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Enzyme Dynamics During Catalysis

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Internal protein dynamics are intimately connected to enzymatic catalysis. However, enzyme motions linked to substrate turnover remain largely unknown. We have studied dynamics of an enzyme during catalysis at atomic resolution using nuclear magnetic resonance relaxation methods. During catalytic action of the enzyme cyclophilin A, we detect conformational fluctuations of the active site that occur on a time scale of hundreds of microseconds. The rates of conformational dynamics of the enzyme strongly correlate with the microscopic rates of substrate turnover. The present results, together with available structural data, allow a prediction of the reaction trajectory.

Although classical enzymology together with structural biology have provided profound insights into the chemical mechanisms of many enzymes (1), enzyme dynamics and their relation to catalytic function remain poorly characterized. Because many enzymatic reactions occur on time scales of micro- to milliseconds, it is anticipated that the conformational dynamics of the enzyme on these time scales might be linked to its catalytic action (2). Classically, enzyme reactions are studied by detecting substrate turnover. Here, we examine enzyme catalysis in a nonclassical way by characterizing motions in the enzyme during substrate turnover. Dynamics of enzymes during catalysis have previously been detected with methods such as fluorescent resonance energy transfer, atomic force microscopy, and stopped-flow fluorescence, which report on global motions of the enzyme or dynamics of particular molecular sites. In contrast, nuclear magnetic resonance (NMR) spectroscopy enables investigations of motions at many atomic sites simultaneously (3, 4). Previous NMR studies reporting on the time scales, amplitudes, and energetics of motions in proteins, have provided information on the relation between protein mobility and function (5–15). Here, we have used NMR relaxation experiments to advance these efforts by characterizing conformational exchange in an enzyme, human cyclophilin A (CypA), during catalysis. CypA is a member of the highly conserved family of cyclophilins that are found in high concentrations in many tissues. Cyclophilins are peptidyl-prolyl cis/trans isomerases that catalyze the interconversion between cis and trans conformations of X-Pro peptide bonds, where “X” denotes any amino acid. CypA operates in numerous biological processes (16, 17). It is the receptor for the immunosuppressive drug cyclosporin A, is essential for HIV infectivity, and accelerates protein folding in vitro by catalyzing the rate-limiting cis/trans isomerization of prolyl peptide bonds (18, 19). However, its function in vivo and its molecular mechanism are still in dispute. X-ray structures of CypA in complex with different peptide ligands show cis-X-Pro bonds (20, 21), except for a trans conformation in the CypA/HIV-1 capsid complex (22, 23). In each case, only one conformer was observed in the crystal, even though both isomers must bind to CypA for catalysis of cis/trans isomerization to occur.

