Protein Structural Information and Evolutionary Landscape by In Vitro Evolution

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Associate editor: Tal Pupko

All custom scripts used in this study can be accessed upon request. All sequencing data that support the findings of this study have been deposited in the National Centre for Biotechnology Information Sequence Read Archive (SRA) and are freely accessible through the SRA accession code PRJNA528665 (http://www.ncbi.nlm.nih.gov/sra/PRJNA528665). The data sets of TEM-1 sequence variants collected in this study and the top contacts predicted in the analyses can be downloaded from the BioSNS website http://laboratoriobiologia.sns.it/supplementary-mbe-2019/.

Abstract

Protein structure is tightly intertwined with function according to the laws of evolution. Understanding how structure determines function has been the aim of structural biology for decades. Here, we have wondered instead whether it is possible to exploit the function for which a protein was evolutionarily selected to gain information on protein structure and on the landscape explored during the early stages of molecular and natural evolution. To answer to this question, we developed a new methodology, which we named CAMELS (Coupling Analysis by Molecular Evolution Library Sequencing), that is able to obtain the in vitro evolution of a protein from an artificial selection based on function. We were able to observe with CAMELS many features of the TEM-1 beta-lactamase local fold exclusively by generating and sequencing large libraries of mutational variants. We demonstrated that we can, whenever a functional phenotypic selection of a protein is available, sketch the structural and evolutionary landscape of a protein without utilizing purified proteins, collecting physical measurements, or relying on the pool of natural protein variants.

Key words: beta-lactamase, AmpG, DCA, direct coupling analysis, evolutionary couplings, Sequel, PacBio, third-generation sequencing, SMRT sequencing, mutagenesis, error-prone PCR, molecular evolution.

Introduction

Deleterious mutations can damage the fold and the function of proteins. These mutations are usually rescued, in the course of evolution, by compensatory mutations at spatially close sites that restore contacts and thus preserve structure and function. This creates a correlation between protein contacts and the mutational space of the residues involved that can be compared with shackles. These shackles, that are called evolutionary couplings, can be observed by looking at the covariation between positions in a multiple sequence alignment. Through them, it is possible to predict the network of contacts that determine protein fold. Recently, direct coupling analysis (DCA) and other techniques based on the interpretation of evolutionary couplings have emerged as powerful novel methodologies that enable to predict protein architecture, fold and interactions (Weigt et al. 2009; Marks et al. 2011; Morcos et al. 2011; Ekeberg et al. 2013; Kamisetty et al. 2013; Ovchinnikov et al. 2014, 2017). These techniques have immensely increased the arsenal of tools at the scientists’ disposal to obtain structural information (Altschuh et al. 1987; Gobel et al. 1994; Pazos et al. 1997). One of the several advantages of an evolution-based approach is also the possibility to obtain structural information of proteins notably difficult to crystalize and/or model, such as membrane (Hopf et al. 2012) or disordered proteins (Toth-Petroczy et al. 2016). DCA has been successfully applied at the proteome scale leading, for instance, to the successful prediction of all the binary protein interactions in Escherichia coli (Hopf et al. 2014) and the retrieval of the structures of entire protein families and subfamilies present in the PFAM database (Uguzzoni et al. 2017).

We wondered if these tools could be applied to other types of evolutionary data such as libraries of proteins evolved in vitro by carefully controlled mutations and selection (Chen and Arnold 1993; Zaccolo and Gherardi 1999). This artificial form of evolution generates a collection of functional variants of the protein of interest by coupling a targeted mutagenesis of the gene to a strong selection pressure for the desired phenotypic trait. The method is widely used in
Molecular Evolution Libraries Mimic Natural Variability

We first thought to perform complete combinatorial two-residue deep mutational scanning to create a library (Olson et al. 2014). However, although powerful, deep mutational scanning does not represent the mutational space that nature would explore during evolution. We used instead error-prone PCR to drive mutagenesis and a phenotypic selection to collect the functional variants to mimic natural evolution. To survive the selective environment, the bacterial cells had to incorporate one of the plasmids of the mutant library. The variant of β-lactamase carried by the plasmid had to maintain functionality after mutagenesis. The first event is favored by a high transformation efficiency allowed by the use of the pUC19 plasmid vector, while the enzyme functionality is expected to decrease with the incremental number of mutations introduced in the lactamase sequence. The mutations generated during mutagenesis are at the same time necessary for evolution but harmful for survival. To obtain a high
mutational load while still guaranteeing a good amount of survivors, we applied a generational approach, where new mutations were built on a collection of mutated but functional sequences from the previously selected generations. The number of mutations and the related final 2.5–3% survival rate was regulated by limiting the error-prone PCR to 20 cycles every generation. To verify the progress of molecular evolution and maintain libraries with a fair amount of complexity, we controlled three parameters throughout 12 generations: the number of transformants in the bacterial growth, the number of mismatching amino acids in a small sample of clones and the information entropy at each amino acid position.

Since each bacterial colony in the selection medium expresses a single-functional variant of the protein, the number of transformants poses a theoretical upper limit to the library diversity. We kept the number of transformants at least in the same order of magnitude of the sequencing capacity of the next-generation sequencing (NGS) platform (between 100,000 and 1 million) to guarantee a good library complexity (supplementary fig. S2, Supplementary Material online). We raised this limit to 400 thousand clones in the last few generations to increase the probability to sequence unique variants. After each generation a small sample of clones underwent sequencing to estimate the number of mismatching nucleobases and amino acids with respect to the ancestor sequence (fig. 2A). After 12 generations of molecular evolution when sequences had a median of 25 mutations in the peptide chain, the system is still showing a nearly linear increment in the number of mutations per generation.

