

Conformer Selection and Intensified Dynamics During Catalytic Turnover in Chymotrypsin**

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During catalytic turnover, enzymes undergo thermally driven conformational fluctuations (dynamics) that are directly linked to catalytic efficiency.^[1–3] In broad terms, this link exists because enzymes must sample specific dynamic modes in order to access high-energy structures along the catalytic reaction coordinate.^[4] Mass spectrometry-based approaches for probing enzyme dynamics are developing rapidly, but have been limited thus far by the application of H/D exchange (HDX) under steady-state conditions, which results in averaging of the data over multiple catalytic states.^[5,6] Models that attempt to define the specific nature of the enzyme dynamics/activity relationship are therefore drawn overwhelmingly from Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion NMR experiments on “active” enzymes.^[3,7,8] This approach is extremely powerful, but is confined to a small set of highly reversible reactions in order to circumvent the issue of substrate depletion during the experiment.^[2,3,9–12]

From the limited set of “CPMG accessible” reactions, two models for catalysis-linked dynamics have been formulated. The “induced fit” model is characterized by a substrate-free (resting) state that samples a different set of dynamic modes compared to the substrate-bound, catalytically active state. This would imply that the dynamics observed in the resting state should be substantially different from those observed during catalysis. A number of studies have reported evidence supporting this model.^[13,14] In the “conformer selection” model, the conformational space sampled by the enzyme is independent of catalysis (i.e., the resting and active-state conformational dynamics are identical). Productive enzyme–substrate interactions occur when incoming substrate “selects” the appropriate conformer for binding. Conformer selection is supported by a substantial number of studies showing no difference in dynamics between free and substrate-bound enzyme.^[2,3,5,9–11] “Hybrid” models have also been proposed in which substrate binding occurs through conformer selection followed by “induced fit-like” substrate-directed conformational sampling during catalysis.^[15] These

models provide crucial insights into virtually all aspects of enzyme function including substrate binding, specificity, allostery and rate-limiting catalytic processes. However, their formulation from a relatively small pool of similar enzyme systems suggests that they may describe only a fraction of possible catalysis-linked dynamic modes.

In this work, we probe conformational dynamics in an active, “CPMG inaccessible” enzyme system using an alternative approach that combines time-resolved electrospray mass spectrometry (TRESI-MS)^[16] and sub-second H/D exchange (HDX) labeling^[17] to monitor dynamics in the pre-steady state. By this approach, catalytic processes are detected as time-dependent intensity changes in mass-to-charge (m/z) peaks corresponding to the accumulation and/or depletion of enzyme intermediates. For each species that becomes populated during the measurement, dynamics are probed simultaneously, by the rate and magnitude of deuterium uptake. In contrast to CPMG NMR spectroscopy, these measurements are not “site specific”, however, they represent a straightforward and broadly applicable method for characterizing dynamics in active enzyme systems. A schematic illustration of the experimental setup is provided in Figure 1.

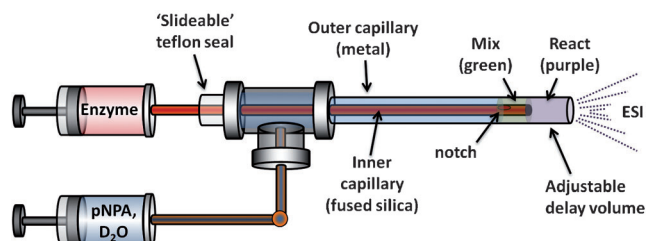


Figure 1. A schematic depiction of the TRESI/HDX experimental setup.^[15] A solution containing chymotrypsin in water (red) is passed through the inner capillary, mixing 1:4 with pNPA in D₂O (blue) in the mixing region (green). The reacting mixture (purple) is then passed through a delay volume before undergoing electrospray ionization. The reaction/HDX labeling time is adjusted by changing the position of the inner capillary within the outer capillary. Details are provided in the Supporting Information.

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Chymotrypsin-catalyzed hydrolysis of *para*-nitrophenyl acetate (*p*-NPA) has long been a model for pre-steady state enzyme catalysis, likely due to the fortuitous release of a chromophoric product (*para*-nitrophenol) upon acylation of the enzyme.^[18] Here we use this reaction to determine if current models are sufficient to define catalysis-linked dynamics in a system that is substantially different from the set of reactions upon which the models were formulated (i.e., different class of enzyme, covalent modification, equilibrium

far to the right, etc.). The detection of dynamics that do not conform to current models would indicate a need to broaden the catalysis-linked dynamics “basis set”.

