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Enzyme conformational dynamics during catalysis and in the 'resting state' monitored by hydrogen/deuterium exchange mass spectrometry

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Abstract **This work reports the use of electrospray mass spectrometry for studying the conformational dynamics of enzymes by amide hydrogen/deuterium exchange (HDX) measurements. A rapid-mixing quench-flow approach allows comparisons to be made between the HDX kinetics of free enzymes with those under steady-state conditions. Experiments carried out on carboxypeptidase B in the absence of substrate and in the presence of saturating concentrations of hippuryl-Arg result in HDX kinetics that are indistinguishable. This finding implies that the conformational dynamics that mediate HDX are not significantly different in the resting state of the enzyme and during substrate turnover.**

Keywords: Steady-state; Carboxypeptidase B; Lysozyme; Conformational change; Induced fit

1. Introduction

It is well established that the native state of proteins is highly dynamic, with motions that span time scales from picoseconds to seconds [1,2]. These motions are coupled to thermal fluctuations of the solvent, and they encompass the movement of individual amino acid residues, all the way to large-scale transitions affecting the entire polypeptide chain [3–5]. Conformational plasticity and dynamics have been implicated in many aspects of protein function. Yet, in most cases the exact relationship between protein structure, dynamics, and function is not fully understood [6–9]. Of particular importance is the role of conformational dynamics during enzyme catalysis. For many enzymes, the catalytic cycle is initiated with an induced fit binding event that involves a large scale conformational change, thereby enclosing the substrate in a cavity where it is in contact with the catalytically active residues and shielded from the solvent [10–13]. Following chemical conversion of the substrate the active site has to open up for product release to occur [2]. Information on these structural changes is normally obtained from static before-and-after X-ray structures of enzymes recorded in the absence and presence of bound inhibitors or substrate analogs. In addition to substrate binding and product release, enzyme dynamics are also thought to play a central role for the actual catalytic conversion step(s) [6–9,14–21].

Insights into protein structural dynamics come from a variety of techniques including computer modeling [7], neutron scattering [18], and NMR spin relaxation experiments [22]. Another important tool that is rapidly gaining in popularity are amide hydrogen/deuterium exchange (HDX) measurements by electrospray ionization mass spectrometry (ESI-MS) [23–26]. In native proteins most of the polyamide backbone forms secondary structure elements such as α-helices and β-sheets that are stabilized by C═O···H–N hydrogen bonds. Backbone amide hydrogens may be replaced with deuterium upon exposure of the protein to a D_2O-containing solvent system. Notably, the occurrence of these HDX events is closely related to the dynamics of the protein. This is due to the fact that HDX is mediated by structural fluctuations leading to transient opening events that briefly disrupt individual hydrogen bonds and expose the resulting free N–H sites to the solvent. In the commonly encountered EX2 regime these opening/closing events occur on time scales ranging from sub-micro to milliseconds, and most exchangeable sites have to cycle through numerous of these transitions before exchange occurs [27–29].

It is an unresolved question in how far the conformational dynamics of a free enzyme are different from those during catalytic turnover. One could envision an enzyme under steady-state conditions cycle through a repetitive series of motions that are linked to substrate binding, chemical conversion, and product release. In the absence of substrate, the same enzyme might be considerably less dynamic, as implied by the term "resting state" that is commonly used to describe these conditions [30]. Several previous studies support the notion of such a mobility enhancement during catalysis [2,31–34]. An alternative view suggests that the thermal motions of enzymes are virtually identical in the resting state and during the catalytic cycle. In this scenario, catalysis can only be performed within the limitations given by the intrinsic dynamics of the protein [20,23,24,26,35–39].

A considerable difficulty in deciding between the two alternative models is that much of the previous literature devoted to this topic is based on rather indirect evidence. The most obvious strategy for addressing this problem, namely a direct comparison of conformational dynamics measured in the absence and in the presence of substrate, has not been practical

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in most cases. One major problem is that experiments on protein dynamics extend over relatively long times, typically hours for most techniques. It is not trivial to prevent the occurrence of substrate depletion on such a long time scale, considering the requirement for relatively high protein concentrations (on the order of 1 mM for NMR and 10 μM for ESI-MS). For example, at a concentration of 1 mM an enzyme with $k_{cat} = 100\ s^{-1}$ will cause the complete conversion of 10 M substrate within only a few minutes! Some workers have circumvented this problem by resorting to studies on equilibrium processes, where the reaction flux in the forward direction matches that in the reverse direction [20,36]. However, this approach is only feasible in a few select cases, because most biochemical processes have equilibrium constants that are far from unity.

The current study explores the use of a customized quench-flow mixing system for monitoring enzyme conformational dynamics by HDX/ESI-MS. By employing slightly basic conditions it is possible to follow the exchange of a significant fraction of amide hydrogens within only a few seconds. Owing to the short duration of the observation time interval it is possible to provide enzymes with a sufficiently large amount of substrate to ensure steady-state conditions during the entire experimental time window. This approach is utilized to monitor the conformational dynamics of carboxypeptidase B in the presence and absence of substrate. It is found that the HDX kinetics of this enzyme under the two conditions are indistinguishable, implying that its structural motions are not enhanced during catalysis. This result is consistent with the view that catalytically important conformational dynamics are intrinsic to the structure of carboxypeptidase B.

