention of activity may indicate that this principle is not general (Vandenbrug et al., 1998). Alternatively, a more careful distinction may need to be made between the flexibility required for activity (which may be local or restricted to particular time scales) and that which is likely to affect stability (which is possibly global). Clearly, a complete understanding of this and related problems will require continued investigation into the forms, amplitudes, and time scales of motions involved in enzyme catalysis.

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