To complement this information, the same parameter was estimated from the sequencing results of the three sequenced generations. The distribution of the number of mismatches per sequence fitted the theoretical Poissonian model expected for a mutagenesis (gen1: $\lambda = 5.12$ s = 0.0054; gen5: $\lambda = 12.54$ s = 0.0084; gen12: $\lambda = 26.9$ s = 0.0159) (fig. 2B). The median number of mutated residues observed when the colonies were picked matched perfectly that obtained from NGS (fig. 2A) and what was expected from a Poissonian model, proving that the handful of colonies picked are representative of the mutations present in the library. We concluded from the observed steady increase in the number of mutations throughout molecular evolution that the final mutation fraction of the evolved protein library can be regulated by increasing the number of generations. Sequencing data also allowed us to calculate the mutation fraction per amino acidic position, defined as the frequency of the observed mismatching amino acids compared with the original pUC19 β-lactamase sequence. After 12 generations of molecular evolution, we started to observe several instances of genetic drifts, in which a mutation became more common than the original residue at a given position (supplementary fig. S3, Supplementary Material online). This phenomenon makes the mutation fractions less informative, since they involve a comparison to the original residue that is now a minority. To circumvent the problem, we measured the Shannon information entropy of each residue, obtaining an approximation of the mutagenesis impact for each position, without the need of a reference sequence (fig. 3A and supplementary fig. S4, Supplementary Material online). The proportion of
mutants and the information entropy of each residue were strongly correlated to each other and with those observed from the UniProt data set (mutant frequency: $\rho = 0.624$, $P < 1e-15$; entropy: $\rho = 0.632$, $P < 1e-15$) (supplementary fig. S5, Supplementary Material online). We also observed that, at each position, both the entropy and the mutation frequency of the molecular evolution libraries are almost always lower than the corresponding ones from natural evolution (fig. 3B and supplementary fig. S5, Supplementary Material online). This is likely a limit to which a molecular evolution library would tend, given enough mutagenesis rounds.

For reference, the Ostermeier’s mutational database from deep mutational scanning experiments on TEM-1 has a similar entropy profile (Firnberg et al. 2014). We constructed a list of viable mutations by pooling together the mutations from Ostermeier’s data able to grow in 32, 256, and 108 μg/ml ampicillin and above in a single collection. The Shannon entropy of the Ostermeier’s database correlates with all our sequenced generations and in particular with the first generation (correlation with gen1: $\rho = 0.560$; gen5: $\rho = 0.530$; gen12: $\rho = 0.494$; all of these with a $P$ value $< 1e-15$). In the first generation, the accumulation of mutations is reduced and the network of interconnected residues is less developed compared with the last generation. Thus our entropy profile is more similar to what can be obtained from a single-residue deep-mutational scanning.

**Single-Molecule Sequencing Overcomes Library Restrictions**

We used the PacBio single-molecule real-time (SMRT) sequencing platform (Sequel) (Eid et al. 2009) that can obtain up to a million readings per sequencing cell (van Dijk et al. 2018) and is compatible with the complexity of a molecular evolution library. The total number of transformants for the three sequenced libraries, that pose a limit to the library complexity, were 200, 260, and 400 K CFUs, respectively, while each sequencing run generated 192, 289, and 157 K raw readings after quality filtering. The sequenced DNA fragment was over 800 base pairs. Other more common NGS platform like Illumina HiSeq or MiSeq are instead characterized by decreasing quality with increasing base position (Kircher et al. 2009) and thus cannot sequence more than few hundreds base pairs. It is possible to scale up the number of sequencing cells, the amount of bacteria that undergo the selection process and the size of the DNA fragment used in mutagenesis to satisfy the requirement of this technique for any desired protein. Our mutational library is the first molecular evolution library sequenced in a third generation sequencer, thus guaranteeing a high volume of high quality single-molecule data. This library is also one of the most mutated TEM $\beta$-lactamase libraries ever produced, where its elements diverge from the ancestral protein for ca. 10% of their original amino acidic composition.

**The Mutational Landscape of the Evolved Library Reflects the Structural Features of TEM $\beta$-Lactamases**

The $\beta$-lactamase structure (PDB entry 1ZG4, Stec et al. 2005) was used as a reference structure to assess the contact predictions and the accuracy of the analysis. TEM1 $\beta$-lactamase is a globular protein with a roughly ellipsoidal shape (fig. 1B) (Jelsch et al. 1993). It can be divided into two subdomains. One is composed of a five stranded beta sheet, the N-terminus and the two last C-terminal helices. The second is a big helical subdomain located on the other side of the sheet. The protein contains a large hydrophobic core between the beta sheets and the helical subdomain, and a second hydrophobic region in the core of the helical domain. The innermost helix of this domain, H2, contains both structural and catalytic residues. The PDB structure lacks the first 23 amino acids,
corresponding to the leader sequence for secretion, which is cleaved during protein maturation to allow protein release.

