Conservation of flexible residue clusters among structural and functional enzyme homologues

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Background: It remains unclear whether structural homologues rely on similar concerted motions to promote enzyme function.

Results: Ribonuclease homologues display similar, contiguous clustering motions that can be modulated by mutagenesis.

Conclusion: Conformational flexibility can be conserved between distant structural homologues.

Significance: Controlling dynamics to modulate function has broad implications in protein engineering and allosteric drug design.

SUMMARY

Conformational flexibility between structural ensembles is an essential component of enzyme function. While the broad dynamical landscape of proteins is known to promote a number of functional events on multiple timescales, it is yet unknown whether structural and functional enzyme homologues rely on the same concerted residue motions to perform their catalytic function. It is hypothesized that networks of contiguous and flexible residue motions occurring on the biologically relevant millisecond timescale evolved to promote and/or preserve optimal enzyme catalysis. In this study, we use a combination of NMR relaxation dispersion, model-free analysis and ligand titration experiments to successfully capture and compare the role of conformational flexibility between two structural homologues of the pancreatic ribonuclease family: RNase A and eosinophil cationic protein (ECP, or RNase 3). In addition to conserving the same catalytic residues and structural fold, both homologues show similar yet functionally distinct clusters of millisecond dynamics, suggesting that conformational flexibility can be conserved among analogous protein folds displaying low sequence identity. Our work shows that the reduced conformational flexibility of ECP can be dynamically and functionally reproduced in the RNase A scaffold upon creation of a chimeric hybrid between the two proteins. These results support the hypothesis that conformational flexibility is partly required for catalytic function in homologous enzyme folds, further highlighting the importance of dynamic residue sectors in the structural organization of proteins.

The role of sequence and structure in defining enzyme function is a broadly accepted biological dogma (1). However, structure-function analyses remain very limited in their ability to predict and design new enzyme
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catalysts. Indeed, information about sequence and structure remains largely insufficient in providing a complete description of the intricate complexities that govern protein folding and enzyme function. Despite recent advancements in predicting folding mechanisms and catalytic function (2,3), current limitations partly stem from our lack of understanding of the molecular mechanisms that define protein behavior in space and time, i.e. as flexible and dynamic macromolecules (4,5). While classical computational design methodologies have typically considered proteins as homogeneously static structures (5), overwhelming theoretical and experimental evidence now advocates a more complex view of their reaction cycle. Among a few other methodologies, NMR relaxation experiments recently uncovered the existence of functionally relevant, low populated conformational states playing essential roles in the thermodynamics of ligand binding, substrate discrimination, active-site reorganization, and product release over the complete time course of an enzyme turnover (6-10). From an enzyme engineering perspective, designing efficient biocatalysts requires the modulation of flexibility events at the atomic scale to exert some control over enzyme function, further validating the need for a better characterization of the motional states found in natural enzymes, thus leading to a superior understanding of their precise role in catalysis (4,5). In many enzyme systems, conformational exchange has been shown to correlate with the timescale of enzyme turnover, suggesting that flexible networks of concerted residue motions are an integral part of the structure-function relationship in numerous protein architectures (7).

Theoretical studies have postulated the existence of functionally relevant clusters of dynamic residues, which appear to be conserved across evolutionary distant clans of enzyme superfamilies (11) and among members of the same enzyme fold (12). While these studies put forth compelling evidence suggesting that enzyme function is at least partly controlled by conformational exchange, the experimental validation of such hypotheses remains largely unexplored. Although the role of flexibility in enzyme function has been amply demonstrated on a case-by-case basis (7,9), is yet unknown whether residue motions on multiple timescales are evolutionary traits conserved among structurally and functionally similar homologues.

Having been studied for more than 50 years as a de facto protein model for biochemical and biophysical analyses (13-15), ribonucleases represent an excellent model system to investigate the evolutionary and dynamic processes that regulate catalytic function among enzyme homologues. RNase A is the founding member of the mammalian and vertebrate superfamily that bears its name, which comprises an extensive network of functionally distinct enzymes sharing invariant structural and catalytic elements (16). These enzymes all catalyze the transphosphorylation and subsequent hydrolysis of single-stranded RNA molecules (Fig. 1), but also carry broad and yet relatively uncharacterized biological activities. Initial sequencing of the human genome identified eight canonical members of this family (RNases 1-8), all of which preserving the same catalytic triad and displaying the analogous kidney-shaped tertiary fold of RNase A (17). These structurally similar enzymes catalyze such diversified activities as neurotoxicity, angiogenesis, immunosuppressivity or anti-pathogenicity, while still preserving varying degrees of ribonucleolytic activity. Though they do not all degrade RNA with the same catalytic efficiency, human ribonucleases require the strict conservation of important catalytic residues to perform their non-catalytic function (16). Interestingly, one of the few exceptions to this rule is eosinophil cationic protein (ECP, or RNase 3), a human canonical member for which a functional active site is not always required for biological function (18), much like in RNase 7 (19). ECP has been the subject of many studies over the past few years, mainly because of its potential use as an antibiotic and as a cytotoxic agent (20). While its biological function remains unclear, ECP has been shown to display antibacterial, neurotoxic, helminthotoxic, antiviral and cytotoxic activities, some of them independent of its ribonucleolytic activity (18).

Short- and long-range concerted residue motions occurring on the timescale of \( k_{cat} \) (~10^{-3} s) have been shown to correlate with
Flexible residue clusters in enzyme homologues of the ribonuclease superfamily. We used NMR relaxation dispersion, model-free analyses and titration experiments to show that both enzymes display similar millisecond dynamic residue clusters near their active sites, with relatively rigid and homogeneous backbone fluctuations on the ps-ns time frame. Interestingly, while motional clusters are structurally conserved between the two homologues, their respective global rates of conformational exchange ($k_{ex}$) vary significantly. As predicted by the absence of the functionally dynamic H48-T82 residue pair in ECP, this enzyme lacks the aforementioned loop1-$\beta$1-$\beta$4 network of coupled residue motions previously shown to be involved in the modulation of product release in RNase A. Additionally, this functionally essential network can be eliminated in RNase A by swapping loop 1 for that of ECP in the RNase A$_{ECP}$ chimera (22).

