A hierarchy of coupling free energies underlie the thermodynamic and functional architecture of protein structures

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ABSTRACT

Protein sequences and structures evolve by satisfying varied physical and biochemical constraints. This multi-level selection is enabled not just by the patterning of amino acids on the sequence, but also via coupling between residues in the native structure. Here, we employ an energetically detailed statistical mechanical model with millions of microstates to extract such long-range structural correlations, i.e. thermodynamic coupling free energies, from a diverse family of protein structures. We find that despite the intricate and anisotropic distribution of coupling patterns, the majority of residues (>70%) are only marginally coupled contributing to functional motions and catalysis. Physical origins of 'sectors', determinants of native ensemble heterogeneity in extant, ancient and designed proteins, and the basis for allostery emerge naturally from coupling free energies. The statistical framework highlights how evolutionary selection and optimization occur at the level of global interaction network for a given protein fold impacting folding, function, and allosteric outputs.

1. Introduction

Protein sequence and structural evolution are driven by a multitude of factors arising from requirements for efficient folding, function, stability, regulation and inter-molecular interactions, to name a few. Every random mutation that modulates the physico-chemical property of an amino-acid could influence any of the biophysical selection factors, thus influencing organismal fitness. Of these, foldability and stability are critical as any mutation that reduces the folded population will quickly in fold (Gassner et al., 1996; Riddle et al., 1997; Kurnik et al., 2012; adjust and accommodate substitutions while still maintaining the native conformational states in the native ensemble. One avenue to generate forces that are modulated (through mutations) resulting in improved catalytic efficiency, promiscuity or adaptability to a particular condition (Dougherty and Arnold, 2009; Goldsmith and Tawfik, 2012; Risso et al., 2018; Gupta and Varadarajan, 2018; Bolognesi et al., 2019; Hochberg (Dougherty and Arnold, 2009; Goldsmith and Tawfik, 2012; Risso et al., 2018; Gupta and Varadarajan, 2018; Bolognesi et al., 2019; Hochberg and Thornton, 2017).

While functional readouts of large-scale mutagenesis studies are becoming increasingly possible, understanding the origins of functional changes on mutations from the perspective of basic thermodynamic forces will enable the targeted design of proteins with altered or specific functionalities. To design natural-protein-like enzymes with marginal stability and specific activity, it is necessary to understand how residues in the native structure are 'thermodynamically coupled' to each other and the magnitude of this coupling, which in turn determines epistasis or context-dependence of mutations (Domíngo et al., 2019; Horovitz et al., 2019). The extent of thermodynamic coupling of a residue arises not merely from the number of direct contacts but also via the second- or third-shell effects through intervening residues and their interactions (Yang et al., 2016; Rajasekaran et al., 2017a, 2017b). Importantly, the statistical nature of the protein with numerous conformational states lends itself to more subtle effects in coupling; specifically, if two residues i and j are always folded in a sub-ensemble or a collection of states, it contributes to a coupling free energy in the thermodynamic sense, even if they are distant in the native structure, as perturbation of i will necessarily influence j (Hilser et al., 1998).

Thermodynamic coupling free energies are, however, challenging to estimate given the diversity and the weak non-covalent nature of the numerous intra-molecular interactions in a protein, and the multitude of conformational states in the native ensemble. One avenue to generate

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this is via molecular simulations (coarse-grained or all-atom), where conformations are accumulated as a function of time to construct a native ensemble. However, sampling limitations and the long time-scales associated with structural transitions in large proteins (milliseconds or slower) would mean that a reasonable statistical description of a protein's conformational space is not straightforward from this method. An alternate possibility is to employ statistical mechanical treatments of protein folding, wherein a fixed ensemble of conformational states is constructed through physically reasonable assumptions thus enabling access to the total partition function and hence the relative population of states. In this regard, earlier attempts at estimating coupling free energies from statistical treatments employed the COREX algorithm and empirical energies derived from the accessible surface area of conformations (Hilser et al., 1998, 2006). On the other hand, the Wako-Saito-Muñoz-Eaton (WSME) model (Wako and Saito, 1978; Muñoz and Eaton, 1999) provides an attractive and alternate avenue to estimate thermodynamic coupling free energies employing a single model are sufficient detailed enough to reproduce changes in stability and folding mechanisms arising from mutations involving charged residues (Naganathan, 2013), apolar residues (Rajasekaran et al., 2017b), post-translational modifications (Gopi et al., 2015) and disordered loop insertions (Gopi et al., 2015).

In this work, we show how the WSME model can be used to simultaneously generate native ensembles, understand folding mechanisms and estimate thermodynamic coupling free energies employing a single structure as an input. Our results highlight the robustness and tunability of interaction network in proteins, and provide a complementary viewpoint to sequence- and function-based methods with implications in protein structural organization, design and allostery.