The profiles of the mutation rate and entropy per residue observed in our molecular evolution libraries are conserved and increase across generations, in line with what is observed in the UniProt data set of the naturally evolved \(\beta\)-lactamase family (fig. 3B). This profile reflects the different mutation propensities of the various residues as well as the interactions with the solvent and the polarity of the local environment. We observed a high degree of conservation in the presence of bulky nonpolar amphipathic residues like tryptophans (W2108, W227, and W286) and methionines (M184, M209, and M268) and in cysteines involved in the sulfur bridge (C75 and C121), whereas small residues show in general an increased variability (supplementary fig. S6, Supplementary Material online). Small nonpolar amino acids such as valine, leucine, and isoleucine form a group of interchangeable residues in several positions (45, 54, 171, 196, and 244). The small polar counterparts glutamate and aspartate can be found replacing one another in others (33, 36, 113, 195, and 269).

A periodic alternating pattern of high and low entropy can be seen in the long alpha helices H1, H9, H10, and H12. This reflects the nature of the two halves of the helices, one being hydrophilic partially exposed to the solvent, the other containing hydrophobic residues packed against the protein core. H2 is different from the other helices because it is located deeply inside the hydrophobic core of the protein and mediates most of the hydrophobic interactions of the protein. This

**Fig. 3.** Sequencing and molecular evolution results. (A) Shannon information entropy (H) per residue position of the sequenced 12th generation library. The colors and annotations follow the secondary structure classification present in the PDB structure 1ZG4 (red: alpha helices, blue: beta sheets, tan: coils). The leader peptide sequence (light gray) is missing in the structure. (B) Comparison of the Shannon information entropy between the UniProt and the in vitro evolved data sets. (C) Relationship between the entropy of the residues obtained in molecular evolution and the mean B factor of the residues observed in the reference structure 1ZG4. Since the reference structure is missing the leader peptide, the first 23 amino acids do not have an associated B factor. (D) t-SNE dimensionality reduction applied to the joined UniProt/error-prone PCR 12th generation library data set. Hamming distance between sequences was used as distance metric. Gray and cyan represent the original data set (gray UniProt, cyan epPCR library). Overlaid on top, the UniProt sequence membership to one of the three main families of type A beta-lactamases retrieved from the corresponding UniProt annotation are displayed in bright colors. The original pUC19 beta-lactamase before molecular evolution is classified as a TEM beta-lactamase (red).
obtained after sequencing the last generation of mutagenesis in all our libraries (fig. 3A and supplementary figs. S3 and S4, Supplementary Material online), since mutations in the hydrophobic core have a high chance to damage the fold and thus impair protein function.

It is also noteworthy the correlation (Spearman correlation: rho 0.53, P < 1e-15) between the mean crystallographic B factors of residues in the reference structure and the information entropy retrieved from the evolved library (fig. 3C). This correlation likely reflects the tendency of residues that are part of ordered structures to be averse to mutation.

Although the mutational landscape of TEM-1 β-lactamase covers a broad range of substitutions, four mutations became more frequent than the original sequence in the last generation in comparison to the natural variants. These anal-

yses suggest that subsequent mutagenesis cycles consistently evolve the sequences in a concerted direction that is similar to that observed in the natural data set. t-SNE also suggests that the cluster of the evolved lactamase is only an extension of the TEM family and does not cluster with other members of class A β-lactamase (fig. 3D). Thus the molecular evolution libraries describe the mutational space of a specific protein and not of a protein family.

From this analysis, we may conclude that the library has retained the most salient characteristics of natural β-lactamase variants and exclusively represents the mutational landscape around the protein of interest. This means that the library provides a snapshot of the early stages of evolution, neither too similar nor too diverse from the original sequence, but exploring the landscape of mutational substitutions in a direction analogous to that followed by natural selection.

The Observed Mutational Events Mimic the Early Stages of Protein Folding

After several generations, we extracted the longest open reading frame from each of the 157K circular consensus reads obtained after sequencing the last generation of mutagenesis and removed those shorter than the wild type protein. We built a multiple sequence alignment (MSA) from the remaining 106K (68.9%) translated peptides and kept only the original 286 positions related to the wild type enzyme. To predict which residue pairs interact, we applied a custom implementation of DCA that applies a pseudolikelihood approximation (Balakrishnan et al. 2011) to this MSA as well as to the MSA obtained similarly from the other two sequenced generations of mutagenesis (see Materials and Methods). We retained the 286 residue pairs (0.72% of the total possible contacts) which showed the highest DCA score and were more than five residue apart in the MSA and compared them to the contact map of the reference structure (gen1: supplementary fig. S9, Supplementary Material online, gen5: fig. 4A, gen12: fig. 4B).