Our current motional investigation demonstrates that both ECP and a chimeric hybrid between RNase A and ECP (RNase A$_{ECP}$ (22)) show very similar dynamic behaviors, further highlighting the possibility of using mutagenesis to control residue flexibility and function through the dynamic conversion of one enzyme (RNase A) into another (ECP). Finally, our conformational investigation of ECP confirms previous RNase A observations suggesting that this fold can be dynamically divided into several independent regional flexible subdomains (or dynamic clusters).

The current study represents one of the very few experimental validations of similar conformational exchange in structurally related enzymes (26,27) and the first to compare similar mesophile homologues, confirming theoretical observations suggesting that specific motions can be evolutionarily conserved within and among structurally similar protein folds. The present work also demonstrates the importance of controlling millisecond dynamics to modulate protein function, a central concept with broad implications in protein engineering and allosteric drug design (4,5,28,29).

**EXPERIMENTAL PROCEDURES**

Cloning, expression and purification— *Escherichia coli* codon-optimized sequences of ECP and RNase A (GenScript) were subcloned...
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into NdeI/HindIII-digested expression vector pET22b(+). 15N-labelled samples were prepared by growing E. coli BL21(DE3) in M9 minimal medium and enzymes were purified as described by Boix (18) (ECP) and Doucet et al. (22) (RNase A). Protein concentration was determined using an extinction coefficient of 17,460 M\(^{-1}\)cm\(^{-1}\) (9,800 M\(^{-1}\)cm\(^{-1}\)) for ECP (18) (RNase A (30)).

Solution NMR experiments—All NMR experiments were recorded at 298 K on samples containing 0.2-0.7 mM 15N-labelled ECP in 15 mM sodium acetate, 10% \(\text{H}_2\text{O}\), pH 5.0, or 0.7 mM 15N-labelled RNase A in 5 mM MES-NaOH, 7 mM NaCl, 0.01% NaN\(_3\), 10% \(\text{H}_2\text{O}\), pH 6.4. NMR experiments were carried out on Varian (Agilent) 500 MHz and 800 MHz NMR spectrometers equipped with triple-resonance cold probes and pulsed field gradients. Backbone resonance assignments for apo ECP were taken from the Biological Magnetic Resonance Data Bank (BMRB entry 15757) and further confirmed with a 1H-15N TOCSY HSQC experiment.

Relaxation dispersion experiments—Backbone amide 15N-CPMG relaxation dispersion experiments were acquired on apo and saturated enzyme complexes using published sequences (31) and methods (22). Interleaved two-dimensional spectra were collected in a constant time manner with \(\tau_p\) CPMG repetition delays of 0.625, 0.714 (×2), 1.0, 1.25, 1.67, 2.0, 2.50 (×2), 3.33, 5.0, and 10 ms, using a total relaxation period of 40 ms. All NMR spectra were processed using NMRPipe (32), in-house CPMG scripts and analyzed with Sparky (33). Global residue fits and model analyses were performed by fitting 500 and 800 MHz CPMG dispersion data to the full single-quantum CPMG equation (34) using GraphPad Prism 5.

Fast timescale motions (ps-ns)—15N-\(R_1\), 15N-\(R_2\) and steady state heteronuclear NOE experiments were performed in an interleaved fashion at 500 MHz, as previously described (35,36). The \(R_1\) experiments were performed with relaxation delays of 10, 50, 100 (×2), 200, 350, 700, 1100, and 1400 ms. The \(R_2\) experiments were performed with relaxation delays of 10, 30, 50 (×2), 70, 90, 110, and 130 ms. The motional parameters on the faster ps-ns timescale were analyzed by fitting the NMR spin relaxation rates to the model-free formalism (reviewed in (37)) using the methodology for the dual optimization of the model-free parameters and the global diffusion tensor proposed by d’Auvergne (38) and implemented in the program relax 2.1.0 (38,39). The crystallographic structure of free ECP was used as starting coordinates (PDB 1QMT) and hydrogen atoms were added in DS Visualizer 3.5 (Accelrys). Values for the 15N chemical shift anisotropy (CSA) and N-H bond length were set at −172 ppm and 1.02 Å, respectively. Model selection for each residue and diffusion tensors were tested, optimized and selected using Akaike information criterion (AIC) as described in the relax manual. Errors were obtained from 500 Monte Carlo simulations.

NMR titration experiments—Freshly prepared 3'-UMP and 5'-AMP ligands were dissolved in ECP or RNase A NMR buffers. 1H-15N sensitivity-enhanced HSQC experiments were acquired at 800 MHz using spectral widths (points) of 1600 Hz (256) and 7000 Hz (8192) in the \(t_1\) and \(t_2\) dimensions, respectively. 1H-15N HSQC spectra were collected for titration points of ligand-enzyme ratios of 0, 0.174, 0.393, 0.691, 1.31, 2.71, 6, and 12. The pH was maintained constant by addition of 0.01M HCl, when necessary.