2. Methods

2.1. Wako-Saito-Muñoz-Eaton (WSME) model

The WSME model employs a binary treatment for the folded status of residues with 1 and 0 representing folded and unfolded conformations, respectively. While many flavors of the WSME model are available (Wako and Saito, 1978; Muñoz and Eaton, 1999; Naganathan, 2012; Gopi et al., 2015; Henry and Eaton, 2004; Bruscolini and Naganathan, 2011), we employ the bWSME model (with b standing for block, a set of consecutive residues (Gopi et al., 2019)) that partitions the protein’s phase space into single stretches of folded residues or blocks (single-sequence approximation, SSA), two stretches of folded residues or blocks (double-sequence approximation, DSA) and DSA with interaction across the folded islands even if the intervening residues or blocks are unfolded (DSAw/L, i.e. DSA with loop). The block-size can vary from 1 to 5, depending on the number of residues in the protein, to reduce the number of microstates that can otherwise number in several hundreds of millions (Fig. 1A and B). For example, generating the residue-level conformational landscape of Kemp Eliminase (299 residues) would require the algorithmic enumeration of more than 661 million states. However, treating a set of 5 consecutive residues as a block reduces the number of conformations to just over 2 million making the calculations less intensive a coarse-graining that does not affect the conformational landscape or the folding mechanism (Gopi et al., 2019).

The total partition function (Z) of the bWSME model is calculated as

\[
Z = \sum_{i=1}^{n} \exp(-\Delta G_i / RT)
\]

where \( n \) is the total number of microstates considered in the model framework (SSA, DSA and DSAw/L), \( R \) is the gas constant and \( T \) is the temperature, which is set to 310 K. Free energy of microstates with folded structure between and involving blocks \( p \) and \( q \) (\( p, q \)) is:

\[
\Delta G_{p,q} = \Delta G_{p,q}^{\text{block}} - T \Delta S_{conf}^{\text{block}}
\]

The stabilization free energy for the microstate (\( p, q \)) is written as,

\[
\Delta G_{p,q}^{\text{block}} = \sum_{j=1}^{q} \sum_{i=1}^{p} \sum_{k-L(i)-j}^{k-L(i)-j} \sum_{l<k} \Delta G_{ij}^{\text{vdW}}
\]

where \( L \) is the set of residues comprising the protein, \( L(i) \) and \( L(j) \) represent the set of residues in blocks \( i \) and \( j \), respectively, while also accounting for interactions between residues within the same block. The stabilization free energy \( \Delta G_{p,q}^{\text{block}} \) includes contributions from van der Waals interactions (a uniform interaction energy \( \xi \) for the vDW contacts identified from the native structure with a 5 Å cut-off; supporting Table S1), charge-charge interactions without a distance cut-off via the Debye-Hückel formalism (at a fixed ionic strength of 0.1 M, with an effective dielectric constant of 29 for soluble proteins (Naganathan, 2012; Naganathan, 2013) and 4 for membrane proteins) and contacts-scaled implicit solvation term (Naganathan, 2012) (\( \Delta G_{p,q}^{\text{vdW}} \) calculated as the heat capacity change per native contact \( \Delta C_p^{\text{vdw}} \) that is fixed to \( -0.36 \text{ Jmol}^{-1}\text{K}^{-1} \) per native contact). The entropic penalty associated with fixing all residues in the microstate (\( p, q \)) is given as,

\[
\Delta S_{p,q}^{\text{conf}} = \sum_{j=1}^{q} \sum_{i=1}^{p} \Delta S_{ij}^{\text{conf}}
\]

here, \( \Delta S_{ij}^{\text{conf}} \) is the entropic cost of fixing residue \( j \) in the native conformation (set to \( -14.5 \text{ Jmol}^{-1}\text{K}^{-1} \) per residue), \( L(i) \) includes the set of residues within block \( i \). An excess entropic penalty (Rajasekaran et al., 2016) (\( \Delta \Delta S = -6.1 \text{ Jmol}^{-1}\text{K}^{-1} \) per residue) is additionally assigned to residues identified as unstructured (coil) by STRIDE (Helwig and Frishman, 2004) and glycine residues. The entropic penalty of fixing proline in the native conformation is set to \( 0 \text{ Jmol}^{-1}\text{K}^{-1} \) per residue, owing to its limited backbone flexibility. Partial partition functions are calculated by lumping together microstates with a specific number of structured residues from which the one-dimensional free energy profiles are generated.
3. Results