2. Materials and methods

2.1. Chemicals

Hen egg white lysozyme, porcine carboxypeptidase B, and hippuryl-Arg (*N*-benzoyl-Gly-Arg) were obtained from Sigma (St. Louis, MO), and deuterium oxide was purchased from Cambridge Isotope Laboratories (Andover, MA). *N*,*N*′,*N*″-triacetylchitotriose (NAG_3) was obtained from MJS BioLynx (Brockville, ON). All chemicals were used without further purification. pH values were measured with an AB15 pH meter (Fisher Scientific, Nepean, ON). pD values reported for D_2O-containing solutions were corrected for isotope effects by using the relation pD = (pH meter reading) + $0.3139\alpha + 0.0854\alpha^2$, where α is the atom fraction of deuterium in the solution, $\alpha = [D]/([D] + [H])$ [11]. All measurements were carried out at room temperature (22 ± 1)°C, unless noted otherwise.

2.2. Rapid mixing HDX/ESI-MS measurements

The experiments were performed by using a custom-built three syringe continuous-flow setup (Fig. 1) [40,41]. The contents of syringe 1 (enzyme in aqueous 250 mM HEPES buffer) and syringe 2 (D_2O with or without substrate) were combined at a first mixing tee in a ratio of 1:4. The resulting solution had a D_2O content of 80% at pD 8.8. This first mixing step serves a dual purpose, it initiates the catalytic conversion by combining enzyme and substrate, and it triggers HDX. After a variable reaction time another mixing step at a second tee exposed the enzyme to acidic quenching buffer from syringe 3 in a 1:1 ratio (500 mM KH_2PO_4/H_3PO_4), resulting in pH value of 2.6 in the final solution. This solution was collected in a Eppendorf cup, the bottom tip of which was dipped in liquid nitrogen to cause immediate freezing. Samples were collected in 100 μL aliquots and stored at −80 °C. The reaction time was determined by the total flow rate from syringes 1 and 2, and by the dimensions of the fused silica capillary (Polymicro Technologies, Phoenix, AZ) connecting the first and the second mixer. Two different inner diameters were used for this capillary connection

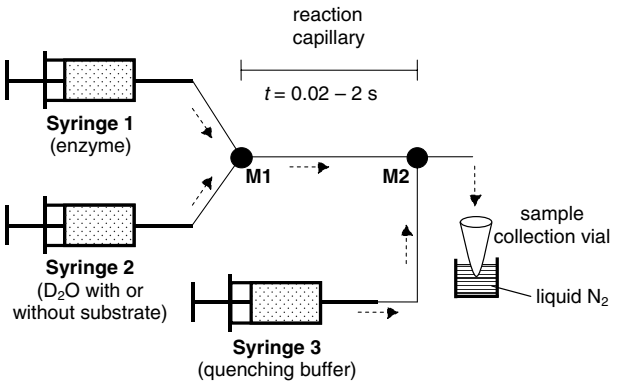

Fig. 1. Continuous-flow rapid mixing setup for monitoring the HDX kinetics of enzymes in the presence and absence of substrate. M1 and M2 represent mixing tees, thin solid lines represent fused silica capillary connections, and dashed arrows indicate the direction of liquid flow. Further details are given in the text.

(75 and 100 μm), with lengths ranging from 3.6 to 76 cm. Reaction times between 20 ms and 2 s were monitored by varying the total flow rate after the first mixer from 200 to 600 μL/min. Longer time points were studied in manual mixing experiments. Experiments on carboxypeptidase B were carried out in the presence of 0.2 M NaCl in syringes 1 and 2 [42].

The extent of HDX for all samples was measured by LC/ESI-MS on an 1525μ HPLC coupled to a Q-TOF Ultima (Waters). Proteins were eluted using a water/acetonitrile gradient in the presence of 0.05% trifluoroacetic acid on a self-packed Poros R2(C8) column (Applied Biosystems, Foster City, CA), resulting in a protein elution time of less than 6 min. The column, injector, and the extensively coiled solvent delivery lines were embedded in an ice-bath. All of the isotopically labeled side chains undergo back exchange during the LC step, only deuterons bound to backbone amide groups can be retained [29]. Control experiments on bradykinin revealed that the extent of amide back exchange under the conditions used here was 26%. Prior to data analysis all ESI mass spectra were converted to a mass scale by using the MaxEnt deconvolution routine provided by the instrument manufacturer.

3. Results and discussion

ESI-MS-based studies typically explore the amide HDX behavior of proteins at near-neutral pH where isotope exchange is relatively slow, thus necessitating experimental time windows of tens of minutes to several hours [23,29]. For the current work it is important to accelerate the exchange kinetics, such that extensive HDX occurs within seconds. For pD values greater than ~5, HDX proceeds under base catalysis, such that the overall exchange rate constant (k_{HDX}) in the EX2 regime can be expressed as $k_{HDX} = K_{op} \times k_{base} \times [OD^-]$. In this equation, K_{op} is the equilibrium constant of the opening event for a given amide, and k_{base} is a second-order rate constant [29,43]. Therefore, a pD increase of one unit will accelerate amide isotope exchange by a factor of 10. HDX in the current work was carried out at pD 8.8, resulting in an acceleration of almost two orders of magnitude compared to neutral conditions. At the same time, this value is still within a range where most enzymes retain most of their catalytic activity.

As a first step, it was evaluated whether the experimental strategy is suitable for monitoring differences in the HDX behavior of enzymes on a time scale of milliseconds to seconds. Lysozyme, a polysaccharide hydrolase, was chosen as model system for these control experiments. This enzyme