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Article

# Correlated Evolution of Low-Frequency Vibrations and Function in Enzymes

Tushar Modi, Paul Campitelli, Matthias Heyden,* and S. Banu Ozkan*

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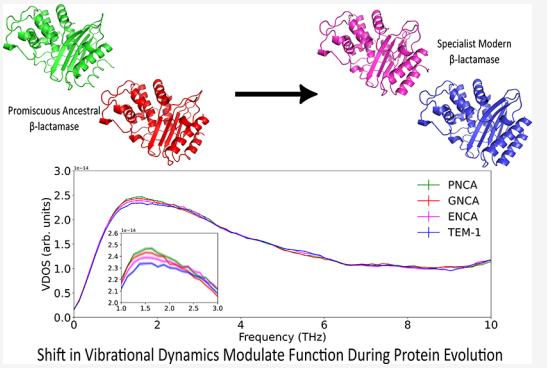
**ABSTRACT:** Previous studies of the flexibility of ancestral proteins suggest that proteins evolve their function by altering their native state ensemble. Here, we propose a more direct method to analyze such changes during protein evolution by comparing thermally activated vibrations at frequencies below 6 THz, which report on the dynamics of collective protein modes. We analyzed the backbone vibrational density of states of ancestral and extant $\beta$-lactamases and thioredoxins and observed marked changes in the vibrational spectrum in response to evolution. Coupled with previously observed changes in protein flexibility, the observed shifts of vibrational mode densities suggest that protein dynamics and dynamical allostery are critical factors for the evolution of enzymes with specialized catalytic and biophysical properties.



Shift in Vibrational Dynamics Modulate Function During Protein Evolution

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## INTRODUCTION

Allostery describes long distance interactions between different regions of a protein that orchestrate conformational dynamics necessary for its function. Classically, allostery is often described in the context of ligand binding at a distal, allosteric site that affects the activity of a functional site through conformational changes, allowing remote functional regulation.[1,2] Such mechanisms are of prime importance in the regulation of biochemical processes relevant for the cellular metabolism,[3] transcription,[4,5] signal transduction,[2,6−8] and so on. However, allosteric mechanisms can be generalized beyond ligand binding. The modulation of local interactions by other phenomena, such as mutations at residue positions distal from an active site of an enzyme, can affect substrate binding and/or catalytic activity.[9−13]

The current understanding of allostery is derived from the ensemble model of proteins[10] inspired from Cooper's model of dynamic allostery.[14] It describes that the modulation of normal modes through allosteric ligand binding can alter the underlying energy landscape within a native ensemble without significantly changing the average conformation of the protein. Recent studies on reconstructed ancestral proteins also suggest that nature may use the same dynamic allostery principles for protein evolution. Comparative computational studies of ancestral proteins and their modern homologues have shown that mutations far from the functional site can alter protein conformational dynamics, resulting in the emergence of new functions or the fine-tuning of pre-existing functions while the 3D fold remains conserved.[15,17−19]

In our previous studies, we have used our protein-dynamics based metric, Dynamic Flexibility Index (DFI)[20] to analyze how changes in the conformational dynamics modulate the function during evolution of several different types of protein systems such as green fluorescent protein (GFP),[17] $\beta$-lactamase,[19,21] and thioredoxin (Thrx).[16] DFI is a position-specific metric that combines molecular dynamics (MD) simulations with linear response theory to quantify the relative flexibility of a residue with respect to the rest of residues in the protein. Using DFI analysis, we have shown that proteins can adapt to new environments or enhance their enzymatic activity via a hinge shift mechanism associated with altered flexibilities of key residues. Particularly, an increased flexibility of certain rigid sites is compensated by the rigidification of other distal flexible sites.[15,18,21] Furthermore, during evolution, rather than directly mutating active site residues, distal mutations modulate the flexibility of active sites to accommodate novel noncognate substrate recognition while conserving the 3D fold.[15,16,18,22] The mutations utilize the anisotropic nature of the 3D network of interactions between protein residues to alter the relative population of the accessible conformations in their native state ensemble.[15,16,18] Indeed, this is nothing but similar to ligand-binding-induced dynamic allostery where changes in the network of interactions due to binding modulate the native state ensemble without having any observable effect on the native state conformation.