3.1. Thermodynamic coupling free energies

The steps involved in calculating the extent of thermodynamic coupling of protein residues from the perspective of the bWSME model are outlined in Fig. 1. The model description, ensemble generation and parameterization are discussed in the Methods section. In the current work, we adjusted the van der Waals interaction energy (\(\xi\)) of every residue to a generating a folding free-energy profile such that the native ensemble is 30 kJ mol\(^{-1}\) more stable than the unfolded ensemble. Fixing the relative stability of the native ensemble, in turn, fixes the probability \(p\) of every microstate following which four sub-ensembles are generated (Liu et al., 2006) for every residue interacting the folded status of any other residue \(j\) – both \(i\) and \(j\) are folded \((\sum p_{i,j})\), both \(i\) and \(j\) are unfolded \((\sum p_{i,j})\), \(i\) is folded and \(j\) is unfolded \((\sum p_{i,j})\), and \(i\) is unfolded and \(j\) is folded \((\sum p_{i,j})\) – with the summation running over all microstates that fit the criteria (Fig. 1C). Positive (\(\Delta G_+\)) and negative (\(\Delta G_-\)) thermodynamic coupling free energies for a residue \(i\), adapted from Chowdhury and Chanda (2010), are defined as (Fig. 1D),

\[
\Delta G_+ = RT \ln \left( \frac{\sum p_{i,j}}{\sum p_{i,j}} \right)
\]

(5)

\[
\Delta G_- = RT \ln \left( \frac{\sum p_{i,j}}{\sum p_{i,j}} \right)
\]

(6)

Note that in the definition of \(K_+\) and \(K_-\), the (un)folded status of all other residues is held fixed while that of \(i\) varies; this referencing facilitates the calculation of the true degree of coupling of \(i\) with \(j\) (also see Fig. 1C). Specifically, if residues \(i\) and \(j\) interact strongly – through direct interactions, via spatially intervening residues, or if they display little change in folding probability on perturbations amongst a collection of microstates – then the equilibrium constant \(K\) will be significantly greater than 1 as the total probability of microstates in which both residues are folded (numerator in equation (5)) will be higher than those in which \(i\) is unfolded and \(j\) is folded (denominator in equation (5)), and hence \(\Delta G_+ > 0\). On the other hand, if \(i\) and \(j\) do not interact directly or through mediated interactions \(K\) will be greater than 1 as the probability of both residues being unfolded is infinitesimally low (under folding conditions), and thus \(\Delta G_- < 0\). The coupling free energies (equations (5) and (6)) can be calculated for every residue \(i\) with respect to every other residue \(j\). The resulting matrix is not symmetric since the impact of residue \(i\) on \(j\) is not the same as \(j\) on \(i\) due to differences in residue environments, and can be symmetrized by averaging the effects.

The balance between positive and negative coupling free energy of a residue will in turn determine the effective coupling free energy between different residues in the native ensemble:

\[
\Delta G_e = RT \ln \left( \frac{K_+}{K_-} \right) = \Delta G_+ - \Delta G_-
\]

(7)

The resulting two-dimensional (2D) matrices (Supporting Fig. S1) can be averaged as a function of residue index to calculate the mean coupling free energies \(< \Delta G_+ >, < \Delta G_- >\) and \(< \Delta G_e >\) (Fig. 1E and F). Intuitively, the effective thermodynamic coupling free energies are a measure of the extent to which local stabilities and hence the conformational status of individual residues are connected to other residues. Strong effective coupling between a pair of residues will mean that they exhibit similar behavior on perturbations and are correlated at the ensemble level, while weak effective coupling would translate to little correlation and by extension larger dynamics. The connection to dynamics can be explicitly made for weakly coupled residues as they are not held in place by multiple stabilizing interactions and thus enabling relatively uninhibited structural movements. It is important to note that changes in protein stability would redistribute the populations, which would in turn alter the magnitude of coupling free energies. However, as long as the calculations are performed under conditions in which the native state is more stable than the unfolded state with finite populations of partially structured states \((\Delta G_+ > 0)\) and \((\Delta G_- > 0)\) at \(T < T_m\), the rank ordering of coupling free energy magnitudes are insensitive to the choices of vDW interaction energies or stabilities (Fig. S2).
As a representative example, consider the Ankyrin repeat protein (Ank4, the four first repeats of the full-length domain, Table S1). The WSME model representation of Ank4 with more than a million microstates captures the expected multi-state folding in the one-dimensional (1D) free energy profile (Fig. 2A). The \( \Delta G_c \) matrix exhibits strong effective coupling close to the main diagonal, as repeat domains are primarily stabilized by nearest neighbor interactions, and weak effective coupling for off-diagonal elements (Fig. 2B). The mean effective coupling, when mapped on to the structure, reveals that 18% of Ank4 residues are very strongly coupled (\( f_c \)) to the rest of the structure (Z-score of \( < \Delta G_c > \) greater than 1) while the rest of the structure is only weakly (Z-score of \( < \Delta G_c > \) less than −1) or moderately coupled (Z-score \( < \Delta G_c > \) between 1 and −1) with an overall standard deviation (\( \sigma \)) in the effective coupling free energy of 3.5 kJ mol\(^{-1}\) (Fig. 2C). It is interesting to note that some off-diagonal regions exhibit finite coupling despite making no direct interactions in the structure. For example, the arrow in Fig. 2B points to a moderate effective coupling of \( \sim 5 \) kJ mol\(^{-1}\) between residues A40 and L120 located in the 2\(^{nd}\) and 4\(^{th}\) repeats, respectively, but separated by 21 Å (\( C_C-C_C \) distance). The residues do not interact directly but are still ‘thermodynamically coupled’ via interactions mediated through the third repeat and due to the high intrinsic stability of the second repeat.