NMR line shape analysis—Analysis of NMR line shapes originating from titrations of ECP with 3’-UMP and 5’-AMP ligands was performed using the NMR line shape analysis module of the Integrative Data Analysis Platform (IDAP) (40). The 1D datasets for fitting were obtained as slices through the fast-exchange peaks in 1H-15N HSQC spectra using Sparky (33) with a custom Python extension (41). The 1D NMR line shape was simulated using Bloch-McConnell equations for a spin exchanging between two magnetic environments as described earlier (42,43). The line shapes were optimized to fit the experimental data by varying the thermodynamic equilibrium constant, the dissociation rate constant as well as the frequency of the bound complex using the IDAP code implemented in MATLAB (MathWorks). The standard errors were estimated from fitting of multiple line shape.
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RESULTS

Ligand binding interactions in ECP and RNase A—The structural and functional binding differences between ECP and RNase A were assessed by NMR titration experiments using the uracil-3′-monophosphate (3′-UMP) and adenosine-5′-monophosphate (5′-AMP) ligands (Fig. 1b,c). 3′-UMP and 5′-AMP are single nucleotide homologues of the end products of the UpA dinucleotide RNA substrate, providing good structural assessments of the individual ribonuclease subsites involved in substrate binding to the active site cavity in both enzymes. Ligands were individually titrated in 15N-labeled proteins and binding was followed by measuring chemical shift variations observed in 1H-15N Heteronuclear Single-Quantum Coherence (HSQC) spectra until saturation was reached. Affinities of 3′-UMP and 5′-AMP binding to ECP were estimated using line shapes observed in the 1H-15N HSQC titration experiments (41-43). Observation of ligand binding to the cognate sites in ECP was complicated by the apparent secondary binding events observed in both titrations, likely due to weak affinity of the nucleotides to a second non-specific site. To estimate affinity of the binding interaction with the cognate site in the 3′-UMP datasets, we selected residues that only responded to the main binding event in ECP, i.e. V125 and V127 (Fig. S1). The 5′-AMP binding has much weaker contribution from the second binding process, so residues demonstrating the most significant shifts were analyzed (H15, A110, and H128). Despite significant scatter of the global dissociation rate constants obtained in the individual fitting, the equilibrium dissociation constant ($K_d$) was accurately estimated in all cases (Fig. S1). Therefore, global fitting to obtain $K_d$ within each group was performed on ECP, resulting in dissociation constants of $340 \pm 30 \mu M$ for 5′-AMP and $460 \pm 100 \mu M$ for 3′-UMP (Table 1).

In both enzymes, the 3′-UMP and 5′-AMP nucleotides preferentially bind to the $B_1$ and $B_2$ subsites of the active site cavity, respectively. This provided a unique opportunity to characterize distinct atomic interactions involved in ligand discrimination in both structural homologues (Fig. 2). A total of 12 residues showed significant (> 0.1 ppm) 1H-15N weighted average chemical shift variations upon 3′-UMP binding to ECP, relative to 17 residues in RNase A (Fig. 2a,b). While the chemically affected residues are mostly located in the vicinity of the $B_1$ subsite and are directly involved in binding and/or catalysis in RNase A (e.g. H12, K41, T45, H119) (Fig. 2b), their functional equivalents in ECP (H15, K39, T42 and H128) do not show significant chemical shift perturbations upon 3′-UMP binding (Fig. 2a). If 3′-UMP does bind to the $B_1$ subsite of ECP as expected, this would illustrate that the high sequence conservation of active site residues in the two homologues does not translate into similar pyrimidine recognition, partly accounting for the significantly reduced binding affinity of 3′-UMP in ECP (Table 1). Pyrimidine contacts with T45 and D83 are required for optimal product release in RNase A (24). However, no D83 equivalent can be found in ECP, and T42 (the T45 equivalent) is completely unaffected by 3′-UMP binding. Additionally, residues displaying important chemical shifts upon 3′-UMP binding are scattered throughout the ECP structure, contrasting with the relatively bundled group of clustering residues in RNase A (Fig. 2b).

Based on their motional behavior (vide infra) and secondary structure elements (44), the two structural homologues were divided into four distinct regional residue clusters encompassing loop 4 (cluster 1), the $\beta_2$ sheet (cluster 2), the $\beta_1$ sheet and adjacent loop 1 (cluster 3), and loops 2 and 6 (cluster 4) (Fig. 3). Interestingly, the contiguous residues displaying important chemical shift variations upon pyrimidine binding to RNase A (T45, F46, T82, T100, Q101, all part of cluster 3) also show important conformational exchange on the millisecond timescale and are known to be involved in the propagation of motions between...
loop 1 and the active site during turnover (22,23). Coupled to the chemical shift differences induced by 3′-UMP binding, this observation strongly suggests that long-scale millisecond motions connecting the active site to loop 1 (cluster 3) are involved in the binding/release of pyrimidine ligands in RNase A. Although long-scale chemical shift variations are also observed in ECP upon pyrimidine binding—e.g. residues Q58, R75 and R77, located respectively 20.2, 18.0 and 14.7 Å away from the ligand—residues of cluster 3 in ECP are not similarly responsive and no contiguous residue network is affected upon ligand binding to this enzyme (Fig. 2). Additionally, residues of the ECP cluster 3 (β-sheet and adjacent loop 1) are almost completely devoid of significant chemical shift perturbations upon 3′-UMP binding to the B2 subsite, suggesting that concerted long-range interactions linking loop 1 to the active site are not functionally important in ECP.