We characterized motions in CypA during catalysis with the use of 15N spin relaxation experiments with and without the substrate Suc-Ala-Phe-Pro-Phe-4-NA (24). Longitudinal ($R_L$) and transverse ($R_T$) auto-relaxation rates, transverse cross-correlated cross-relaxation rates ($R_{ccc}$), and $\{^{1}H,^{15}N\}$ nuclear Overhauser enhancements (NOE) were measured for all backbone amides in CypA (25). Though all parameters are sensitive to “fast” motions (pico- to nanoseconds), only $R_L$ is sensitive to “slow” conformational exchange (micro- to milliseconds) (3–8). A progressive substrate-induced shift for several CypA amide resonances (Fig. 1) indicates catalysis-linked motions. It shows (i) that these amides experience different magnetic environments in free CypA (E) and in CypA bound to substrate (ES) and (ii) that the chemical shift changes of the amide signals in CypA upon titration with the substrate Suc-Ala-Phe-Pro-Phe-4-NA (A) At a constant CypA concentration of 0.43 mM, spectra were recorded at 0 mM (blue), 0.38 mM (orange), 1.01 mM (green), and 2.86 mM (red) substrate. The signal of R55 is progressively shifting upon addition of increasing amounts of substrate, indicating fast conformational exchange during catalysis. The observed chemical shifts are population-weighted averages of E and ES, and thus shift towards the position of the ES complex with increasing amounts of substrate. In contrast, the signal of V139 is not affected by catalysis. (B) The chemical shift differences between free CypA and in the presence of 2.86 mM substrate were mapped onto the structure (1RMH) (27) with the use of a continuous color scale.
exchange rates between these states are faster than the difference in their frequencies, i.e., the time scale of exchange falls in the range of $10^{-5}$ to $10^{-3}$ s. In the presence of substrate, at least three different states of CypA exist in equilibrium (Scheme 1). Exchange between these will increase the value of $R_2$ by an amount $R_{2\text{ex}}$ above that observed in free CypA, provided that the nuclear spin experiences different chemical shifts in at least two of the three states (8). Direct measurements of $R_2$ revealed that during catalysis CypA undergoes microsecond conformational exchange in specific regions of the protein. A significant increase in $R_2$ was observed for 10 out of 160 backbone amide nitrogens (26) (Figs. 2 and 3), which together define a contiguous region in the structure (Fig. 3). The measured $R_{2\text{ex}}$ for a particular amide does not necessarily indicate motion of that amide itself. $R_{2\text{ex}}$ can be caused by slow time scale motions of nearby atoms. Addition of substrate induces only minor changes in picosecond dynamics, as evidenced by $R_1$, $\eta_{15N}$, and $\{1H\}^{13}N$ NOE (27). Thus, measurement of $R_2$ identifies “hot spots” of micro- to millisecond dynamics associated with either or both of the processes involved in catalysis: binding and isomerization.

Can the microscopic reaction steps be separated? The minimal reaction scheme (Scheme 1) consists of three microscopic reaction steps: binding of (i) cis and (ii) trans isomers and (iii) the catalytic step of substrate isomerization on the enzyme. We separated the effects of binding and isomerization by monitoring changes in $R_2$ as a function of substrate concentration. The relative contributions to $R_2$ from exchange due to binding and isomerization have different dependencies on the total substrate concentration. For most residues, $R_2$ first increases and then decreases with the addition of substrate (Fig. 2, D and E, and Fig. 4A). This pattern of maximum chemical exchange at intermediate substrate concentrations—where E, ES$\text{cis}$, and ES$\text{trans}$ are all significantly populated—is diagnostic of a predominant effect due to binding (see Eq. 1). In contrast, $R_2$ increases monotonically with substrate concentration for R55 (28) (Figs. 2B and 4B). This increase in $R_2$ with a concomitant increase in populations of ES$\text{cis}$ and ES$\text{trans}$ pinpoints a significant conformational exchange contribution to $R_2$ from interconversion between ES$\text{cis}$ and ES$\text{trans}$.

Do the exchange dynamics observed for the enzyme correspond to the microscopic catalytic steps of substrate turnover? To shed light on this fundamental question, we determined rate constants for the conformational changes on the enzyme and compared them with rate constants of substrate interconversion. For residues that report only on binding, a simple two-state exchange model (including the free and a single bound state) can be applied, enabling the use of closed analytical formulae to determine the binding constant, off-rate, and chemical shifts. The exchange contribution ($R_{2\text{ex}}$) to $R_2$ may be approximated as (29):

$$R_{2\text{ex}} = P_E P_{ES} \frac{2}{k_{\text{ex}} \tau_{\text{ip}}} \left( \frac{k_{\text{ex}} \tau_{\text{ip}}}{2} \right) \tan\left( \frac{k_{\text{ex}} \tau_{\text{ip}}}{2} \right) \tag{1}$$

where $P_E$ and $P_{ES}$ are the fractional populations of the free (E) and bound (ES) states, $\delta\omega$ is the chemical shift difference between E and ES, $k_{\text{ex}}$ is the exchange rate, and $\tau_{\text{ip}}$ is the delay between refocusing pulses in the Carr-Purcell-Meiboom-Gill (CPMG) experiment. Expressing Eq. 1 in terms of the free substrate concentration, $[S]$, and an effective dissociation constant, $K_{D}^{\text{eff}} = P_E [S]/P_{ES} = k_{\text{off}}/k_{\text{cat}}$, and making the substitution $k_{\text{ex}} = k_{\text{off}} + k_{\text{cat}}[S] = k_{\text{off}}(1 + [S]/K_{D}^{\text{eff}})$, one gets