We further tested our approach on the popular model system, villin head piece domain. The model predicts a folding free energy profile with a shallow intermediate like state at \( \sim 18 \) structured residues (Fig. 2D). The constructed \( \Delta G_c \) matrix reveals a strong coupling between the first two helices (residues 1–20) while the third helix is only weakly coupled to the first two (white rectangle in Fig. 2E). This weak coupling likely contributes to the population of a partially structured state wherein the third helix samples conformations independent of the first two helices (Fig. S3), in good agreement with triplet-triplet energy transfer experiments (Reiner et al., 2010). The fraction of strongly coupled residues is estimated to be 14% with a smaller standard deviation (just 1.4 kJ mol\(^{-1}\)) compared to the Ank4 protein, which is likely to do with the smaller size of the protein (Fig. 2F).

### 3.2. Functional connect of marginally coupled residues

The simpler helical architecture of Ank4 and Villin allows for a straightforward interpretation of the effective coupling matrix. In contrast, most proteins exhibit a complex topology with an intricate distribution of energetics. We therefore constructed the native ensemble for Barstar, the inhibitor of Barnase, which not only exhibits a complex \( \alpha-\beta \) topology but also displays a multi-state folding mechanism (Sarkar et al., 2013). The current version of the model captures the multi-state nature of Barstar folding with at least 3 intermediates or partially structured states in the native side of the folding barrier (Fig. 2G) in agreement with an earlier work (Naganathan et al., 2015). The effective coupling matrix derived from the native ensemble shows a non-intuitive grouping of strong positive and strong negative effective coupling with no apparent trend (Fig. 2H). However, when mapped on to the structure, the majority of strongly coupled residues cluster together and more importantly form a strong ‘base’ to enable binding of Barnase via the weakly coupled helices 2 and 4 (green arrow in Fig. 2I that points to the direction of Barnase binding). In other words, functional sites are only marginally coupled (either weakly or moderately coupled) to the rest of the structure and appear to be supported by the strongly coupled residues.

To explore this feature further, we constructed the native ensembles, coupling matrices and average coupling free energies of three domains: (a) Cnu, a protein that acts as a sensor of environmental changes in
uropathogenic *E. coli*, (b) RNase H, an enzyme that cleaves DNA-RNA hybrids, and (c) T4 lysozyme, an enzyme that breaks down peptidoglycan in bacterial cell walls. The free energy profiles range from downhill to multi-state (Fig. 3A, D, 3G) while being consistent with experiments, as shown in earlier works (Rajasekaran et al., 2017b; Narayan et al., 2017; Cecconi et al., 2005; Narayan and Naganathan, 2014; Llinás et al., 1999). Specifically, the C-terminal helix of Cnu is observed partially structured at 310 K (note the low positive coupling for residues 60–71 in Figs. 3B and 1E) (Narayan et al., 2017), the RNase H folding pathway agrees with the order of formation observed in HX experiments (Narayan and Naganathan, 2014), while the C-terminal half of the structure is predicted to fold first for T4 lysozyme again in accordance with experiments (Rajasekaran et al., 2017b). In each of the three cases, the number of strongly coupled residues that form a part of the binding or catalytic site are 4/14, 3/12 and 7/31, respectively (red in Fig. 3B, E, 3H). Thus, more than 70% of the strongly coupled residues do not contribute to function directly. However, structural mapping points to a clustering of the strongly coupled residues while placing them right next to the ligand binding site and forming a base to support binding and catalysis (green arrow in Fig. 3C, F and 3I point to the direction of ligand binding), similar to the observation in Barstar. The fraction of strongly coupled residues ranges between 8 and 20% for the four proteins with a standard deviation of ~3–6 kJ mol$^{-1}$.