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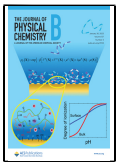

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In this study, we extended our analysis of ancestral enzymes and their modern homologues to the protein vibrational density of states (VDOS) at THz frequencies, which reports on thermally activated vibrational modes. The spectrum of THz vibrations described by the VDOS represents a dynamic signature of the protein potential energy surface, which is sensitive to alterations of protein dynamics, e.g., upon ligand binding.[23,24] A straightforward decomposition of the VDOS into contributions from individual residues further provides information that is complementary to protein flexibility by including dynamic information on the picosecond and subpicosecond time scale. Combined, the DFI and the VDOS allow us to identify evolutionary mechanisms that utilize dynamic allostery to modulate or evolve enzyme functionality.

## METHODS

We started with equilibrium molecular dynamics simulations of each protein at constant temperature and pressure at 300 K and 1 bar for 1.5 $\mu$s using the AMBER molecular dynamics simulation package[25] following the protocol described in.[15]

The first 1.0 $\mu$s of these trajectories was considered as equilibration, and we used the final 0.5 $\mu$s to identify the 10 most relevant conformations using a $k$-means clustering algorithm based on the protein backbone root mean squared deviation (RMSD). In each case, the minimum RMSD between distinct cluster centers was confirmed to be larger than 0.8 Å. For each of the clusters, we randomly selected 1000 starting configurations (coordinates and velocities) to initiate independent simulations in the microcanonical ensemble for our analysis.

For each of the resulting 10,000 starting configurations, we performed 100 ps constant energy simulations where the velocity information for each atom was recorded every 8 fs. We focused the analysis of the VDOS on vibrations of the protein backbone, specifically the $C_\alpha$ atoms, to isolate collective protein dynamics from local vibrations of amino acid side chains. To obtain contributions to the protein VDOS, we first computed ensemble averaged velocity time auto correlation function for the $C_\alpha$ atom of each protein residue $i$ with a maximum correlation time of 4 ps.

$$C_{\alpha,i}(t) = \langle \vec{v}_i(0) \cdot \vec{v}_i(t) \rangle \tag{1}$$

The corresponding contributions to the total protein VDOS were then obtained via the Fourier transform of the individual correlation functions.

$$I_{\alpha,i}(\omega) = \frac{1}{3k_B T} \int_{-\infty}^{\infty} C_{\alpha,i}(t)\, e^{i\omega t}\, dt \tag{2}$$

Ensemble averages were defined as averages over all correlation time windows within the 10,000 NVE simulations obtained for each protein. For the intensity of the VDOS at each sampled frequency, we computed the standard error of the mean, which allowed us to verify the statistical significance of differences in the $C_\alpha$ VDOS between ancestral and extant enzymes.

The individual per-residue $C_\alpha$ VDOS can be simply added to obtain the total VDOS of all protein $C_\alpha$ atoms, which describes the full spectrum of vibrational modes of the protein backbone.

## RESULTS AND DISCUSSION

We previously performed extensive comparative protein dynamics studies of ancestral and extant homologues of $\beta$-lactamase and Thrx enzymes using the DFI analysis. In both cases, minor variations in enzyme structure during evolution were insufficient to explain major changes in enzymatic activity, substrate specificity and thermal stability. Instead, we observed pronounced changes in the flexibility of key residues not associated with the catalytic site. These findings indicated dynamic allostery as a critical mechanism for the emergence of new or the adaptation of existing enzyme functions during evolution.[15,16,22]

Here, we performed additional MD simulations of the reconstructed ancestral proteins and their modern counterparts for both enzyme families to further investigate how mutations alter the dynamics of the protein native state ensemble during enzyme evolution. Specifically, we analyzed the VDOS of protein $C_\alpha$ atoms and the contributions from individual residues to identify global and local changes in the vibrational dynamics at frequencies below 6 THz. Notably, vibrations in this frequency range are thermally activated at room temperature ($h\nu \leq k_B T$) and include collective vibrations of secondary structure elements and protein domains.[26,27] In essence, the VDOS describes a distribution of kinetic energy over the frequency domain and can be interpreted as the number of vibrational modes or degrees of freedom per frequency unit. The integral of the VDOS describes the sum of all vibrational modes/degrees of freedom, which are normalized for comparisons.