$$R_{2\text{ex}} = \frac{2}{k_{\text{off}} \tau_{\text{ip}}} \frac{[S]}{K_{D}^{\text{eff}}} \left( 1 - \frac{2}{k_{\text{off}} \tau_{\text{ip}}} \right) \tan\left( \frac{k_{\text{off}} \tau_{\text{ip}}}{2} \right) \tag{2}$$

where $[S]$ is a function of $K_{D}^{\text{eff}}$ and the total concentrations of substrate and enzyme, $k_{\text{off}}$ is the off-rate, and $k_{\text{cat}}$ is the on-rate (30). Hence, $K_{D}^{\text{eff}}$, $k_{\text{off}}$, and $\delta\omega$ can be estimated by nonlinear regression (31). The data for residues K82, L98, N102, and A103 are fit well by the two-state model, yielding values of $K_{D}^{\text{eff}}$ between 0.95 and 1.20 mM, and values of $k_{\text{off}}$ between 10,700 and 14,800 s$^{-1}$ (Fig. 4A). These values agree within uncertainties with those measured from line shape analysis of the substrate (32). Next, we obtained quantitative estimates of the rate constants of protein dynamics by simulating the $R_2$ rates for the full three-state model of Scheme 1. Excellent agreement between the simulated and experimental data was obtained with the use of the rate constants of cis/trans isomerization determined separately from line shape analysis (32) together with reasonable chemical shift differences between the three states (33) (Fig. 4). The results confirm the qualitative evaluation of $R_2$ outlined above: for

![Scheme 1. Three-state model of CypA catalysis. E is the free enzyme, and ES$\text{cis}$ and ES$\text{trans}$ are the two Michaelis-Menten complexes with the substrate in the cis and trans conformations, respectively. $K_\text{D}$ is the dissociation constant, $k_{\text{off}}$ the off-rate, $k_{\text{on}}$ the on-rate, $k_{\text{cat}}$ and $k_{\text{cat}}^{\text{eff}}$ are the rate constants of isomerization. Superscripts cis and trans identify the cis and trans isomer, respectively. CypA catalyzes the cis/trans isomerization of the Phe-Pro peptide bond of the substrate used here with a turnover rate of several hundreds per second (43, 46).](image-url)
most residues the binding processes dominate the exchange contribution, whereas for others a contribution from the isomerization step is required to yield satisfactory fits.

The backbone amide nitrogen of R55 clearly experiences conformational exchange associated with the isomerization and binding process. Because the backbone amide group is distant from the substrate binding site, it is unlikely that the observed exchange merely reflects a conformational change of the bound peptide in a rigid active site. Rather, the results provide evidence for conformational fluctuations in the enzyme. Notably, R55 is essential for catalysis (34). Its side-chain guanidino group is hydrogen bonded to the prolyl nitrogen of the substrate, and, thus, promotes isomerization by weakening the double-bond character of that peptide bond (20, 21, 23, 34). The good agreement between the 15N relaxation results and those from substrate line enzyme shape analysis implies that the observed conformational dynamics of the enzyme are strongly and quantitatively correlated with the chemical steps of substrate interconversion. Furthermore, the conformational fluctuations of the enzyme are likely to be collective motions, because similar rate constants were inferred for several backbone amides.