Purine binding to the B2 subsite also yields important chemical shift differences between these two structural homologues. While the chemical environment of only 4 residues is significantly perturbed upon 5′-AMP binding to ECP, 14 residues are affected when this ligand binds to RNase A (Fig. 2c-d). The magnitude in the 1H-15N weighted average chemical shift variations is also stronger in RNase A than ECP. Only the immediate vicinity of the B2 subsite is chemically affected in presence of 5′-AMP in ECP, including the important catalytic residues H64 and H128. While H128 is required for the hydrolytic step of the reaction, H64 (located in loop 4) directly interacts with P0 during turnover and is thought to act as the functional equivalent to K66 in RNase A (45). The most striking differences observed upon 5′-AMP binding to ECP and RNase A occur for residues of clusters 1 and 2 (Fig. 3), more specifically for residues in loop 4 (cluster 1). While residues of loop 4 show considerable chemical shift variations upon 5′-AMP binding to RNase A, only H64 is affected when this purine binds to ECP. This loop faces the active site and acts as an arm that holds the purine ligand in position (Fig. 2d). Interestingly, loop 4 is the only dynamic cluster experiencing conformational exchange in both the free forms of ECP and RNase A, albeit with distinct conformational exchange rates (vide infra).

Fast timescale analysis (ps-ns)—While most proteins interact and catalyze reactions on a time frame much slower than nanoseconds, the kinetics and thermodynamics underlying such larger conformational motions may rely on faster timescale dynamics (21). In an effort to decipher the importance of such fast motions in ECP and to compare them with the previously characterized ps-ns motions in RNase A (35), we fitted the 15N-R1, 15N-R2 and heteronuclear NOE spin relaxation data to the new approach for the dual optimization of the model-free parameters and the global diffusion tensor proposed by d’Auvergne and Gooley (38,39). The spin relaxation behavior of 112 out of the 121 non-proline residues of ECP could be reliably quantitated. Residues F43, R45, N53, Q58, N70, R73, F76, C83 and L129 were removed from the analysis because of low signal intensity, spectral overlapping and/or because they were absent from the 1H-15N HSQC. The average values of the relaxation rates are $R_1 = 1.13 \pm 0.13$, $R_2 = 14.04 \pm 1.78$, and NOE = 0.478 ± 0.71 (Fig. S2).

Much like in RNase A, the ECP heteronuclear NOE values at the N- and C-terminus of the protein are similar to those in the rest of the protein. This was expected because of the structural similarity in the protein architecture, which relies on the N-terminal helix and C-terminal sheet packing for binding and activity (35).

The model-free calculated order parameters ($S^2$) are used to characterize the amplitude of the internal motions of the 1H-15N vector on the ps-ns timescale, and thus provide a measure of atomic scale residue flexibility on this particular time frame (46-48). As a result, a completely unrestricted 1H-15N bond vector would show a $S^2$ value of 0 while a fully rigid one would display an order parameter of 1. The generalized $S^2$ parameters determined from model-free fitting are plotted as a function of residue number in Fig. 4. The calculated order parameters are relatively high and uniform across the sequence, much like with RNase A (35). However, the average value of $S^2 = 0.740 \pm 0.052$ indicates that ECP is a bit more flexible than the very rigid RNase A on the ps-ns timescale, which displays an average $S^2 = 0.910$.
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Interestingly, the catalytic residue K38 in ECP, which is strictly conserved among pancreatic RNases, displaces one of the highest order parameters in the protein \( S^2 = 0.848 \pm 0.026 \), along with its very rigid neighbor N39 \( S^2 = 0.907 \pm 0.055 \). K38 is thought to be involved in the stabilization of the excess negative charge on the phosphoryl oxygens in the transition state during RNA cleavage (15), which may require rigidity in ECP. On the contrary, the two most flexible residues, V78 \( S^2 = 0.571 \pm 0.062 \) and D84 \( S^2 = 0.452 \pm 0.039 \), are at the beginning and the end of the \( \beta \)2 strand, respectively. Neither was shown to play any particular role in enzyme function.

For a number of residues that do not fit to simple calculation models, a \( R_{ex} \) exchange parameter is calculated to achieve an adequate fit to the model-free analysis. This \( R_{ex} \) parameter accounts for contributions to \( R_2 \) that potentially describe motions occurring on the slower \( \mu \text{s-} \text{ms} \) timescale (Fig. 4). Much like with RNase A, a large number of residues throughout the ECP sequence require the addition of this exchange contribution (26 out of the 112 residues analyzed), suggesting the existence of motions occurring on the slower \( \mu \text{s-} \text{ms} \) timescale in this enzyme. This observation is in line with our slow timescale analysis of ECP performed by \(^{15}\text{N-CPMG} \) (vide infra), most notably for residues of loop 4 (cluster 1, Fig. 3), which display some of the most important \( R_{ex} \) values in the protein (Fig. 4). Interestingly, the highest \( R_{ex} \) observed is for residue H64 \( R_{ex} = 4.22 \pm 1.53 \text{ s}^{-1} \), the structural equivalent to K66 in RNase A, which is also one of the most flexible residues of RNase A on the millisecond timescale (Fig. 5c). Overall, while ECP is a bit more flexible than RNase A on the ps-ns time frame, both enzymes show relatively rigid and homogeneous backbone fluctuations on this particular timescale, in addition to similar flexible residue sectors on the millisecond timescale (vide infra).

Conserved networks of flexible millisecond residue clusters—We investigated the apo and ligand-bound millisecond motions experienced by both structural homologues using solution NMR relaxation dispersion experiments \((^{15}\text{N-CPMG}) \) (10). \(^{15}\text{N-CPMG} \) experiments allow the characterization of an equilibrium exchange process experienced by the \(^1\text{H}-^{15}\text{N} \) bond vector by recording the transverse relaxation rate constant, \( R_2 \), as a function of \( \tau_{cp} \), an inter-pulse delay in the CPMG pulse train (49). \(^{15}\text{N-CPMG} \) experiments thus enable the detection of millisecond dynamics in proteins caused by internal conformational exchange, ligand binding, and/or the chemical reaction (for reviews see (9,50-52)). When \( R_2 \) values are plotted as a function of \( 1/\tau_{cp} \), the characteristic dispersion of \( R_2 \) values reveals residues experiencing conformational exchange on the millisecond timescale (Fig. 5). On the contrary, \(^{15}\text{N} \) nuclear spins that do not experience motions on this particular time frame do not show any dispersion (flat line profiles). In addition to this qualitative assessment, the fitted relaxation dispersion curves can further be quantitatively analyzed to extract structural information of the excited state (differences in chemical shifts, \( \Delta \omega \)) as well as population dynamics (exchange rates, \( k_{ex} \), and equilibrium populations, \( p_{A}, p_{B} \)) (see ref. (49) and references therein).