Low-frequency vibrations are associated with large masses (i.e., collective motion of large secondary structure elements or protein subdomains), weak restraining forces resulting in large amplitude motion, or both. In contrast, higher frequency vibrations are associated with either local vibrations of smaller units (i.e., side-chains or individual functional groups) and/or larger restraining forces that limit the amplitude. Thus, comparing the VDOS of two homologous proteins provides us with information on the collective character of vibrational modes and their associated forces, which is complementary to the DFI analysis. In the following, we compare previous findings from our DFI analysis with the total $C_\alpha$ VDOS for ancestral and extant members of the studied enzyme families.

**Modulation of Vibrational Spectra During Protein Evolution.** The total $C_\alpha$ VDOS of the ancestral and extant enzymes in the $\beta$-lactamase (Figure 1) and Thrx families (Figures S1 and S2) exhibit common features known also for other proteins.[24,28] Most prominent is a broad band of vibrations with a maximum between 1.5 and 2 THz. Thus, the VDOS for ancestral and extant enzymes appear similar at first glance. However, even small variations such as shifts of vibrational modes from high to low frequencies are consequential, because the mode density in this frequency region (i.e., the number of affected degrees of freedom) is high. Furthermore, while the underlying vibrations are likely anharmonic,[29] the quantum harmonic oscillator partition function, which can be used as an analytical model system to describe thermodynamic properties of vibrations, is sensitive to shifts in frequency in this low-frequency region.[30,31] Therefore, we highlighted subtle variations between the VDOS of the distinct enzyme variants near the maximum, which exceed the level of statistical uncertainty despite their small magnitude. All VDOS shown include statistical error bars, but due to extensive sampling in our simulations, the error bars are too small to be easily recognized (see Figure 1).

The ancestral precambrian $\beta$-lactamases (the Gram-negative bacteria ancestor, GNCA, and Gram-positive and Gram-


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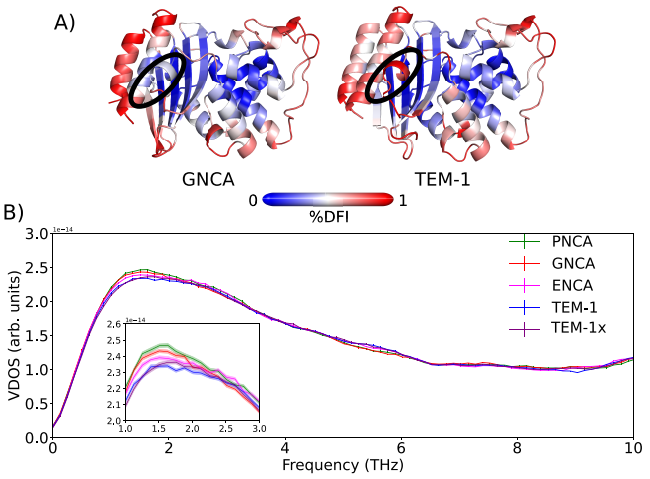


**Figure 1.** Evolution of local flexibilities and low-frequency vibrations in $\beta$-lactamases. Mutations accumulated through evolution fine-tune enzymatic function without considerable changes in structure. (A) Instead, the mutations alter local flexibilities as shown by the DFI-coded color of the secondary structure cartoons for ancestral GNCA and extant TEM-1 $\beta$-lactamases. The color describes relative variations of the per-residue DFI in both enzymes. The comparison of the ancestral and extant enzymes highlights localized changes in flexibility that accompany evolutionary changes in enzyme function. (B) The vibrational spectra characterized by the protein $C_\alpha$ VDOS indicates increased mode densities at low frequencies (1.5 THz) for ancestral enzymes PNCA, GNCA, and ENCA, which are converted into less collective higher frequency modes for the extant enzyme TEM-1 and its modern homologue. The VDOS in the vicinity of the dominant vibrational band from 1.5 and 2 THz is highlighted in the inset. Minor statistical uncertainties are shown as error bars in the main plot and as shaded regions in the inset.