Interpretation of the results in light of structural data provides additional insights into CypA catalysis. We focus here on residues that show conformational exchange dynamics only during catalysis, thus excluding the loop from residues 68–77 that also undergoes conformational exchange in resting CypA (35). Several residues (101–103 and 109) with catalysis-linked exchange are physically close to the substrate bound in cis (Figs. 3A and 3B). However, L98 and S99 are distant from the peptide in the cis conformation, yet undergo exchange during the catalytic cycle and show chemical shift changes upon titration with substrate that are comparable to those observed for residues 101–103 and 109 (Figs. 1 and 3). Though the structure of the enzyme in complex with the corresponding trans isomer of the peptide is not known, our results suggest that L98 and S99 may be interacting with the trans peptide. Thus, we propose a reaction trajectory that involves a rotation of the COOH-terminal peptide segment around the prolyl peptide bond while the NH-terminal part remains bound to the enzyme (37). This conformational rearrangement brings all residues exhibiting exchange, except R55, T68, and K82, into close proximity with the peptide (Fig. 3B). K82 is located within a loop remote from the active site (21). Our relaxation data clearly indicate the involvement of K82 in substrate binding, as confirmed by a reduction in substrate affinity for the mutant Lys82→Ala82 (K82A) (38). As shown in Fig. 1B, additional backbone amides show chemical shift changes during turnover. However, these changes are smaller than those observed for the aforementioned residues; hence, their exchange contribution to R2 is below the present detection limit (Eq. 1).

Taking all results together, we can envision the enzymatic cycle of CypA as follows (Scheme 1 and Fig. 3): the substrate exists in the cis and trans conformations free in solution. Both isomers can bind to CypA. For the cis isomer, the areas around 101–103, 109, 82, and 55 are important in the binding process, which is close to diffusion-controlled. After binding, the enzyme catalyzes a rotation of the prolyl peptide bond by 180°. For this to occur, the substrate tail COOH-terminal to proline likely swings around to contact the area around residues 98 and 99 (Fig. 3), whereas the NH-terminal tail of the substrate stays fixed. In other words, the enzyme holds on to the substrate through this binding interaction. The isomerization step takes place with a rate constant of about 9000 s⁻¹, and motions of the protein coincide with the rate of substrate rotation. The major player in catalysis is R55, for which the observed changes in backbone conformation are likely to be coupled with motions of the catalytically essential side chain. After the isomerization step, the enzyme releases the substrate, which is now in trans, with a rate constant of about 13,000 s⁻¹.

The approach outlined here allows the identification of the dynamic “hot spots” during catalysis and reveals that the time scales of protein dynamics coincide with that of substrate turnover. However, it does not provide a detailed physical picture of the motions during catalysis. To do this, side-chain dynamics need to be included and, ultimately, all the relaxation data need to be used in molecular dynamics calculations. The application of relaxation measurements during substrate turnover promises to be of general use in understanding the dynamic behavior of enzymes and its relation to catalysis.

References and Notes
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Role of Nucleoporin Induction in Releasing an mRNA Nuclear Export Block

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Signal-mediated nuclear import and export proceed through the nuclear pore complex (NPC). Some NPC components, such as the nucleoporins (Nups) Nup98 and Nup96, are also associated with the nuclear interior. Nup98 is a target of the vesicular stomatitis virus (VSV) matrix (M) protein–mediated inhibition of messenger RNA (mRNA) nuclear export. Here, Nup98 and Nup96 were found to be up-regulated by interferon (IFN). M protein–mediated inhibition of mRNA nuclear export was reversed when cells were treated with IFN-γ or transfected with a complementary DNA (cDNA) encoding Nup98 and Nup96. Thus, increased Nup98 and Nup96 expression constitutes an IFN-mediated mechanism that reverses M protein–mediated inhibition of gene expression.

The Nup98 and Nup96 proteins are encoded by a single gene. The primary transcript is alternatively spliced, and the translation products are autocatalytically proteolyzed at one specific site (1–3). Nup98 interacts with an intranuclear protein and transport factors (5, 6). It is involved in nuclear import and export of proteins and RNAs (7–11) and is the target of the VSV M protein–mediated inhibition of mRNA export (12). A cDNA clone coding for part of the COOH-terminal sequence of Nup98 has been detected among mRNAs that were specifically induced by IFN-γ.

We found two classical elements, GAS and ISRE, that mediate increased gene expression by IFN (14, 15). When U937 cells were incubated with IFN-γ for up to 12

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