The first generation library was clearly unable to provide any meaningful result, while the fifth generation showed an interesting pattern. We may observe that the predictions of interacting residues made from the fifth generation library tend to cluster and are crowded in the area near the diagonal. Of particular interest are the elements at the N-terminus (residues 1–60) where the prediction clearly overlaps with the interactions made by the first few N-terminal secondary structure elements (helix H1 with the first two strands of the central beta sheet). Other important clusters can be seen in correspondence to the branching points from the diagonal (near the diagonal around residues 100, 160, 200, and 260). This set of contacts running perpendicular to the diagonal reflects the presence of hairpins. The clustering at the branching point should be expected because the branching point is where the chain inversion takes place. Since loops are more flexible and solvent exposed than other secondary structure elements (Schlessinger and Rost 2005), their composition is less critical for protein fold and allows a broader range of variation. More variations in amino acid composition increase the probability to observe a covariation pattern in DCA. Long-range contacts, the most important interactions to reconstruct the tertiary structure of a protein, were also observed (fig. 4A). Most of them overlap with the structural trace, with only five exceptions that do not match the reference (M180 is the position that shows the strongest genetic drift and is very noisy). Two of these long-range contacts predict the interactions between the N- and C-terminal helices (Q274-K30, R73-Q37). The other two mediate the interaction of the terminal helices with the beta sheet: T261-L38 mediates the interaction between the middle strand B9 of the sheet and the N-terminal helix H1; S281-E46 mediates the interaction between the C-terminal helix H12 with the first beta strand B1. M180-F58 is a contact between the loop between B6 and H9 and the lateral beta strand B2. This is an interesting area because F58 stands at the very beginning of one of the two hinge regions that connect the two domains of the protein. The last two contacts are at the opposite ends of the innermost helix H2 that carries both a catalytic and structural function. Q240-L89 is an interaction between H10, the last helix of the helical domain, and the loop at the end of H2, while S240-M67 mediates the interaction of H2 with a conserved serine in the loop between the beta strands B7 and B8.
The data are still too sparse to define clear cut interaction zones and tend to cluster around the diagonal. The protein prediction also shows a cluster of points (residues in positions 40–70 against residues around positions 100) that do not reflect any structural contact.

We tried to improve the prediction power of the analysis by increasing the number of mutations with successive generations of molecular evolution, but we observed only a strong enrichment of proximal interactions (near the diagonal of the contact map), at the expense of long-range contacts. The strongest predictions from these mutational data appear to mimic the interactions observed during the early events of protein folding (Rose 1979), where the first and stronger connections are established between adjacent

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**Fig. 4.** DCA predictions of the beta-lactamase contact map. (A) DCA plot showing the top $L$ ($L = 286$, the length of the protein amino acid chain) contact predictions by DCA obtained from the fifth generation of molecular evolution. The graph is an $L \times L$ grid where each axis represents the amino acid positions of the lactamase chain, from the N- to C-terminals. Each point represents the pair of residues described by its coordinates. The graph is separated in two halves. In the lower half black dots represent pairs of residues that have at least a pair of their respective nonhydrogen atoms $<8.5\,\text{Å}$ apart in the reference crystallographic structure (PDB ID: 1ZG4). These positions are considered residues in contact with each other. In the upper half the top $L$ DCA predictions from the molecular evolution data set are plotted above the gray-mirrored silhouette of the crystallographic contacts. Pairs where the respective residues are less than five positions apart in the lactamase alignment are excluded from this ranking to promote visualization of long-range interactions. In the graph the color indicates the shortest path (as the lowest $L1$ norm in the graph grid space) connecting the point to a contact pair position (a pair of residues that have nonhydrogen atoms $<8.5\,\text{Å}$ apart in the reference structure). (B) DCA plot showing the top $L$ ($L = 286$, the length of the protein amino acid chain) contact predictions by DCA obtained from the 12th generation of molecular evolution. (C) Plot of the top $L$ DCA predictions of the UniProt data set. (D) Plot of the top $L/2$ partial correlations of residue positions on DCA score obtained from the 12th generation of molecular evolution.
secondary structure elements. In general, the predicted contact distribution was nonrandom and contacts tended to crowd near the extremities of helices ignoring highly conserved areas like H2 (residues 67–83). Other minor crowding was observed around two big loop regions (90–100 and 160–170). The N-terminal crowding of contacts is likely the consequence of the degeneration and duplication around the starting site that was already observed during Sanger sequencing, while the C-terminal density is probably caused by sequential mutated positions in sequences where a C-terminal frameshift creates a block of strongly correlated positions without significantly affecting the functionality of the protein. The sparse number of contacts in conserved areas like H2 reflects the difficulty of creating a robust prediction when observing an inadequate number of mutations (fig. 3A).

We thus face an interesting problem: the more contacts a residue is involved in, the more harmful a mutation becomes and we observe a limited number of variations. Since the mutational space at each position dictates the prediction power, the contacts formed by the most important residues will also be the ones harder to predict.

### Improving the Prediction Power in Key Areas and Retrieval of Long-Range Interactions

To improve the accuracy and the spread of the predictions, we applied a correlation-based approach identical to that proposed for fitness (Schmiedel and Lehner 2019). Residues in structural proximity are often deeply interconnected and likely to share the same environment. Consequently they produce similar interaction patterns. Exploiting this similarity, we could obtain interactions from conserved positions by calculating partial correlation of the protein positions on the DCA patterns. This is because highly interconnected positions will have a characteristic association pattern across the protein easily recognizable by partial correlation, even if the original DCA predictions are inaccurate.

To validate this approach, we calculated the partial correlation with the UniProt data set (supplementary fig. S10, Supplementary Material online). As expected, the predictions from partial correlation are similar to the coupling scores obtained by DCA (fig. 4C) and are in general less densely packed around the diagonal albeit showing a few more incorrect predictions.