negative bacteria ancestor, PNCA) differ from their modern counterpart in their function. The ancestors are moderately efficient promiscuous enzymes capable of degrading a diversity of $\beta$-lactam antibiotics. In contrast, the modern $\beta$-lactamases TEM-1 and its closer ancestral homologue in enterobacteria (ENCA) are specialized to degrade a simple substrate, penicillin, with a higher efficiency. This generalist to specialist enzyme evolution involved more than 100 mutations that alter the flexibility profile of the enzyme (Figure 1A), while conserving the 3D fold and the catalytic residues. In Figure 1B, we observed that PNCA and GNCA $\beta$-lactamase exhibit a higher mode density in the VDOS at 1.5 THz compared to TEM-1 and ENCA $\beta$-lactamases. The excess mode density at 1.5 THz for the ancestral enzymes is accompanied by a decreased mode density between 5 and 6 THz. This shift indicates a higher collectivity (increased reduced mass) of vibrations and/or weaker force constants of specific vibrational modes.

To extend our data set for modern $\beta$-lactamases, we repeated our analysis for simulations of a second modern TEM-1 homologue (PDB: 1xpb, here referred to as TEM-1x), which functions similar to TEM-1 (i.e., shows preferential activity against penicillin). The $C_\alpha$ VDOS of TEM-1x is nearly identical to the $C_\alpha$ VDOS of TEM-1, supporting our hypothesis that changes in the $C_\alpha$ VDOS are associated with changes in function.

Differences in the $C_\alpha$ VDOS between the promiscuous ancestral and specific modern $\beta$-lactamases support our previous findings[15,16,22] that mutations at distal sites modulate substrate specificity via dynamic allostery. Similar results are observed in other studies, which showed that vibrational modes of the enzyme native state ensemble are sensitive to, for example, ligand binding.[24,32,33]

We also observed changes in the $C_\alpha$ VDOS for enzyme variants in the Thrx family, which have evolved their functions to adapt to lower temperatures and less acidic conditions (higher pH) while conserving their 3D fold.[16] In Figure S1, we compared the extant Thrx enzyme from *Escherichia coli* (*E. coli*) to the Last Bacterial Common Ancestor (LBCA), the Last Common Ancestor of the Cyanobacterial, Deinococcus, and Thermus group (LPBCA), and the Last $\gamma$-Proteobacteria Common Ancestor (LGPCA) Thrxs. Interestingly, the extant *E. coli* enzyme exhibits an increased mode density in the entire low-frequency region from 0 to 10 THz. The latter is accompanied by a decrease in the mode density at higher frequencies (beyond 10 THz) and thus indicates an increased fraction of thermally activated, low frequency modes in the extant bacterial enzyme compared to its ancestors. We further compared the modern human enzyme to its Archeal Eukaryotic Common Ancestor (AECA), the Last Eukaryotic Common Ancestor (LECA), and the Last Animilia and Fungi Common Ancestor (LAFCA) in Figure S3. Here, a frequency shift of low-frequency modes at 1 THz in AECA toward higher frequencies for the LECA and LAFCA enzymes can be observed, which in the extant human enzyme leads to a wide shoulder of enhanced mode density at 3 THz. In contrast to the evolutionary changes in the VDOS of bacterial enzymes toward *E. coli*, the evolution of the extant human enzyme resulted in a decrease of the lowest frequency modes around 1 THz and an increase in the number of intermediate frequency modes at 3 THz, which still remain thermally activated at room temperature.

In summary, we find increased mode densities in the low-frequency tail of the dominant vibrational band in ancestral homologues of $\beta$-lactamase and human Thrx, which can be associated with global collective modes. With increasing specialization in extant enzymes, low-frequency collective modes are replaced by higher-frequency modes, which indicate less collective vibrations, larger effective force constants, and narrower potentials.