Our results demonstrate that ECP and RNase A reveal four clusters of dynamic residues closely mapping the structural clusters shown in Fig. 3. We also observed that conformational exchange in these clusters is significantly different between the two proteins (Fig. 5). In both homologues, similar contiguous residues experiencing conformational exchange interact with their structural neighbors to form bundles of cross-talking networks that transfer millisecond motions over structurally distinct clusters and subdomains of the protein. Although ECP and RNase A display different catalytic activities and binding properties, these two structural homologues nevertheless constrain millisecond dynamics to these four dynamic clusters instead of displaying randomly scattered ms dynamics throughout the protein structure. These localized dynamic clusters suggest that conformational exchange may have evolved through the formation of confined and contiguous networks of coupled motions to support common function and/or that ms dynamics may play distinct roles in the biological function of these structural and functional homologues. This clustering of ms dynamics into contiguous yet independent motional sectors may thus serve as a point of
comparison to analyze functional differences between enzyme homologues.

A direct comparison of the dynamic clusters between RNase A and ECP indicates that some residue clusters experiencing conformational exchange in one protein homologue are almost entirely silenced in the other. This can be observed for the core residues of clusters 2 and 3. For instance, 12 residues of cluster 2 show considerable conformational exchange in ECP (Fig. 5a), a region that is almost completely devoid of ms dynamics in RNase A (Fig. 5c). Simultaneous fitting of the all-timescale, two-state Carver-Richards equation (34) to the spin-relaxation data obtained at 500 and 800 MHz for all ECP residues of cluster 2 yielded a global exchange rate constant \(k_{ex}\) of \(728 \pm 104\) s\(^{-1}\). Inversely, while cluster 3 motions are entirely absent from ECP, 11 nearly contiguous residues linking the active site to loop 1 in RNase A show a global \(k_{ex}\) of \(1438 \pm 125\) s\(^{-1}\) (Fig. 5c). Cluster 1 is the only protein sector showing important conformational exchange in both structural homologues. Relaxation dispersion is observed for 5 and 6 residues of loop 4 in RNase A and ECP, respectively. While the global exchange rate of loop 4 \(k_{ex} = 1548 \pm 82\) s\(^{-1}\) parallels the product release and catalytic rates in RNase A \((k_{off} = k_{cat})\), the global exchange rate for loop 4 is more than three times slower in ECP \(k_{ex} = 504 \pm 44\) s\(^{-1}\) and appears to be unrelated to \(k_{cat}\) (18).

Interestingly, cluster 3 in RNase A encompasses the same long-scale residues experiencing chemical shift variations upon pyrimidine binding (Fig. 2b), in line with previous hypotheses linking motions of this cluster to product release in RNase A (22-25). However, efforts to correlate free and 3'-UMP- or 5'-AMP-bound \(^{15}\)N chemical shift variations \((\Delta \delta \text{ ppm})\) with \(^{15}\)N chemical shift differences between the major and the minor excited state obtained from \(^{15}\)N-CPMG \((\Delta \omega \text{ ppm})\) remained inconclusive (Fig. S3). In an ideal case, \textit{i.e.} when the excited state of the enzyme corresponds to the product-bound form obtained by a shift in the reaction equilibrium (in this case saturation with 3'-UMP or 5'-AMP product analogues), one should expect a perfect linear correlation between the \(^{15}\)N chemical shift differences of the NMR titrations \((\Delta \delta \text{ ppm})\) and the \(\Delta \omega \text{ ppm} \) obtained from \(^{15}\)N-CPMG. The absence of such linear correlation implies additional perturbations caused by the arrival of the ligands and/or that the conformational exchange we observe by \(^{15}\)N-CPMG is unrelated to transitions between free and product-bound states in both ECP and RNase A. The observation of exchange at certain sites may be reflecting or providing a measure of ground state destabilization that is essential for catalysis but unrelated to the functional importance of the excited state in product release. The integrity of such motional networks nevertheless remains critical for optimal enzyme catalysis in both homologues, as was previously demonstrated elsewhere (22-25,41) and in the present study (vide infra).

The RNase \(A_{ECP}\) chimera: translating RNase A dynamics into those of ECP—Some of us have previously shown that cluster 3 residues in RNase A propagate motions from loop 1 (residues 14-24) to \(\beta\) strands 1 and 4, both of them containing the important active site residues T45 and D83 involved in RNA pyrimidine stabilization at the active site (22-25). These motions correlate to those of the distant loop 1 and affect the rate-limiting step of RNase A catalysis by limiting the rate of product release. This was demonstrated by creating a chimeric hybrid between RNase A and ECP in which loop 1 in the former is replaced by loop 1 of the latter (generating the RNase \(A_{ECP}\) chimera), affecting both the millisecond dynamics and activity of the enzyme (22). Not only is loop 1 conformationally restrained and much shorter in ECP than in RNase A (6 residues in the former \textit{vs.} 12 in the latter), ECP also lacks the important H48-T82 residue pair that we showed essential for the propagation of ms motions in this dynamic cluster (23). As mentioned above, cluster 3 residues are completely devoid of conformational exchange in ECP (Fig. 5a), highlighting a different dynamic landscape in this protein.