The partial correlation approach applied to the molecular evolution data set gave very different results compared with the original coupling score (fig. 4D) and resulted more similar to what observed in the UniProt data set, where the predicted interactions were more broadly distributed and both the terminals and the diagonal far less crowded (long-range contacts >50 residues: gen5: 55; gen12: 13; gen12 [partial correlation]: 31; Uniprot: 71). The accuracy of the prediction was relatively low (supplementary fig. S11, Supplementary Material online), even though several times bigger than the random expectation. This inaccuracy was caused by low precision and not by a low trueness to the underlying values as proven by the low value of shortest path from a true contact observed for the predicted pairs (supplementary fig. S12, Supplementary Material online). Along the contact map diagonal, we observed densities in correspondence to strong secondary structure interactions, like the proximity between N-terminal sheets and helix H1 represented in the graph by the cluster of contacts near residues 25–60. Other off-diagonal crowding (around residues 90–170) could be observed in the helical domain in correspondence to the interactions formed by the bending of the peptide chain in a turn. These interactions and similar ones, formed between the C-terminal half of the five stranded sheet and the C-terminal helices of the protein (200–285), were also visible in the original DCA score (fig. 4B) and the most evident areas along the diagonal of the UniProt data set where the predicted interactions clustered (fig. 4C). Long-range contacts, represented in the contact map by data points far from the diagonal, were significantly different if we evaluated the interactions obtained by partial correlation and those predicted by the original DCA score. Partial correlation prediction showed several off-diagonal prediction points, mainly associated with highly interconnected regions or between elements of the hydrophobic core. In particular, we observed several contacts of H2 (67–83) with other elements of the helical domain (H2 to H10, residues 65–210). This demonstrated the centrality of H2, even though the region is per se characterized by a small mutational landscape (fig. 3A). The analysis identified also another cluster in the helical domain describing the proximity of helix H10 (199–210) to helix H5 (117–126).

Overall, we were able to obtain a contact map that matches effectively that of the crystal structure without any prior structural information. Our analysis demonstrated the possibility to obtain evolutionary couplings from a collection of sequences evolved in vitro. DCA highlighted the strongest evolutionary signal of proximal interactions (around the diagonal of the contact map) while partial correlation extracted information on the relations between secondary structure elements. These results demonstrate that molecular evolution can be used as a powerful tool for structural prediction.

### Discussion

The study of evolutionary couplings is an emerging frontier of bioinformatics, able to retrieve the network of interactions that dictate protein fold and function (Weigt et al. 2009; Marks et al. 2011; Morcos et al. 2011; Ekeberg et al. 2013; Kamisetty et al. 2013; Ovchinnikov et al. 2014, 2017). The innovation brought by the technique is the ability to produce structural information without the need of experimental structure determination, relying only on the traces left by evolution on protein sequence. The correlations are obtained from the continuous polishing process that the flow of time exerts on sequence to optimize/retain function. This makes any structural information retrieved by the analysis like a fossil imprint of an in vivo interaction.

The current computational techniques based on evolutionary couplings require thousands of sequences to provide statistically meaningful results (Morcos et al. 2011; Marks et al. 2012; Ekeberg et al. 2013). Thus, current evolutionary coupling methods are limited to ancient and universal protein families, for which sequence data are available across a huge variety of
species. This is a major limitation: a large number of human proteins, for instance, do not have ancient phylogenetic origin (Lander et al. 2001). They are therefore not amenable to evolutionary coupling methods based on phylogenetic databases and can only be tackled by experimental approaches.

Another advantage of mutational libraries with respect to the classical phylogenetic data is the representation of a sequence instead of a family, since the Markovian models that retrieve the sequences for the alignments in the standard analysis do not differentiate close paralogs from true orthologs. This poses a serious limitation for protein families rich in paralogs like globulins, for which it is nearly impossible to obtain information for a specific member of the family. The ability to represent a protein instead of a family is a new feature that can enable to distinguish a different level of details during the biological interpretation of the data.

Here, we presented a strategy (CAMELS, Coupling Analysis by Molecular Evolution Library Sequencing) that overcomes this limitation and lays the bases to develop a general method to gather structural information on protein contacts without performing experimental structural studies or the need for thousands of natural variants of the target protein across natural evolution. We provided a unique pipeline from the molecular to the computational levels using most advanced techniques and solved a number of crucial technical problems. Because DCA is good at capturing compensating mutations, a high mutational load in the collection of functional sequence variants is recommended. When a single harmful mutation appears in the sequence, the protein will likely not be functional and bacteria that carry that specific variant will die. However, if a second mutation able to compensate the damage is also present in the sequence, the function of the protein can be restored and the host cell survives. At the time that the selection is introduced, both mutations must already be present in the sequence, hence the more mutations are inserted in each round of mutagenesis, the better. We favored this coincidence by increasing the selective pressure in the generations that we sequenced. This way, if a single mutation is harmful but still barely allows survival in a low selective pressure, there will still be few generations in which the second compensating mutation could occur before the strong selection of the last generation reaps all the sequences carrying mutations that are not compensated.

The CAMELS method is in principle generalizable: by generating hundreds of thousands of mutagenic functional variants, it permits to focus on any protein and builds the foundation for a targeted structural analysis. This may allow to investigate by DCA-like methods evolutionary younger proteins, like eukaryotic-only or vertebrate-only proteins or
human proteins of neurobiological interest, ultimately solving species-specific questions that need species-specific answers.