A distinct and somewhat opposing trend is observed for bacterial Thrx enzymes. Here, the mode density for the extant enzyme in *E. coli* is increased across the entire low-frequency range. Notably, the extant Thrx enzyme in *E. coli* also stands out from other enzymes in its phylogenetic tree on the basis of its very low catalytic rate and thermal stability, which will be discussed later.[16]

**$C_\alpha$ VDOS Captures Shifts in the Dynamics of Individual Protein Residues during Evolution.** In previous work, the analysis of local protein flexibility using the DFI indicated a hinge-shift mechanism in the evolution of protein function.[15,17,18] Specifically, the mutations accumulated during enzyme evolution led to increased local flexibilities in specific sites, while formerly flexible sites are rigidified. This effectively shifts the location of hinges in the protein structure that are critical for collective dynamics in the enzyme. Here, we further analyzed the $C_\alpha$ VDOS of individual protein residues to explore whether these dynamical changes are also reflected in the local contributions of these sites to the low-frequency vibrational spectrum of the protein.


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First, we focused our analysis on conserved hinge residues, which not only maintained the identity of their amino acid side-chain during evolution but also conserved a low flexibility. We defined such sites via a %DFI score of less than 0.2, which indicates a hinge in the ancestral and extant enzymes. Such residues have a high significance for orchestrating motions throughout the protein. In Figure 2, we show that the

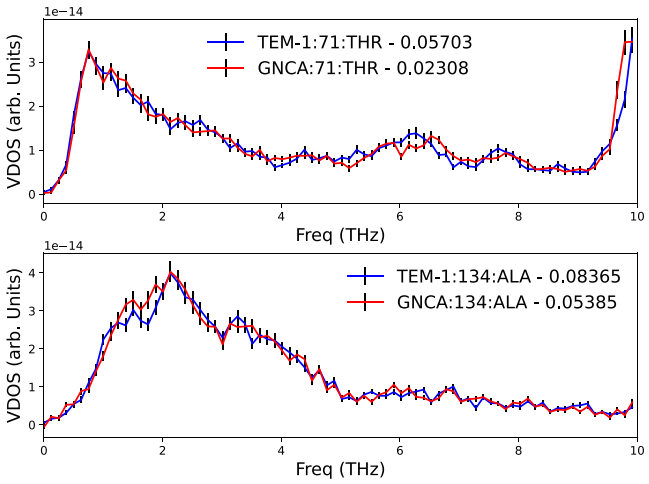


**Figure 2.** $C_\alpha$ VDOS for conserved $\beta$-lactamase residues with low flexibility (%DFI score) in the ancestral (GNCA) and extant (TEM-1) enzymes. Shown are the $C_\alpha$ VDOS for Thr71 (top panel) and Ala134 (bottom panel) and the corresponding %DFI scores are indicated in the legends. Error bars indicate the standard error of the mean at each frequency.

corresponding $C_\alpha$ VDOS for two selected residues are essentially unchanged in the ancestral GNCA and the extant TEM-1 $\beta$-lactamase. On a single residue level, minor changes of the VDOS intensity at a given frequency are less relevant than for the total $C_\alpha$ VDOS of the entire enzyme analyzed in Figures 1, S1, and S2, because only three degrees of freedom are involved. A similar analysis for Thrx enzymes is shown in Figure S4.

The observation that conserved low flexibility for a given residue results in a conserved contribution to the vibrational spectrum, demonstrates the complementarity of the information provided by the DFI and the $C_\alpha$ VDOS.

Second, we compared the $C_\alpha$ VDOS of $\beta$-lactamase residues that underwent a large change in local flexibility during evolution, while the identity of the side-chain remained conserved. These residues have either lost flexibility, thereby becoming a rigid hinge (i.e., $\%DFI_{GNCA} > 0.3$ and $\%DFI_{TEM\text{-}1} < 0.2$) or have gained flexibility (i.e., $\%DFI_{GNCA} < 0.2$ and $\%DFI_{TEM\text{-}1} > 0.3$). The results for residues Ala187 and Ile279 are shown in Figure 3, and the corresponding DFI are indicated in the legend.