Originally not apparent from the original chimera study (22), one of the most impressive observations of the global dynamic comparison between ECP and RNase A is the motional similarity between ECP and the swapped mutant of RNase A (RNase \(A_{ECP}\), Fig. 5a,b). Despite conserving 90% sequence identity with RNase
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A, the RNase A_{ECP} chimera almost perfectly mirrors the ms dynamics of ECP. Indeed, the conformational exchange of cluster 3 residues is completely dampened in the RNase A_{ECP} hybrid. Conversely, millisecond dynamics are acquired for contiguous residues in cluster 2 (Fig. 5b), a sector that is almost completely devoid of ms motions in RNase A (Fig. 5c). Even a few residues of cluster 4 now experience conformational exchange in this chimeric hybrid, a protein cluster that is otherwise very rigid in RNase A.

Consequently, replacing the distant loop 1 in RNase A not only reduces ligand binding by one order of magnitude (22), it also affects the entire conformational flexibility of the enzyme by translating the dynamic landscape of WT RNase A into that observed in WT ECP. These results demonstrate that mutagenesis can be used to selectively modulate conformational exchange in proteins, a prerequisite to exert efficient control over motional residue networks affecting function in protein engineering and drug design applications (4,5,29).

Relationship between ligand binding and internal dynamics—To clarify the effect of ligand binding on the collective dynamics of each structural homologue, we also performed $^1$H-$^1$N-CPMG experiments in the presence of saturating concentrations of 3′-UMP and 5′-AMP (Fig. 6). For both enzymes, ligand-bound complexes show that conformational exchange is still segregated to the same four clusters as those observed for the unbound forms. Using relaxation dispersion measurements, it may be possible to show how internal conformational equilibrium is shifted by addition of a ligand. This “population shift” (6) has been demonstrated in RNase A by comparing frequency differences between major and minor dynamic states between the free and ligand-bound enzyme (41). This analysis requires very high quality relaxation data because the frequency difference of exchanging conformers is most difficult to obtain in the fast-exchange regime (53). To attempt this analysis in ECP, we performed global fittings of residues in each dynamic individual clusters (1 and 2) such that $k_{ex}$ and populations were shared while the frequency difference was individual for each residue. The results were filtered on the basis of the coefficient of determination ($R^2 > 0.9$) to ensure that only good fits are used in the analysis. Due to spectral overlap induced by ligand binding and variable data quality, only residues C62 and T67 (cluster 1) were available in the free and ligand-bound forms for comparison. For C62, we determined that the frequency difference between major and minor conformers was in the 95% confidence interval of 320–450 s$^{-1}$ for apo ECP and 300–530 s$^{-1}$ for the ECP-3′-UMP complex. For T67, the corresponding ranges for the same complexes were 180–240 s$^{-1}$ and 190–290 s$^{-1}$, respectively. Similarity of the frequency differences between conformations in the free and bound forms of ECP may indicate that the same type of population shift is induced by the ligand in ECP as the one observed in the earlier RNase A report (41).

Our NMR results nevertheless point out to significant motional distinctions in the behavior of ECP and RNase A upon ligand binding. The most striking effect is the gaining and dampening of conformational exchange in the aforementioned motional clusters regardless of the RNA ligand investigated. Indeed, while 3′-UMP and 5′-AMP considerably rigidify the ECP structure upon binding (Fig. 6a,b), the opposite is true for RNase A, for which an increase in the total amount of residues displaying relaxation dispersion can be observed (Fig. 6c,d). A total of 11 (8) and 14 (9) residues rigidify upon 3′-UMP and 5′-AMP binding to ECP (RNase A), respectively (Figs. S4 and S5). However, the increase in conformational exchange upon ligand binding is considerably more pronounced in RNase A than ECP. This is particularly true for the 3′-UMP complex (Fig. 6c), in which ligand binding induces conformational exchange for all residues of $\alpha_2$ facing the hydrophobic core of the enzyme (residues 25-34, cluster 4). Globally, only 1 and 3 residues gain ms dynamics when 3′-UMP and 5′-AMP bind to ECP, respectively. This number jumps to 12 and 8 residues when the same two ligands bind to RNase A. The increase in RNase A ms dynamics upon ligand binding is also supported by the disappearance of several $^1$H-$^1$N HSQC backbone resonances due to line broadening in the hinge region of cluster 3 (Fig. 6c,d, grey spheres). Overall, these observations
are consistent with the previously suggested ligand-dependent induction of hinge motions between the two β-sheets (54), a distinctive feature of RNase A that is undetectable in ECP.

The comparison between residues experiencing nitrogen chemical shift variations and the effect they exert on conformational exchange upon ligand binding also yields interesting distinctions between ECP and RNase A (Figs. S4 and S5). For instance, the strictly conserved catalytic lysine (K41 in RNase A, K38 in ECP) is thought to be involved in the stabilization of the excess negative charge on the phosphoryl oxygens in the transition state during RNA cleavage (15). Surprisingly, while the flexible K41 in RNase A shows significant chemical shift variations in the presence of 3’-UMP, K38 is not at all affected by ligand binding and does not show any conformational exchange in ECP (Fig. S4). While K41 gains conformational exchange in the RNase A-nucleotide complex, this result is not observed in ECP. This different binding and dynamic behavior for such an important catalytic residue may partly explain the important differences observed in the catalytic rates of these two RNases (18), which is also supported by the restricted $^1$H-$^{15}$N bond vector of K38 on the ps-ns timescale. Also, it is interesting to point out that F46, one of the most critical aromatic residues for RNase A stability and folding (55-57), is both flexible in the apo and 3’-UMP-bound forms of RNase A (Fig. S4). Although very highly conserved in the RNase family and positioned in the exact same orientation, no such flexibility is observed for F43 in ECP. These observations suggest that very similar functional homologues may still use local dynamics for very different purposes in defining function and stability.