What are the current limits of CAMELS? The most important one is the inability to fully reconstruct the protein structure in the current proof of concept formulation of the technology. Nevertheless, CAMELS provides local and long-range contacts. Improvements might be envisaged to overcome this limitation in future work. The main factors that could allow a complete structure determination are likely the number and distribution of mutations, the sequencing depth, and the strength of the selective pressure. All these parameters can be easily scaled up, under straightforward conditions, that were, however, beyond the scope of the present proof-of-concept study.

The data revealed a strong correlation between the mean crystallographic B factor of residues in the reference structure and the information entropy retrieved from the evolved library. This happens because residues that are part of ordered regions as in the protein core are adverse to mutation and thus the most conserved ones. Harming these zones would affect the fold and function. A drastic increase in the number of mutations would help to generate variability in these conserved key residues that could be translated in better evolutionary couplings. The same logic applies by forcing a distortion in the mutation propensities to favor the generation of mutations in key areas. The problem of this approach is that we would distort the landscape obtained by unbiased epPCR. Modifying the driving force of the mutagenesis from epPCR to deep mutational scanning could provide a diverse landscape that might produce more precise couplings for the reasons mentioned. A critical comparison between the landscapes and couplings produced by epPCR and deep mutational scanning may be important to improve the technique in future implementations. Increasing the sequencing depth and the corresponding scale of selection is also an easy albeit currently expensive solution. This would likely increase the statistical power and allow low-entropy regions to show enough variation to be translated in couplings.

We noted that the fifth generation produced more long-range contacts in the standard DCA prediction in respect to the 12th generation. A possible explanation could be that the fifth generation produced nearly twice the number of reads of the 12th generation that in turn could alter the number of mutations in key areas. Another interpretation is that accumulation of mutations over the generations could create a broad compensatory effect that partially hinders the results. The correlated mutations accumulating throughout evolution are a mixture consisting of directly and indirectly interacting mutations. Since most random mutations are destabilizing, under the severe mutational load exerted on TEM-1 by selective pressure, most of the variants in the library should have a compromised stability. This strong selection pressure toward fixation of stabilizing mutations might thus favor the accumulation of correlated mutations of residues that do not directly interact. A more systematic investigation of this point using different target proteins will be important in the future.

An important requirement of CAMELS is phenotypic selection, a necessity that makes the method truly evolutionary: like in the natural environment, selection is always based on the target protein function. As a consequence, selection must be designed on a case-by-case basis. For some proteins (such as TEM1 β-lactamase), a phenotypic selection scheme is readily designed. More in general, selection schemes based on interactions could be considered that is probably the best approach to generalize the method. CAMELS could easily be modified, for instance, for the study of protein–protein interactions, exploiting selection schemes for interacting proteins coupled to SMRT sequencing, which would allow observation of protein pairs in a single-sequencing read. Selection schemes based on signaling by the mutated target protein could also be envisaged.

The next obvious step will be to exploit standard and generic selection methods that rely on the folding and binding properties of the mutant proteins in the library, regardless of their functional activity. We have, for instance, already planned to use selection schemes to select for interacting partners, using a strategy, we already pioneered for screening more stable antibodies against a given target (Visintin et al. 1999; Chirichella et al. 2017). We could apply CAMELS to two coevolving interacting proteins, which could then be coselected by a two-hybrid scheme for preserving their mutual binding. This strategy will provide information on the direct or indirect structural determinants for protein–protein interacting domains. This would be a revolutionary breakthrough that is not restricted to specific cases. It should be noted however that to successfully apply CAMELS, a good selection is critical. Although testing the function of a protein may seem a simple requirement on paper, the difficulty of developing an effective screening strategy cannot be underestimated especially for understudied proteins.

An elegant recent study successfully used deep mutagenesis to attempt determination of an unknown structure of a large complex human receptor in a physiologically relevant active conformation (Park et al. 2019). However, this example was somewhat limited to the specific structure of the protein. It will be interesting to apply CAMELS to members of the GPCR family, exploiting the signal transduction propriety of the receptors coupled to a screenable selection readout.

Finally, one of the biggest obstacles to in vitro evolution approach was the precarious equilibrium between mutagenic strength and selection survival rate. We solved this issue with a generational approach. We can further envisage future applications of the method to a continuous evolution in a specialized bioreactor. Overall, the CAMELS method provides a solid methodology that bypasses the most limiting factors of evolutionary coupling analysis techniques and opens a new page in structural biology and evolution.

Materials and Methods

Plasmid Construction and Cloning

The backbone plasmid vector pUC19 (Norrander et al. 1983) (ATCC 37254) from ThermoFisher Scientific (SD0061) was modified to add flanking XhoI and NheI restriction sites to
the already present AmpR ORF to be able to easily clone in later steps the mutagenized AmpR. To construct the plasmid, both the \( \beta \)-lactamase gene and the complementary plasmid vector fragments were amplified with oligonucleotides carrying the Xhol and Nhel restriction sites (Xholbla-fw: tgaagaacctcaggagagtATGAGTATTC, Nhelbla-rv: acttttgctttcgagttttcaatattattgaag). They were then digested with the restriction enzymes and ligated with T4 ligase (supplementary fig. S12, Supplementary Material online). The 5’ restriction site was placed just behind the Shine–Dalgarno sequence and the ability to metabolize ampicillin was assessed by growth of \( E. \) coli carrying the plasmid in selective media. The new plasmid is named pUC19a (supplementary fig. S13, Supplementary Material online). The AmpR gene of pUC19 (GenBank: M77789.2) expresses a TEM-1 (class A) \( \beta \)-lactamase whose structure can be viewed in the 1ZG4 PDB entry.