Functional sites induce strong co-evolution of residues around them even up till the second and third shell to maintain activity above a certain threshold (Lockless and Ranganathan, 1999). The physical origins of such ‘sectors’ around the active site have remained elusive. Since thermodynamic coupling analysis provides an alternate avenue to study long-range correlations, we generated the native conformational ensemble of PDZ, a popular model system for allostery and in which the concept of sector was first established (Lockless and Ranganathan, 1999). The free-energy profile of PDZ points to a two-state-like system (Fig. 3J) with a native ensemble in which the C-terminal hairpin only partially folded (arrow in Fig. 3K). Strongly coupled residues constitute only 15% of the total number and again cluster together in the region between the ligand binding site and the C-terminal hairpin (dots in Figure 3L). Remarkably, none of the strongly coupled residues, except for I31 (I336 in the PDB numbering scheme), fall within the sector region (Fig. 3K). In other words, sector residues are only marginally coupled to the rest of the structure, a feature that is likely driven by the requirement of dynamics...
to function, and in this case, to accommodate the ligand. In fact, MD simulations point to the ligand binding regions (all of which form a part of the sector) exhibiting large dynamics in the native ensemble (Bozovic et al., 2020; Kumawat and Chakrabarty, 2020) consistent with our observations of marginal coupling. A similar observation can also be made in the enzyme dihydrofolate reductase (DHFR) where the strongly coupled residues constitute one face of the protein acting as a base while the marginally coupled residues are involved in ligand-cofactor binding and catalysis (Fig. S4). Only 20% of the sector residues in DHFR (Reynolds et al., 2011) (7 out of 36) form a part of the strongly coupled residues subset (32 residues), again highlighting that sector residues identified around the ligand binding site are predominantly characterized by marginal thermodynamic coupling to the rest of the protein.

3.3. Marginal coupling and structural transitions

The marginal thermodynamic coupling of specific helices in both Villin and Cnu manifest as conformational changes in equilibrium. Do larger proteins exhibit a similar distribution of coupling energies favoring conformational transitions?

To test this, we studied the 253-residue ligand binding domain of glucocorticoid receptor (grLBD) that binds multiple ligands (both small molecules and proteins). The WSME model predicts a broad native well for this domain indicative of numerous conformations in equilibrium (Fig. 4A, left column) mirroring experimental observations (Köhler et al., 2020; Suren et al., 2018). Mapping the effective thermodynamic coupling free energies onto the structure reveal a feature similar to the smaller domains presented earlier – strongly coupled residues are physically close in space (Fig. 4B and C). The experimentally observed diversity in ligand binding is exclusively driven by marginally coupled residues that constitute a remarkable 89% of residues (225 of the 253 residues). The N-terminal lid (1–25 residues) and C-terminal helix that undergo conformational transitions to enable binding are weakly coupled (< ΔGc > ~5 kJ mol⁻¹ or less; Fig. 4B, Fig. S5A), consistent with the experimentally observed partially structured states involving these segments (Köhler et al., 2020; Suren et al., 2018).

Adenylate Kinase is another protein that undergoes a dramatic ‘open’ to ‘close’ transition on binding AMP (Schrank et al., 2009; Aviram et al., 2018) whose conformational features are not directly evident from the one-dimensional free energy profile (Fig. 4D). However, the residue-level effective thermodynamic coupling free energy constructed from the native ensemble displays strong positive < ΔGc > in the N-terminal half of the structure (close to the regions where AMP binds), while the ‘LID Domain’ that undergoes a large conformational transition is negative coupled (Fig. 4E). The strong negative coupling arises when the folding status of specific residues are not correlated to others due to weaker interactions and partial unfolding resulting in < ΔGc > dominating over < ΔG > (Fig. S5B). Mapping of effective coupling free energies onto the structure provides a clear view of the demarcation of the two domains from a structural-functional viewpoint and their anisotropic distribution (Fig. 4F).

The observation that only ~30% of protein residues are strongly coupled to the rest of the structure also holds true for a collection of 12 domains that span all three classes of structure (Fig. S6). Surprisingly, such anisotropic distribution is also seen in the membrane protein, the β2-adrenergic receptor, a GPCR (G-Protein Coupled Receptor) involved in various physiological processes. Activation of the β2-adrenergic receptor has been shown to cause major structural changes on the

Fig. 4. Larger proteins display a similar anisotropic distribution of coupling free energies. The color code is maintained the same as Fig. 3 for the proteins glucocorticoid receptor (panels A, B, C), adenylate kinase (panels D, E, F) and β2 adrenergic receptor (panels G, H, I). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
intracellular side of the GPCR involving movements in transmembrane (TM) helices 5, 6, and 7. TM6, in particular, undergoes a 14 Å movement away from the center of the helical bundle (Latorraca et al., 2017). The free energy profile generated from an ensemble of ~1.5 million microstates signals a complex folding mechanism (Fig. 4G). Interestingly, the $<\Delta G_c>$ values point to zero or negative coupling in TM5 and TM6 indicative of partially structured states in equilibrium involving residues in these helices (Fig. 4H, Fig. S5C). The strongly coupled residues are distributed across the structure but still form a spatially contiguous set accounting for 16% of the residues (Fig. 4H and I). Except for D85 in TM2, all other residues that form the binding pocket for partial inverse agonist carazolol exhibit marginal thermodynamic coupling.