Deconvoluted mass spectra from three time-points in a typical TRESI-HDX run are shown in Figure 2. Accumu-

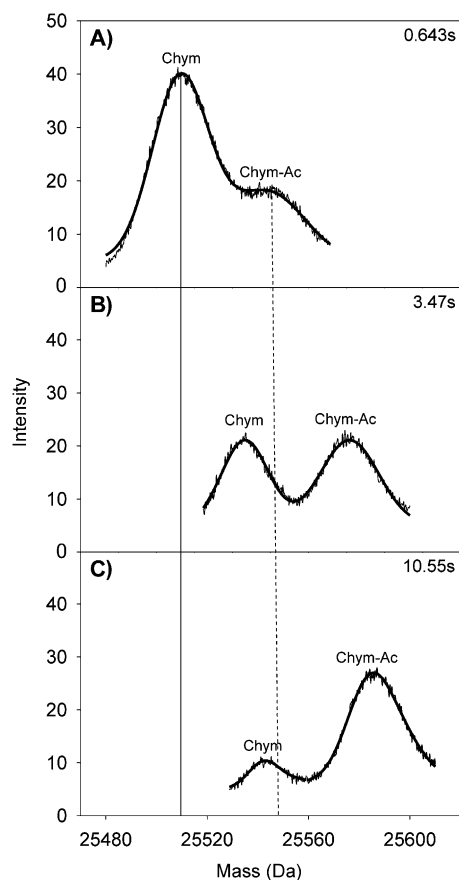


Figure 2. Mass distributions for chymotrypsin simultaneously undergoing acylation and HDX. Peak centroids were determined by fitting a bimodal normal distribution to the data. For clarity, the initial centroids for the free enzyme (Chym) and acyl-enzyme intermediate (Chym-Ac) are denoted by a solid and dotted line, respectively. Time-dependent changes in peak intensity are associated with catalysis while changes in peak position are a result of continuous labeling HDX.

lation of the acyl-enzyme intermediate is evident from the time-dependent increase in the intensity of the higher mass peak I_{heavy} on the right, and concomitant depletion of the free enzyme, corresponding to the lower mass peak on the left. A single exponential fit to I_{heavy} versus time gives an observed acylation rate constant $k_{2(\text{obs})} = 0.69 \pm 0.09 \text{ s}^{-1}$, which is in line with previous measurements.^[18]

The effects of HDX are observed in both peaks as a continuous shift to higher mass as solvent-exposed backbone amide protons undergo exchange with solvent deuterium. The rate of exchange at each of these sites is governed by the frequency with which the amide proton is involved in hydrogen bonding. Thus, the global HDX rate is in fact a globally averaged measure of the frequency of hydrogen

bond breaking, which translates roughly into how fast the protein is sampling conformational space. The amplitude of the HDX shift is a measure of the number of sites that become available for exchange on the time-scale of the measurement; effectively the size of the observed dynamics.

In order to obtain global HDX rates for free and acylated chymotrypsin, all pairs of m/z peaks corresponding to the two species were fit to a bimodal normal distribution, yielding the exact peak centroids. Plotting the centroids as a function of time gives the global exchange kinetics, which are superimposed on a heat map in Figure 3 A and shown in a normal-