**Error-Prone PCR**

Mutagenesis of the AmpR gene was achieved with epPCR (Wilson and Keefe 2001) in a mutation prone buffer with manganese ions, low magnesium, unbalanced dNTPs concentrations, and a low fidelity DNA polymerase. Both low magnesium and the presence of manganese ions affect the efficiency of magnesium ions as cofactors of the polymerase by competition or by sheer low availability, while the unbalanced dNTP concentration favors mutations by scarcity of substrate and the deliberate usage of a low fidelity polymerase further increases the mutation rate. The reaction mix contained Tris–HCl pH 8.3 10 mM, KCl 50 mM, MgCl2 7 mM, dCTP 1 mM, dTTP 1 mM, dATP 0.2 mM, dGTP 0.2 mM, 5’ primer (bla_mut-fw: tgaagaacctcaggagagtATG), 3’ primer (bla_mut-rv: acttttgctttcgagttttcaatattattgaag), Taq G2 DNA polymerase (Promega M784A) 0.05 U/\( \mu \)l (added just before reaction starts), and a template DNA 20 pg/\( \mu \)l, MnCl2 0.5 mM (added just before reaction starts), and a Taq G2 DNA polymerase (Promega M784A) 0.05 U/\( \mu \)l (added just before reaction starts). The error-prone PCR was carried out in serial reactions of four cycles in 100 \( \mu \)l in the recommended supplier reaction conditions and with an annealing temperature of 62 °C. In the first reaction tube, the DNA template was a gel purified Xhol/Nhel digested \( \beta \)-lactamase fragment 20 pg/\( \mu \)l, while subsequent reactions were fed with 10 \( \mu \)l of the previous PCR product.

**Library Construction**

The purification and digestion protocols before library construction changed slightly among generations. However, the optimized version of the pipeline employed in the last generations proceeded as follows: gel purify ~80 \( \mu \)l of the PCR reaction mixture underwent a cumulative amount of 20 cycles of error-prone PCR, avoiding carrying over other reaction byproducts as much as possible. PCR was performed in standard reaction conditions to amplify the product and guarantee that the two strands of the amplicons did not contain mismatching base pairs. This step helped reducing the ambiguity in base calling during the circular consensus analysis. The purified PCR product was digested with Xhol and Nhel restriction enzymes for 3 h in CutSmart buffer (NEB). One hour before the end of the reaction, an appropriate amount of calf intestinal phosphatase (CIP) (NEB M0290S) was added following the supplier’s instruction. Adding CIP during insert digestion strongly reduced the formation of insert concatemers, guaranteeing a single \( \beta \)-lactamase variant per plasmid. After gel purification to remove the CIP, the ligation between the fragment and the Xhol/Nhel digested backbone of pUC19a was performed in a 1:1 insert: vector ratio. Formation of backbone concatemers was expected and unavoidable, but did not hinder the selection efficiency.

**Selection**

The ligated library was purified and then transformed by electroporation in ElectroMAX DH5\( \varepsilon \)-E competent cells (Invitrogen No. 11319019). We employed ultralow gelling agarose (SeaPrep, Lonza No. 50302) 0.3% in Luria Broth (LB) medium with ampicillin 25 \( \mu \)g/ml to grow the bacteria (Elsaesser and Paysan 2004; Fantini, Pandolfini, et al. 2017) obtaining between 0.4 and 3 million surviving colonies per liter. The bacterial growth in the 5th and 12th generation was performed in LB medium with ampicillin 100 \( \mu \)g/ml to increase the stringency of the selection before the sequencing. After 40 h growth, the bacterial pellet was retrieved by centrifugation 7500RPM at RT and the plasmids extracted by a maxi prep.

**Sequencing**

Construction of the libraries and sequencing on PacBio Sequel platform were carried out by Arizona Genomics Institute (AGI). After sequencing, the library was processed with the PacBio official analysis software SMRTLink to obtain the circular consensus (using cc52) of the reads. In this step, the sequences where the consensus was built from less than ten sequencing polymerase passes or when the predicted accuracy was <100 ppm (Phred 40) were filtered out from the data set. The result was then mapped to the wild type \( \beta \)-lactamase Xhol-Nhel digestion fragment of pUC19a with bowtie2 (Langmead and Salzberg 2012) to retrieve the coding strand and the start site of the lactamase. After in silico translating the data set, protein collection was further refined keeping only the elements coding a protein of 286 amino acids (as the wild type) and then aligned using MAFFT (http://mafft.cbrc.jp/alignment/software/) last accessed November 14, 2019) (Katoh 2002) to construct the MSA. The 12th generation had issues with the in silico translation step caused by degeneration of the N-terminus as well as the starting site, resulting in a big amount of sequence with a premature termination codon. To circumvent the problem, the longest open reading frame, identified with a custom script, was considered the correct genetic sequence and the translated products were filtered to keep the sequences coding for proteins of at least the wild type length. This procedure was required to remove from the alignment bad quality reads, unrelated sequences and protein variants carrying a frameshift which would generate a strong correlation noise between adjacent amino acid positions. It is interesting to...
notice that classical evolutionary data supplied to the algorithm do not have this problem, and thus this is a new issue brought by the mutagenic data. This is probably because frame shifted sequences do not carry a sufficient neutrality to the system to be retained throughout natural evolution, while in the small landscape generated by the mutagenesis, every protein that satisfies the selection criteria of activity will be part of the collection.