3.4. Natural vs. designed and extant vs. ancient proteins

In this section, we showcase the advantages of the WSME-model derived thermodynamic coupling free energies from the perspective of protein design, evolution and function. One of the earliest designed proteins is Top7 from the group of Baker and co-workers who identified that it folds via multi-phasic kinetics while concluding that designed proteins are likely characterized by a ‘rough’ folding landscape as they do not go through the process of evolution to weed out conflicting interactions (Walters et al., 2007). Here, we compare two proteins of similar topology S6 and Top7 (Fig. 5A), with the former being a natural protein, to explore differences in coupling patterns, if any. S6 displays a two-state-like folding behavior in contrast to Top7 whose profile appears multi-state-like with a significantly lower free-energy barrier (Fig. 5B), consistent with experiments (Olofsson et al., 2007) and coarse-grained simulations (Zhang and Chan, 2010). Mapping the $<\Delta G_c>$ onto the structure, we find that Top7 has a larger fraction of strongly coupled residues with a smaller standard deviation compared to S6 (Fig. 5A). Importantly, the strongly coupled residues are more uniformly distributed in Top7 compared to S6 suggesting that the differences in folding behaviors could also be a consequence of energy functions in design algorithms that excessively stabilize pockets of structure locally to build up higher order structures, thus ensuring their high stability.

At the other end of the spectrum are ancient or ‘resurrected’ proteins whose sequences are generated via ancestral sequence reconstruction methods (Hochberg and Thornton, 2017). Structures of thioredoxins (Trxs), conserved oxidoreductases in cells, are available for both extant and ancient proteins (Ingles-Prieto et al., 2013) allowing for a direct comparison of coupling behaviors and hence their design principles. Though the extant and ancient Trxs from the bacterial branch display no specific structural differences (Fig. 5C), the ancient proteins that are supposed to have existed 4 billion years ago (LBCA, the last bacterial common ancestor, for example) display an order of magnitude faster

![Fig. 5. Insights into structural partitioning of coupling free energies in designed proteins and enzymes. (A, B) Mapping of coupling free energies onto the structure (panel A) for S6 and the designed protein Top7 together with their free energy profiles (panel B). The red arrow in panel B indicates the large difference in barrier heights between the two proteins. (C, D) Structural mapping of the coupling free energies of thioredoxins from E. coli and the resurrected LBCA ancestor (panel C), and their corresponding free energy profiles (panel D). The red arrow signals the more ‘disordered’ native ensemble for the LBCA ancestor. (E) Free energy profiles of the designed Kemp Eliminases HG3 and HG3.17. The red arrow signals the large difference in barrier heights between the two and the overall tilting of the HG3.17 towards the folded state (black). (F) The difference in coupling free energies between the two variants HG3 and HG3.17. The positions of additional mutations in HG3.17 are shown in green circles while the red arrows displays the non-intuitive changes in coupling free energies at positions far from the mutated sites. (G) Mapping of the coupling free energy differences onto the structure. The transition state analog is shown in cyan. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)](image-url)
disulfide bond reduction rate (Perez-Jimenez et al., 2011). The native ensemble of the LBCA Trx is predicted to be less structured than the extant counterpart from E. coli (arrow in Fig. 5D) with the latter exhibiting strong coupling across the entire structure (with a larger $f_2$) in contrast to the ancient cousin that is weakly coupled (with a smaller $f_2$) (Fig. 5C). This observation is in line with earlier computational treatments that point to ancient proteins being more flexible (a consequence of weak coupling) that in turn contribute to their rapid catalytic turnover and even promiscuity (Zou et al., 2015; Del Galdo et al., 2019).

An alternate approach to enhance enzyme activity involves ‘directed evolution’ that has been exploited to improve the activity of many enzymes (Arnold, 2015). Particularly, the activity of Kemp Eliminase that catalyzes the deprotonation and ring opening of 5-nitrobenzisoxazole resulting in 4-nitro-2-cyanophenol has been improved by several orders of magnitude starting from the original de novo design (Khersonsky et al., 2010; Rothlisberger et al., 2008; Broom et al., 2020). Here, we compare two of the representative enzymes, HG2 S265T (HG3) and HG3.17 E47N/N300D (HG3.17) with the latter being the evolved variant, that differ in their catalytic activity by nearly 700-fold due to accumulation of additional mutations closer and far from the active site. We find that these additional mutations tilt the folding landscape towards the folded state by enabling the population of more partially structured states in the native ensemble, visible from the lower free energies of states all along the reaction coordinate and with the folding transition state ensemble being more stable by 15 kJ mol$^{-1}$ in the evolved variant (Fig. 5E). The population of partially structured states translates to weaker coupling free energies in the evolved variant and this can be observed via the positive $\Delta G_\text{C} > 0$ values ($\Delta G_\text{C}>\text{HG3} - \Delta G_\text{C}>\text{HG3.17}$) across nearly the entire structure (Fig. 5F and G). It is interesting to note that the differences are not concentrated around the mutated site, but can span different regions of protein (arrows in Fig. 5F) including the second- and third-shells around the ligand binding site (Fig. 5G).