DISCUSSION

Most of the work investigating the importance of conserved functional dynamics among enzyme family homologues was historically achieved through computational analyses. These studies typically use coarse-grained models such as normal mode analysis (NMA) to infer catalytic timescale motional similarities between protein folds (58). Generally, these studies also involve the direct comparison of simulated data to crystallographically- and/or NMR-resolved enzymes in apo and ligand-bound forms, inferring dynamic information through—but not limited to—residual dipolar coupling measurements and/or Debye-Waller factors (B-factors) (59). The latter provides a rough approximation of residue flexibility in protein crystals, albeit with no clear definition of dynamic timescale.

Theoretical studies have delivered a breadth of relevant information on slow timescale conservation of functionally relevant motions in many enzyme families and protein folds, including—but not limited to—the amino acid kinase family (59), aspartate proteases (11), oxidoreductases, and peptidyl-prolyl isomerases (12). Nonetheless, experimental validation of such theoretical observations remains elusive and very few experimental studies have yet provided a clear portrait of catalytically relevant dynamic conservation between structural and functional protein homologues. The present work partly provides such validation by showing that two members of the pancreatic-like ribonuclease family sharing similar catalytic mechanisms and protein folds also retain similar ps-ns dynamics and conformational exchange on the ms timescale. The present study also validates the existence of dynamic clusters—similar to the evolutionary sectors of Ranganathan and coworkers (60,61)—which transcend the classical definition of primary, secondary, or tertiary protein architectures. We observe that millisecond dynamic clusters are sequestered into distinct protein subdomains and, as previously suggested, appear to be structurally encoded and ‘fine tuned’ by the protein fold (Fig. 5) (60). The presence of a dynamic subdomain (cluster 3) that transfers millisecond motions essential to product release in RNase A is absent from ECP. Despite conserving the same dynamic cluster architecture as that of RNase A, experimental evidence illustrates that ECP is a more conformationally restrained homologue on the ms timescale.

Previous theoretical studies have suggested the existence of collective motions in
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the pancreatic-like ribonuclease family using molecular dynamics simulations (62,63). Until very recently, such analyses remained confined to the very fast timescales that can be probed by MD simulations (ps-ns), a time frame that is still very far from enzyme turnover (ms). To overcome such barrier, Ramanathan and Agarwal recently used quasi-harmonic analysis (QHA) to model large-scale conformational fluctuations occurring on slower timescales in this enzyme family, providing the most complete theoretical comparison of slow dynamics between ribonuclease homologues so far (12). Focusing on the motional comparison of three pancreatic-like ribonuclease homologues from human, bovine and rat, the authors show remarkable interspecies similarities in the slow motions of surface loops and distal motifs in this enzyme family, observations that transcend sequence identity. The present investigation offers the first experimental validation of the existence of such theoretical networks between two members of the pancreatic ribonuclease family.

The conservation of clustering sectors of coevolving residues among family members and functional homologues has been suggested as a potentially important structural determinant defining biological function in proteins. Ranganathan and coworkers have used statistical coupling analyses of sequence alignments to argue that the classical hierarchy of primary, secondary, tertiary, and quaternary structures used to define protein architecture remains largely insufficient to explain the three-dimensional cooperativity observed between coevolving residues among protein homologues (60). Much like the confined assemblies of dynamic residue clusters highlighted in the present study, the investigators argue that non-random correlations in the physical connectivity between coevolving groups of residues in the 3D structure of the S1A serine protease family underlies the biological function of its members, a property they also observed in the PDZ, PAS, SH2 and SH3 domain families (60). These groups of coevolving residues—termed sectors (60,61)—show striking physical connectivity in the 3D structure of protein families, while completely transcending classical subdivisions of primary, secondary or tertiary subdomain architectures. These sectors, which show stark three-dimensional similarity with the dynamic clusters observed in the present work, are shown to be structurally independent and confined to functional regions among the serine protease family, encompassing residues involved in binding or catalysis (60). Most interestingly, Ranganathan and coworkers also observe a direct correlation between these coevolving sectors and the networks of residues undergoing conformational fluctuations associated with enzyme catalysis in dihydrofolate reductase (DHFR) (61). Similar to the experimentally characterized clusters in the present work, the authors show that dynamic motions associated with catalysis in DHFR extend well beyond the active site environment, dynamically connecting surface sites on both ends of the enzyme through long-range networks of dynamic residues that crosstalk on the catalytic timescale (61).

The present study, coupled to our previous demonstration that RNase A relies on a 20 Å communication link between the active site and loop 1 for optimal catalysis (22-25), suggests that the Ranganathan sectors of coevolving residues may represent a conserved mechanism to initiate allosteric regulation on protein surfaces (61,64). We experimentally uncovered a similar architecture between two homologues of the ribonuclease fold, further lending support to the potential evolutionary conservation of motional networks defining function in protein families. Systematic analysis of correlations between dynamics and function for important protein families is likely to provide novel insights into the structure-function-flexibility relationship that defines protein architectures. This knowledge is required for successful de novo enzyme design (4,5,29) as well as for the development of new allosteric drugs (28).
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**Table 1.** Binding affinities of 3′-UMP and 5′-AMP to RNase A and ECP.

|         | $K_d$ 3′-UMP (µM) | $K_d$ 5′-AMP (µM) |
|---------|------------------|------------------|
| RNase A | 9.7 ± 0.9a       | 124.0 ± 0.9b     |
| ECP     | 460 ± 100c       | 340 ± 30c        |

*a* Taken from ref. (65); *b* Taken from ref. (66); *c* Estimated from NMR line shape analysis (see Experimental Procedures).
FIGURE LEGENDS

FIGURE 1. Substrates and products of a ribonuclease reaction. (a) Schematic representation of a single-stranded RNA molecule binding to the active site of eosinophil cationic protein (ECP). Phosphate and base binding subsites for RNA substrates are defined as Pn and Bn, of which the P0-2 and B1-2 subsites are shown (18). The phosphodiester bond scission occurs at the P1 subsite (arrow) and is universally catalyzed by two strictly conserved histidine residues: His15 and His128 (ECP numbering). In ECP, uracil is preferred over cytosine in the B1 subsite (U > C), a preference that is reversed in RNase A (C > U). Adenosine is almost universally required at the B2 subsite. (b) Schematic representation of the uracil-3'-monophosphate ligand (3'-UMP). (c) Schematic representation of the adenosine-5'-monophosphate ligand (5'-AMP).