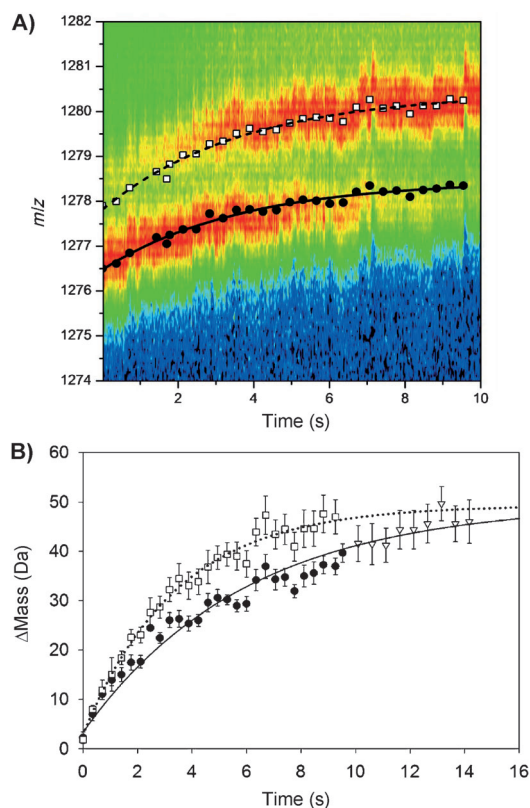


Figure 3. A) A heat map showing catalysis and HDX for the 20+ charge state of chymotrypsin. Catalysis is observed as decreasing (free enzyme) or increasing (acyl-enzyme) heat while the rate of shift to higher m/z gives the global HDX kinetics. Fits to the HDX kinetics were obtained by plotting the peak centroid position as a function of time for each m/z . Closed circles represent the free protein while the open squares correspond to the acyl-enzyme intermediate. B) HDX kinetics averaged over all charge states and three replicates. Series assignments are the same as in (A), expressed in units of ΔMass , which normalizes the data. The data represented by gray triangles were acquired in separate experiments without substrate in order to monitor deuterium uptake for the free protein beyond the point at which the free enzyme population is depleted in the reaction. The amplitude measurement without the additional data was identical, but had a substantially higher error, $49 \pm 11 \text{ Da}$.

ized 2D format in Figure 3 B. Satisfactory fits to the data were achieved using a single exponential expression, which yielded a global exchange rate constant of $0.17 \pm 0.04 \text{ s}^{-1}$ for the free enzyme (filled circles and gray triangles) and $0.29 \pm 0.03 \text{ s}^{-1}$ for the acylated species (open squares). No charge-state

dependence on the HDX rate was observed. The significantly higher rate of deuterium uptake in the acyl-enzyme reflects a higher rate of conformational sampling that occurs specifically in the species undergoing catalytic turnover. This “intensified” conformational searching is a unique feature in that it does not agree with conformer selection, where the frequency of conformational exchange is independent of catalysis, nor with “hybrid” models that include directed conformational searching after substrate binding (see below).^[19,20]

While the free enzyme and acyl-enzyme intermediate undergo exchange at different rates, the exchange amplitude is identical to within error at 51 ± 5 Da and 50 ± 2 Da, respectively. Two criteria must be met to ensure that the identical amplitude measurement is not the result of averaging between several states. The first is that turnover (i.e., return to the free state after having adopted the intermediate state) must be negligible on the time-scale of the measurement. Modeling the reaction using the known values for K_d , k_2 and k_3 ,^[18] indicates that 3.5 % of the enzyme population will complete turnover within the experimental time-window of 10 s, which is sufficiently low to have a negligible impact on the data. Secondly, the rate of acylation must be high relative to the exchange measurement so that the acyl-enzyme signal arises from a population that has undergone HDX predominantly as the acyl-enzyme. This type of averaging is inconsistent with the detection of distinct HDX rates in the free and acyl-enzyme, moreover, the rate constant for acylation is approximately 2 times the rate of deuterium uptake in the acyl-enzyme.

Thus, the identical amplitude values can be interpreted as evidence that productive substrate binding and catalytic turnover are not accompanied by a significant net gain or loss of dynamic modes. This finding is consistent with the conformer selection model, however, it rules out “directed” conformational sampling since the any significant restriction of the active enzyme to “on-pathway” dynamics would constitute a net loss of (off-pathway) dynamic modes, manifested as a lower HDX amplitude for the acyl-enzyme. Ultimately, it would be of great interest to definitively determine if the dynamic modes exhibited by the free and acyl-enzyme were identical using a site-specific analysis.^[21,22] However, significant technical challenges arise when considering the methods available for such studies at present. This system is not amenable to CPMG-NMR as discussed earlier. MS-based methods that employ protein digestion would offer no means to distinguish between peptides originating from the free enzyme and peptides originating from the acyl-enzyme (apart from those few that happen to contain the acylated residue) and studies using non-ergodic fragmentation are limited to smaller proteins at present.^[23]

In summary, by investigating a system outside the “CPMG NMR accessible” set of enzyme-catalyzed reactions, we have uncovered evidence for a new “intensification” model of catalysis-linked dynamics. The features of this model are substrate binding through conformer selection, followed by promotion of catalysis by accelerated, but undirected conformational sampling during turnover. This model implies a general lowering of the energetic barriers between con-

formational sub-states upon productive substrate binding. The evidence was obtained using an alternative MS-based approach that, while providing less structural detail than CPMG-NMR, is suitable for a much wider range of enzyme systems. Our results add a protease to the growing list of enzymes that exhibit conformer selection in substrate binding,^[24,25] and highlight the importance of broadening the range of enzymes used to model catalysis-linked dynamics. With other enzymes clearly showing induced fit behavior,^[13] it is evident that the nature of the link between dynamics and catalytic efficiency is system-dependent and thus unlikely to be well predicted by models drawn from a small subset of enzyme catalyzed reactions. The development of a “unifying” model for catalysis-linked dynamics will therefore require a broad-based approach.

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