### 3.5. Insights into allostery from thermodynamic coupling free energies

The results above signal that mutational effects can contribute to non-intuitive variations in coupling free energies across the structure, hinting at the origins of epistatic effects. Moreover, since coupling estimates explicitly account for populations of various substates in the native ensemble and their thermodynamic interconnectivity, they provide an alternate framework to understand and address allostery as originally proposed by Freire, Hilser and co-workers (Hilser et al., 1998, 2006; Liu et al., 2006). In this section, we explore the extent to which different residues are coupled in proteins from the perspective of the WSME model and without explicitly introducing mutations or modeling ligand binding effects.

The protein PDZ is also a popular model system to study allostery given its small size (100 residues), $\alpha$-$\beta$ topology and its ability to bind a peptide ligand derived from its binding partner CRIPT. NMR experiments show that truncation of the C-terminal helical region, which also harbors a phosphorylation site on Y92 (Y307 in the PDB numbering) and that does not directly interact with the binding site, reduces the binding affinity of the peptide by ~8 kJ mol$^{-1}$ (Petit et al., 2009). To understand the origins of such long-range coupling, we extracted the per-residue coupling free energies of Y92 with all other residues in the protein. Fig. 6A shows that Y92 is positively coupled to the binding site residues with a mean coupling free energy of ~6 kJ mol$^{-1}$. Mapping the pair-wise coupling free energy (and not the average) on to the structure reveals that the entire protein is differentially coupled to the C-terminal helix (Fig. 6B). This observation signals the likely origins of pervasive chemical shift perturbations and order parameter variations in the entire PDZ domain on phosphorylation, truncation of C-terminal helix, ligand binding and mutations (Petit et al., 2009; Gautier et al., 2018; Chi et al., 2008; Hultqvist et al., 2013).

CypA, a peptide-/prolyl isomerase, is another popular model enzyme that has been extensively employed to study allostery (Doshi et al., 2016; Holliday et al., 2017). The mapping of effective coupling matrix on to the structure reveals that the first 40 residues of CypA are more strongly coupled to the rest of the structure (Fig. 6C and D). This observation is also consistent with MD simulations that point to a strongly coupled network in this region of the protein (Doshi et al., 2016). This region also harbors one of the hotspot residues V29, the mutation of which to Leucine reduces the isomerization activity by 30% despite being far from the active site. Extracting only the contribution of V29, we find that it is differentially coupled to different protein regions (as in PDZ) and more coupled to a farther active site residue (R55, ~25 Å) than H126 that is closer (~13 Å) (Fig. 6C). Similarly, CheY, a regulator of chemotaxis in E. coli displays an allosteric behavior wherein the phosphorylation of D56 regulates the binding of the flagellar motor protein FliM at a site ~10 Å away from the phosphorylation site.

![Fig. 6](image.png) Fig. 6. Coupling of experimentally known sites to active site residues in PDZ (Y92; panels A, B), CypA (V29; panels C, D) and CheY (D56; panels E, F). In panels A, C, and E, the star and the shaded block highlight the position in consideration, i.e. the residue whose coupling with other residues is considered (a residue is infinitely coupled with itself and hence no coupling free energy value is shown). The yellow circles are the active site residues in panels A and C, while they represent the known allosteric quartet in CheY. Coupling free energies mapped on to the respective structures are shown in panels B, D, and F with the spheres representing the corresponding active site residues in panel B and D, and the allosteric quartet in panel F. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
away (McDonald et al., 2013). We find that Y105, a part of the binding site, is moderately coupled (~7 kJ mol$^{-1}$) to D56 emphasizing its role as an allosteric site (green arrow in Fig. 6E and F). (Itoh and Sasai, 2011; Amor et al., 2016) However, as observed in Fig. 6F, it need not be the only site that is connected to D56 as any mutation or perturbation around it (even distant) will invariably perturb the interaction energetics and hence the coupling.