FIGURE 2. 1H-15N chemical shift variations induced by 3'-UMP and 5'-AMP binding to ECP and RNase A. Weighted average chemical shift differences (Δδ) for (a, b) 3'-UMP and (c, d) 5'-AMP are mapped on the primary sequence of (a, c) ECP and (b, d) RNase A. The 1H-15N weighted average composite chemical shift differences (Δδ) were calculated between free and ligand-saturated enzymes according to the following equation (67): \( \Delta \delta = \left( \frac{\Delta \delta_{HN}^2 + \Delta \delta_{N}^2}{25}/2 \right)^{1/2} \). The position of 3'-UMP and 5'-AMP (black sticks) is displayed on the three-dimensional structure of RNase A (PDB 1O0N) and is predicted on ECP (PDB 1H1H) based on a structural overlay with RNase A. Yellow spheres represent residues with Δδ > 0.1 ppm.

FIGURE 3. Dynamic residue clusters in ECP and RNase A. The two ribonuclease homologues are divided into four distinct residue clusters covering the following secondary structure elements (44): loop 4 (cluster 1, purple), β2-sheet (cluster 2, yellow), β1-sheet with adjacent loop 1 (cluster 3, orange), and loops 2 and 6 (cluster 4, green). The four color-coded clusters are highlighted on the 3D structure of (a) ECP (PDB 1H1H) and (b) RNase A (PDB 1O0N), with encompassing residues listed. The 3'-UMP ligand is shown in red. (c) Expresso (68) sequence alignment of ECP and RNase A showing the position of conserved residues and secondary structure elements forming the dynamic clusters. Sequence numbering is that of RNase A.

FIGURE 4. Model-free calculated S2 and Rex parameters plotted on the sequence of ECP. The generalized order parameters (S2) provide a measure of atomic scale flexibility of the 1H-15N bond vector on the ps-ns timescale. The predicted Rex parameter accounts for contributions to R2 that potentially describe motions occurring on the slower µs-ms timescale.

FIGURE 5. Conformational exchange is confined to four independent yet contiguous dynamic clusters in structural and functional ribonuclease homologues. Conformational exchange was investigated in the apo forms of ECP, RNase A and the chimeric hybrid RNase AEC, in which the RNase A loop 1 (D146STSAASSSNNY25, in red) was replaced by that of ECP (S17LNPPR22, ECP numbering) (22). Catalytic timescale (ms) residue motions were probed using 15N-CPMG relaxation dispersion experiments at 500 and 800 MHz (298K) for the apo forms of (a) ECP, (b) RNase AEC, and (c) RNase A. Residues were considered for further analysis only if the difference in measured R2 (ΔR2 (1/τcp)) values at fast (τcp=0.625 ms) and slow (τcp=10 ms) pulsing rates was greater than 2 s-1, similar to previous reports (22,35). Blue spheres represent residues displaying 15N-CPMG dispersion with ΔR2 (1/τcp) > 2 s-1. Residues are highlighted on the 3D structure of (a) ECP (PDB 1H1H) and (b, c) RNase A (PDB 1O0N). Representative relaxation dispersion curves (800 MHz, 298K) are shown for structurally equivalent positions of ECP (RNase A) found in cluster 1: H64 (K66) and L68 (Q69); cluster 2: A110 (A109) and D130 (D121); and cluster 3: F48 (F46) and H82 (D83). Flexible residues experiencing 1H-15N bond vector motions on the millisecond timescale display a relaxation dispersion curve while residues...
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with no motion on this particular time frame display flat relaxation profiles. Swapping loop 1 of RNase A by that of ECP converts the dynamic clusters of the former into those of the latter. RNase A$_{ECP}$ data was taken from ref. (22).

**FIGURE 6.** Conformational exchange induced by 3′-UMP and 5′-AMP binding to ECP and RNase A. Catalytic timescale (ms) residue dynamics were probed using $^{15}$N-CPMG relaxation dispersion experiments for (a, c) 3′-UMP-bound and (b, d) 5′-AMP-bound forms of (a, b) ECP and (c, d) RNase A. Blue spheres: residues showing $^{15}$N-CPMG dispersion profiles with a $\Delta R_2 (1/\tau_{cp}) > 2$ s$^{-1}$; orange spheres: residues showing no conformational exchange relative to the apo form (i.e., dampened ms dynamics upon ligand binding); green spheres: residues gaining conformational exchange [$\Delta R_2 (1/\tau_{cp}) > 2$ s$^{-1}$] upon ligand binding; grey spheres: assigned residues in the apo form that cannot be assigned in the ligand-bound form due to line broadening. Residues are highlighted on the 3D structure of ECP (PDB 1H1H) and RNase A (PDB 1O0N). The 3′-UMP and 5′-AMP ligands are shown in red.
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Figure 1

a

b
c

Figure 2

ECP

RNase A

Δδ (ppm) / 3'-UMP

Δδ (ppm) / 5'-AMP

Residue

Residue

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Figure 3
Figure 4
Figure 5

Figure 6
Conservation of flexible residue clusters among structural and functional enzyme homologues

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