Upon comparison, we observed that residues with a gain in flexibility (Ile279) exhibit a red-shift of vibrational modes at THz frequencies (i.e., a decrease of the mode density at high frequencies (3−4 THz) and an increase at low frequencies (2 THz)). Vice versa, residues with a net loss of flexibility (Ala187) exhibit a blue-shift of vibrational modes at THz frequencies (i.e., a decrease of the mode density at low frequencies (2−3 THz) and an increase at high frequencies (5 THz)). This behavior was also observed for conserved Thrx residues in bacterial and human lineages that have undergone

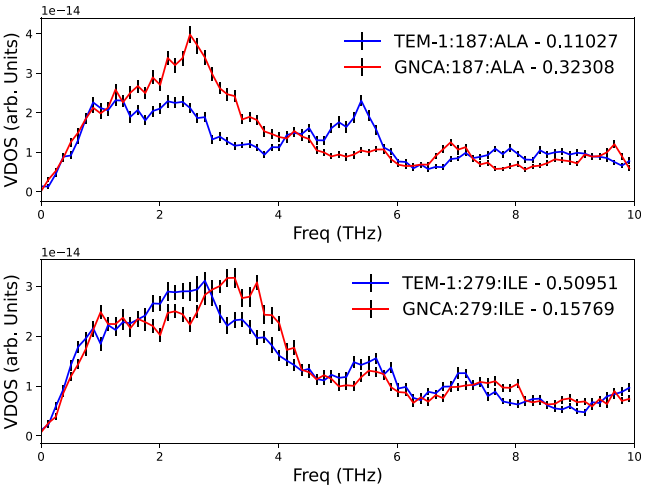


**Figure 3.** $C_\alpha$ VDOS for conserved $\beta$-lactamase residues with distinct flexibilities (%DFI score) in the ancestral (GNCA) and extant (TEM-1) enzymes. Shown are the $C_\alpha$ VDOS for Ala187 (top panel), which exhibits a loss of flexibility in the extant enzyme, and Ile279 (bottom panel), which exhibits an increased flexibility in the extant enzyme. The corresponding %DFI scores are indicated in the legends. Error bars indicate the standard error of the mean at each frequency.

flexibility shifts during evolution (see Figure S4). These findings support the interpretation of frequency shifts in the local VDOS of individual residues as a complementary measure of flexibility and contribution to protein dynamics.

However, changes in the $C_\alpha$ VDOS are not always straightforward to interpret in terms of changes in local flexibility or collectivity of vibrations. An example is shown in Figure S5 for the residues Lys74 and Val261 in the GNCA and TEM-1 $\beta$-lactamases. Here, the changes in the vibrational spectrum are more complex and include superimposed red- and blue-shifts of multiple vibrational bands at distinct frequencies as well as differences in the overall mode density in the THz frequency range.

Therefore, we used an approximate expression of for the vibrational entropy to obtain a quantitative expression that includes contributions from all vibrational frequencies from the $C_\alpha$ VDOS of a given residue. For this purpose, we considered the VDOS as a continuous spectrum of harmonic oscillators, which allowed us to calculate the vibrational entropy from the quantum harmonic oscillator partition function (see the SI).[30,31] The use of the quantum harmonic oscillator model for anharmonic vibrations likely results in an underestimation of the entropy for each degree of freedom, while the implied treatment of independent oscillators ignores correlations and thus overestimates the entropy. The result should thus not be interpreted as an exact measure of vibrational entropy, but as a quantitative parameter that allows us to interpret complex changes in the $C_\alpha$ VDOS of individual residues during evolution.

Using this approach, we computed single residue vibrational entropies along the full sequence of the ancestral and extant $\beta$-lactamases (PNCA, GNCA, ENCA, and TEM-1) as well as for the ancestral and extant Thrx proteins from bacterial (LBCA, LGPCA, LPBCA, *E. coli*), Archea (AECA, LACA), and human (LAFCA, LECA, human) branches of the phylogenetic tree. Based on this information, we compiled the residue-level vibrational entropies for both enzyme families to identify


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correlated changes during evolution and investigate their relevance for enzyme function.

For this purpose, we used principal component analysis and isolated the three Eigenvectors with the largest variance. In the three-dimensional space defined by the selected principal components (PC), we then computed the distances between the distinct enzymes and performed hierarchical clustering. The resulting dendrograms, which illustrate the closeness of the individual enzymes in the space defined by the three PCs, are shown in Figure 4A,C.