To compare our data to the natural occurring mutations of TEM β-lactamase, we created a reference data set by running a small seed of TEM β-lactamases in Hmmer (Finn et al. 2011) on the UniProt database (https://www.uniprot.org; last accessed November 14, 2019). An alternative data set that could be used as a control is the Pfam family of β-lactamase 2 (PF13354). Since the Hmmer data set from Uniprot is a collection of sequences that specifically matched the profile of the TEM family while Pfam family is a more general β-lactamase collection, we preferred the former as reference for our analysis.

**Direct Coupling Analysis**

The predicted contact pairs were obtained using a custom implementation (Fantini, Malinverni, et al. 2017) of the asymmetric version of the DCA (Weigt et al. 2009; Morcos et al. 2011) that applies the Pseudo-likelihood method to infer the parameters of the Potts model (Balakrishnan et al. 2011; Ekeberg et al. 2013):

\[
P(X) = \frac{1}{Z} \exp \left[ \sum_{i}^{N} h_i(X_i) + \sum_{i,j}^{N,N} J_{ij}(X_i, X_j) \right],
\]

where \(X\) is a sequence of the MSA and \(Z\) is the partition function.

Sequences were reweighed using an identity threshold that reflects the mutation rate of the generation analyzed to remove parental inheritance (intended as “phylogenetic” bias created during mutagenesis) and sampling biases in the MSA. The first generation was too similar to the wild type to apply any sampling correction without unreasonably reducing the number of effectively nonredundant sequences (Morcos et al. 2011). The 5th generation used a 95% identity threshold and the 12th generation a 90%. A standard L2 regularization was added following the original regularization described in Ekeberg et al. (2013) (\(\lambda = 0.01\)). The code used the scoring scheme for contacts where the DCA scores were computed as the Frobenius norm of the local coupling matrices of the Potts model. Dunn et al. (2008) average product correction (APC) was subtracted to remove background correlation. The N top scoring contact predictions (N equals the MSA sequence length) were compared with the contact map of the reference structure (1ZG4) constructed considering two residues to be in contact if at least a pair of their respective heavy-atom (nonhydrogens) was <8.5 Å apart (Ekeberg et al. 2013). As it is standard practice, we removed predictions along the diagonal of the contact map if the residue pairs were less than five positions apart to promote enrichment of long-range predictions. Increasing this threshold to 8 or more did not change significantly the contact map prediction (data not shown). We used the shortest path (SP) distance (Malinverni et al. 2015) defined as the L1 norm in the contact map lattice to join DCA predictions and the closest structural contact to visualize the agreement between predictions and empirical observations.

**Partial Correlation**

Partial correlation is a measure of the correlations between two variables after removing from both the possible correlations they might have with another set of confounding variables. In other words partial correlation is the correlation between the residuals obtained from the regression of the variables of interest to the confounding variable set. In the present work, partial correlation is used to infer the correlation between each set of two rows of the DCA score matrix, removing the correlation these rows might have to all other rows of the matrix. Each row of the DCA matrix is a vector of the strength of association between the residue represented by the row and every other residues of the protein. Thus the partial correlation of the DCA matrix represents the similarity of the profile of these vectors, considering and removing the correlation with the profile of all the other residues. To obtain the partial correlation matrix from the symmetrical DCA score matrix, we first set all the diagonal elements of the matrix to 1 and then approximated the partial correlation between rows with the pcor.shrink function of the corpcor R package. The package implements a James–Stein estimator for the covariance matrix. The details of the method are explained in (Schäfer and Strimmer 2005) and in the manual of the package (https://cran.r-project.org/web/packages/corpcor/corpcor.pdf; last accessed November 14, 2019).

**Other Bioinformatic Tools**

Graph generation was performed with R version 3.2.3 (December 10, 2015). Poisson regression was performed with the fitdistplus R package, while PCA was performed with the base R procmod function. Mutation rates and Shannon information entropies were calculated with custom scripts. t-SNE was performed in Matlab version R2018b using the Hamming distance as metric.

**Supplementary Material**

Supplementary data are available at Molecular Biology and Evolution online.

**Note Added in Proof**

While this paper was in review, a similar approach was deposited in bioRxiv (Stißler MA, Poelwijk FJ, Brock K, Stein RR, Teyra J, Sidhu S, Marks DS, Gauthier NP, Sander C. 2019. Protein structure from experimental evolution. bioRxiv:667790).

**Acknowledgments**

We thank our colleagues from Scuola Normale Superiore Federico Cremisi and Alessandro Cellerino for valuable comments. We are also grateful to Martina Goracci and Ottavia Vitaloni for times when, thanks to their wisdom and expertise, we were able to make major breakthroughs. We are also
in Debted to Duccio Malinverni for providing the software implementation of the asymmetric version of the DCA and to Alessandro Viegi for administrative support throughout the project. The project was funded by institutional funds from Scuola Normale Superiore, a BlueSky grant from University of Pavia and the UK Dementia Research Institute (REI 3556) which is funded by the Medical Research Council, Alzheimer’s Society and Alzheimer’s Research UK.

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