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Structural determinants of the rate of protein evolution in yeast

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Abstract

We investigate how a protein's structure influences the rate at which its sequence evolves. Our basic hypothesis is that proteins with highly designable structures (structures that are encoded by many sequences) will evolve more rapidly. Recent theoretical advances argue that structures with a higher density of inter-residue contacts are more designable, and we show that high contact density is correlated with an increased rate of sequence evolution in yeast. In addition, we investigate the correlations between the rate of sequence evolution and several other structural descriptors, carefully controlling for the strong effect of expression level on evolutionary rate. Overall, we find that the structural descriptors that we consider appear to explain roughly 10% of the variation in rates of protein evolution in yeast. We also show that despite the well-known trend for buried residues to be more conserved, proteins with a higher fraction of buried residues nonetheless tend to evolve their sequences more rapidly. We suggest that this effect is due to the increased designability of structures with more buried residues. Our results provide evidence that protein structure plays an important role in shaping the rate of sequence evolution, and provides evidence to support recent theoretical advances linking structural designability to contact density.

Introduction

Protein sequences evolve largely through the gradual accumulation of amino acid substitutions, and the extent of sequence divergence is quantified by the number of non-synonymous substitutions per site, dN. Over forty years ago, Zuckerkandl and Pauling (1965) observed that dN for homologous proteins is proportional to the time since divergence, indicating that dN measures a roughly constant average rate of fixed amino acid substitutions. However, it has also long been clear that different proteins accumulate substitutions at markedly different rates: Zuckerkandl and Pauling (1965) remarked how the large values of dN for hemoglobin were "spectacularly at variance" with the small values of dN for cytochrome c. The availability of full genome sequences now allows for much more extensive comparisons of rates of protein sequence evolution, and such analyses have confirmed the widely differing rates noted by Zuckerkandl and Pauling (1965). For example, dN varies approximately a thousandfold between the fastest and slowest evolving proteins in yeast (Drummond et al. 2005).

Zuckerkandl and Pauling (1965), and subsequently Ohta and Kimura (1971) and others (King and Jukes 1969), argued that variation in dN was due to differences in the selective

constraints on proteins' sequences. The basic argument is that most sequence divergence is due to the fixation of mutations with little or no effect on a protein's function, and so the rate at which substitutions accumulate is proportional to the average fraction of mutations that are effectively neutral (Ohta and Kimura 1971; Brookfield 2000). This argument has now gained widespread acceptance, and numerous studies have used high-throughput genomic data to attempt to pinpoint the biological constraints that underlie the different rates of sequence evolution. Numerous properties have been found to correlate with a protein's dN, including the dispensability or essentiality of its encoding gene (Hirsh and Fraser 2001; Jordan et al. 2002; Zhang and He 2005; Wall et al. 2005), the number of other proteins with which it interacts (Fraser et al. 2002; Lemos et al. 2005), its length (Marais and Duret 2001; Lemos et al. 2005), its centrality in the protein interaction network (Hahn and Kern 2005), and its expression level (Pal, Papp, and Hurst 2001; Drummond et al. 2005; Drummond, Raval, and Wilke 2006). However, a casual analysis of these correlations is complicated by the fact that most of these biological properties are also correlated with each other (Drummond, Raval, and Wilke 2006). At this point, the only clear conclusions are that by far the most dominant trend is for highly expressed proteins to evolve slowly (Pal, Papp, and Hurst 2001; Drummond et al. 2005; Drummond, Raval, and Wilke 2006), and that the other correlations are either much weaker or potentially due to confounding factors (Hurst and Smith 1999; Pal, Papp, and Hurst 2003; Bloom and Adami 2003; Jordan, Wolf, and Koonin 2003; Hahn, Conant, and Wanger 2004; Agrafioti et al. 2005; Drummond, Raval, and Wilke 2006).

The fact that expression level correlates with dN much more strongly than properties reflecting a protein's biological role is consistent with protein mutagenesis experiments showing that deleterious mutations usually act by hindering the formation of a properly folded protein rather than specifically altering a protein's function (Shortle and Lin 1985; Pakula, Young, and Sauer 1986; Loeb et al. 1989; Bloom et al. 2005, 2006). Therefore, dN should be largely determined by the fraction of mutations that prevent adequate protein expression and folding. Highly expressed proteins are under an increased requirement for fidelity in expression and folding due to the costs of misfolded proteins, meaning they have a smaller fraction of effectively neutral mutations and so evolve more slowly (Drummond et al. 2005). A protein's biophysical properties can also influence the fraction of mutations that allow for adequate expression and folding — for example, protein mutagenesis experiments have shown that increasing a protein's thermodynamic stability dramatically increases its tolerance to mutations (Bloom et al. 2005, 2006). Another factor that has received little consideration with respect to its effect on dN, but which may significantly affect a protein's mutational

tolerance, are the characteristics of the native structure itself.