4. Discussion

We find that residue environmental effects, from a combination of energetic heterogeneity and conformational diversity, could be condensed into effective thermodynamic coupling free energies providing a unique picture on the structural-thermodynamic design principles of proteins. It is important to emphasize here that such residue level effective thermodynamic coupling free energies arise from differences in positive and negative coupling free energies that are in turn related to the distribution of states in the native ensemble. Strongly coupled residues cluster to specific regions, independent of protein topology, size or secondary structure content, revealing that protein stability determinants are distributed in an anisotropic manner. Remarkably, only ~8–24% of the residues are strongly coupled to the rest of the structure, i.e. nearly 70% of the protein residues are available to perform functional roles including ligand binding and catalysis. The strongly coupled residues are observed to form a structural ‘base’ with minimal fluctuations on average (a consequence of strong coupling) to ‘present’ the active site and binding residues that by necessity should be dynamic (moderate or weak coupling) to accommodate the ligand, at least in the systems studied in the current work. This is consistent with the marginal stability and minimal cooperativity of proteins (Malhotra and Udgaonkar, 2016; Muñoz et al., 2016) (as only a small fraction of residues exhibit strong long-range coupling) and the expected selection for large flexibility in functional sites (Petrovic et al., 2018; Wand, 2013; Henzler-Wildman et al., 2007). It should be possible to test this observation of differential dynamics by performing long time-scale MD simulations and checking for the correlated folded status of every residue versus every other residue that could, in principle, be directly related to the coupling free energies we extract. However, the challenges are that the conformational sampling should be extensive (not restricted to a local minimum) and estimates of pairwise folded status in the unfolded minimum should also be known, so as to enable precise quantification of coupling free energies.

It is of interest to note that the earliest works employing the COREX ensemble model found a similar contiguous stretch of coupled residues (Hilser et al., 1998) and structural-functional connection (Freire, 1999) from the residue-level stability constants without accounting for the folded status of other residues), further underscoring the robustness of our observations. However, it is still possible that some proteins present a rigid surface for binding with the binding partner being selected for larger dynamics on the binding site, an aspect that can be studied only on a case-by-case basis. An understanding of such co-evolving coupling free energies across two binding partners would therefore be the next important step towards rationalizing the anisotropic distribution of protein stabilities across the structure. We also present here the elusive physical basis for the presence of ‘sectors’ in proteins. More than 80% of sector residues in both PDZ and DHFR exhibit marginal thermodynamic coupling despite forming a near-contiguous pattern around the binding site residues. Therefore, the selection for dynamics likely contributes to the co-evolution of sector-like functional regions while highlighting the power of sequence-based approaches to infer dynamical patterns without explicitly considering them.

The activity and substrate specificity of proteins can be manipulated via an array of high-throughput approaches with the endpoint being a mutation or a collection of mutations that meet the set criteria. Functional studies also show that multiple combinations of mutations (and just not one set) could rescue non-functional variants (Leander et al., 2020) and context-dependence contributes to varied allosteric outcomes (Tang and Fenton, 2017; Wang et al., 2020). But it is still not clear as to how the identified mutations affect the folding conformational landscape and hence, the interaction work or the distribution of conformational states to modulate activities. We provide a plausible answer to this by comparing the folding landscapes of diverse but related pairs of proteins and find significant differences in coupling free energies despite very little changes in the overall structure; importantly, the differences are not localized to the mutated site but are distributed throughout the structure. Moreover, experimental and computational works show that even single-point mutations and perturbations involving ligand binding can contribute to widespread changes in hydrogen-exchange protection factors, NMR order parameters, chemical shifts (Petit et al., 2009; Henzler-Wildman et al., 2007; Roche et al., 2013; Whitley et al., 2008; Naganathan, 2019; Tseng and Kalodimos, 2012; Kumar et al., 2018; Boehr et al., 2006) and structural measures of connectivity in a variety of systems (Zheng et al., 2006; Atilgan et al., 2010; Rajasekaran and Naganathan, 2017; Guarnera and Berezovsky, 2019). Taken together with the results of the current work, it can be concluded that the majority of residues in a protein are thermodynamically coupled to different extents contributing to non-intuitive context-dependence of mutations and allosteric effects. Thus, natural selection works at the level of global interaction network with mutations in the second- or third-shell (or even distant) around the active site fine tuning the catalytic activity and specificity, the likely outcome of which is the observed long-range evolutionary coupling of active site residues (Jack et al., 2016).

To summarize, the thermodynamic framework of the WSME model provides information on not just the folding mechanism and relative barrier heights, but also the extent of native ensemble heterogeneity that manifests as a hierarchy of coupling free energies. The results presented here are consistent with the alternate thermodynamic treatments of Freire and Hilser, and provide a structural viewpoint on proteins that is complementary to sequence- and function-based approaches. In addition, given our observation that the majority of protein residues are coupled to each other in a given protein, it is necessary to think of selection at the level of the global interaction network to understand not just allostery but also in the evolution of enzymatic activity and selectivity; these features can be studied in the proposed model framework by introducing mutations and exploring the extent to which coupling free energies are modulated. The current methodology can also be extended to study the impact of disease-causing mutations even in large systems and in the absence of structural information on the mutant by merely generating coupling free energy maps.

CRediT authorship contribution statement

Athi N. Naganathan: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. Adithi Kannan: Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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