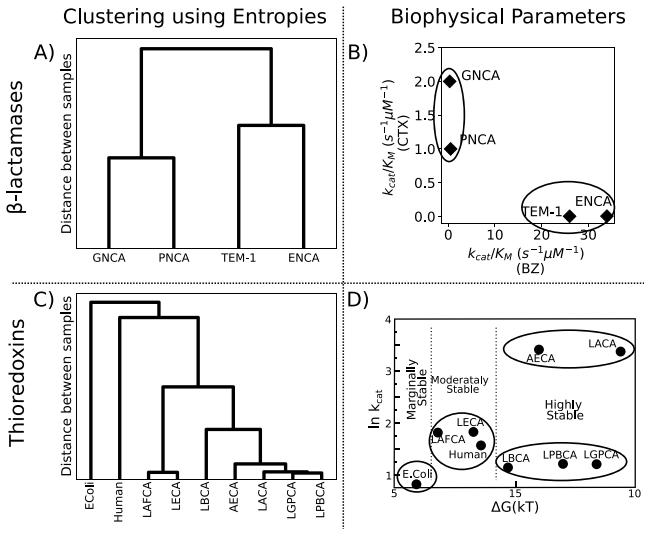


**Figure 4.** Comparison between hierarchical clusters defined by vibrational and biophysical properties for ancestral and extant $\beta$-lactamase and Thrx enzymes Hierarchical clusters obtained after projection of the residue-resolved vibrational entropy for each enzyme into a low-dimensional space of principal components identifies groups of $\beta$-lactamases (A) according to their substrate-specificity (B) and Thrx enzymes (C) according to their thermal stability (D).

The hierarchical clustering based on variations of the vibrational entropy within the enzyme sequence reproduces similarities of biophysical parameters of the enzymes related to enzyme function and stability. For the $\beta$-lactamases, we used the catalytic efficiency, $k_{cat}/k_M$, for the hydrolysis of two distinct substrates, the antibiotics benzylpenicillin (BZ) and cefotaxime (CTX),[19] to characterize the distinct enzymes as shown in Figure 4B.

The GNCA and PNCA $\beta$-lactamases are both promiscuous in their activity toward these two antibiotics and exhibit a moderate catalytic efficiency for the hydrolysis of CTX ($k_{cat}/k_M \approx 1\ s^{-1}\ \mu M^{-1}$) and BZ ($k_{cat}/k_M \approx 1\ s^{-1}\ \mu M^{-1}$). This similarity in their function is captured by the hierarchical clustering analysis in Figure 4A based on the per-residue vibrational entropy. In contrast, the TEM-1 and ENCA $\beta$-lactamases are both highly specific in their activity to hydrolyze the antibiotic BZ as shown by their high catalytic efficiency ($k_{cat}/k_M \approx 20\ s^{-1}\ \mu M^{-1}$), while no significant activity is observed for the hydrolysis of CTX. Both enzymes are again grouped together by our hierarchical clustering analysis based on per-residue vibrational entropies, which indicates that the $C_\alpha$ VDOS, and the vibrational entropy derived from it, contains functionally relevant information. An identical result was obtained in previous work on $\beta$-lactamases, in which the per-residue DFI was used for the hierarchical clustering analysis.[19] Thus, residue-resolved DFI and VDOS provide complementary information on enzyme dynamics relevant for protein function in $\beta$-lactamases, specifically substrate specificity and catalytic efficiency.

For the Thrx enzymes, we used the unfolding free energy, $\Delta G$, to describe enzyme thermal stability, and their catalytic rate constant for disulfide reduction, $k_{cat}$, as biophysical parameters as shown in Figure 4D. The ancestral Thrx enzymes from Archea (AECA and LACA) and bacteria (LBCA, LPBCA, and LGPCA) exhibit a high thermal stability and were grouped together in the hierarchical clustering analysis. The Archea LACA and AECA enzymes feature much larger catalytic rate constants than any of the other enzymes. The hierarchical clustering based on vibrational entropies identifies them as similar to the bacterial Thrx ancestors with the exception of LBCA. This indicates that the VDOS of the Thrx enzymes reports primarily on thermal stability and is less sensitive to $k_{cat}$. The ancestral (LECA and LAFCA) and extant human Thrx enzymes exhibit a moderate thermal stability and the ancestral human enzymes form their own group in the hierarchical clustering analysis. The extant human and *E. coli* Thrx enzymes exhibit distinct vibrational properties from any of the ancestral homologues based on our clustering and each forms its own singular group. The extant *E. coli* enzyme stands out because it exhibits the lowest thermal stability and lowest $k_{cat}$ of all Thrx enzymes studied here.

The insensitivity of the hierarchical clustering based on vibrational properties to the $k_{cat}$ of Thrx enzymes contrasts a previous study, which applied a comparable approach using residue-resolved DFI's.[16] In that case, the hierarchical clusters grouped Thrx enzymes based on both, the thermal stability and $k_{cat}$.[16] Consequently, the information provided by the VDOS and the DFI is complementary but not identical.

The previous work based on residue-resolved flexibilities (DFI) and the present analysis of low-frequency vibrations show that protein dynamics, in addition to protein structure, are a key factor for enzyme evolution.[34−37] The present study showed specifically that changes in catalytic activity (i.e. substrate specificity) and enzyme thermal stability are strongly correlated with changes in the vibrational spectrum at THz frequencies within distinct enzyme families.

## CONCLUSION

In this study, we used the low-frequency vibrational spectrum of the protein backbone at THz frequencies, quantified via velocity time correlation functions and the corresponding VDOS of $C_\alpha$ atoms, to analyze changes in protein dynamics during enzyme evolution. We illustrated that the VDOS at THz frequencies provides a measure of protein dynamics complementary to other parameters describing local or global protein flexibility, such as the DFI. For this purpose, we compared ancestral and extant enzyme homologues in two enzyme families, $\beta$-lactamases and thioredoxins. In both cases, evolution has significantly altered the catalytic and biophysical properties of the enzymes, while the three-dimensional structures are maintained. The analysis showed that the residues which exhibit a gain in their flexibility (through DFI analysis) during evolution show a corresponding gain in the intensity of modes at lower frequency (within 1−4 THz) for the systems studied here. Similarly, an opposite behavior is observed in the VDOS for residues with a loss in flexibility (see Figure 2).


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These results showed that the vibrational spectrum at THz frequencies is sensitive to changes in protein flexibility and collectivity of vibrational modes, which are reflected by frequency shifts in the vibrational mode density. Moreover, we demonstrated that residue-level entropies derived from the vibrational spectra can be used to classify enzymes with distinct catalytic activities and thermal stability.

Analyzing and characterizing evolutionary changes in protein dynamics in conjunction with associated changes in enzymatic function can only be the first step toward understanding the underlying mechanisms. The latter requires the identification of collective degrees of freedom that determine protein dynamics on time scales that exceed the length of standard all-atom simulations. However, the residue-level analysis based on the DFI and the VDOS of $C_\alpha$ atoms discussed in this work provides an excellent starting point by identifying dynamical hotspots in the protein sequence (i.e., residues that contribute significantly to changes in dynamics).

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpcb.2c05983.

Calculation of per residue entropic contribution using their VDOS; total $C_\alpha$ VDOSs of the ancestral and extant enzymes in human and bacterial branches; comparison of modern human enzyme to its AECA, LECA, and LAFCA; corresponding $C_\alpha$ VDOS for two selected residues are essentially unchanged in the ancestral GNCA and the extant Thrx; changes in local flexibility or collectivity of vibrations for the residues Lys74 and Val261 in the GNCA and TEM-1 $\beta$-lactamases (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Authors

**Matthias Heyden** − *School of Molecular Sciences, Arizona State University, Tempe, Arizona 85287, United States*; Email: mheyden1@asu.edu

**S. Banu Ozkan** − *Department of Physics, Arizona State University, Tempe, Arizona 85287, United States*; orcid.org/0000-0002-9351-3758; Email: Banu.Ozkan@asu.edu

### Authors

**Tushar Modi** − *Department of Physics, Arizona State University, Tempe, Arizona 85287, United States*

**Paul Campitelli** − *Department of Physics, Arizona State University, Tempe, Arizona 85287, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jpcb.2c05983


### Notes

The authors declare no competing financial interest.

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