The relationship between a protein's native structure and its mutational tolerance has been extensively studied in the context of protein folding and design. These studies typically attempt to characterize a structure's *designability*, defined as the total number of amino acid sequences that fold into that structure (Li et al. 1996; Kussell 2005). Because more designable structures are encoded by more sequences, proteins that fold into highly designable structures tend to be more tolerant to mutations, and thus should evolve their sequences more rapidly (Fig. 1). Most studies of protein designability have utilized simple computational models in which designability can be directly estimated by an extensive or exhaustive enumeration of different sequences (Li et al. 1996; Govindarajan and Goldstein 1996, 1997; Tiana, Broglia, and Shakhnovich 2000; Irbäck and Troein 2002; Miller et al. 2002; Chan and Bornberg-Bauer 2002; Wingreen, Li, and Tang 2004; Zhang, Sun, and Zou 2005; Wroe, Bornberg-Bauer, and Chan 2005). The main conclusion of these model protein simulations is that designability varies widely among structures; however, the simplicity of the models makes it difficult to extrapolate any quantitative measures of designability to real proteins. At the other end of the computational spectrum, a variety of studies have used state-of-the-art atomistic simulations to attempt the estimate the designabilities of real proteins (Zou and Saven 2000; Kono and Saven 2001; Voigt et al. 2001; Koehl and Levitt 2002; Larson et al. 2002). But these atomistic simulations are computationally expensive, and their accuracy is not known since there are no experimental measurements of protein designability with which they can be compared. Recently, England and Shakhnovich (2003) have proposed a general theory that relates designability to the pattern of contacts between residues in the native structure. Their approach is based on the argument of Wolynes (1996) and Shakhnovich (1998) that a structure's designability can be estimated as the number of sequences that fold into that structure with an energy below some threshold. By assuming that the energy of a structure is due to pairwise interactions between residues, England and Shakhnovich (2003) show that the designability D is given by a series in traces of powers of the structure's contact matrix $\mathbf{C}$,

$$D = \sum_{n=2}^{\infty} (\mathrm{Tr}\ \mathbf{C}^{\mathrm{n}})\, a_n. \tag{1}$$

This theory has been verified with simulations on simple model proteins (England and Shakhnovich 2003; England, Shakhnovich, and Shakhnovich 2003; Tiana et al. 2004), but unlike earlier simulations it has a theoretical basis and so can in principle be applied broadly. However, the coefficients a_n in Eq. 1 cannot be calculated, so the designability can be estimated by truncating the series after the first term. (An alternative method of estimating the

series in Eq. 1 as the maximum eigenvalue of the contact matrix gives comparable results, as discussed below.) The first term of Eq. 1 is just equal to the contact density (the average number of contacts per residue) (England and Shakhnovich 2003; England, Shakhnovich, and Shakhnovich 2003), and so this truncation recovers the predictions of Wolynes (1996) and Shakhnovich (1998) that designability is approximated by contact density.

Here we use this predicted relationship between contact density and designability as the basis for exploring the contribution of protein structure to evolutionary rate in yeast. While numerous earlier studies have shown that the conservation of residues at individual sites is influenced by structural characteristics such as secondary structure or solvent exposure (Overington et al. 1992; Koshi and Goldstein 1995; Thorne, Goldman, and Jones 1996; Goldman, Thorne, and Jones 1998; Mirny and Shakhnovich 1999; Bustamante, Townsend, and Hartl 2000; Dean et al. 2002; Marsh and Griffiths 2005; Dokholyan and Shakhnovich 2001), our work looks at how a protein's structure affects its global rate of sequence evolution. We examine the correlation of dN with contact density and several other structural descriptors (fraction of buried residues, secondary structure composition, length, and fold classification), while statistically controlling for the effect of expression level. Our work shows that dN is influenced by protein structure in a way that suggests that proteins with more designable structures evolve their sequences more rapidly.

Materials and methods

All 33,449 protein structures present in the protein data bank (PDB) on Nov. 13, 2005 were downloaded as mmCIF files. The downloaded files were parsed to get the sequences of all of the proteins, except for 1quz, 1jhq, 1zhe, 1quz, 2ad1, 1zir, and 2etg, which could not be parsed effectively. These parsed sequences included only those residues with coordinates—residues without coordinates were excluded from the sequences. Non-glycine residues that lacked any side chain atoms were also excluded from the sequences. This procedure yielded a total of 73,121 protein sequences.

The sequences of all *Saccharomyces cerevisiae* open reading frames (ORFs) were downloaded from ftp://genome-ftp.stanford.edu/pub/yeast/data_download/sequence/ genomic_sequence/orf_dn on Oct. 19, 2004. All of the genes that could be translated were considered. This procedure yielded 5865 proteins. To match protein structures with these yeast proteins, we BLASTed (Altschul et al. 1990) each protein against all of the PDB protein sequences. Any matches with a BLAST E-value of at least 10^{-5} were then aligned using CLUSTAL W (Thomp-

son, Higgins, and Gibson 1994), and if the number of identities in the total length of the alignment was $> 80\%$ then the match was saved. If there were multiple such matches to a protein, then the best match was saved as the hit for that protein. This process yielded 275 matches. Since the number of proteins with solved structures is small compared to the number of proteins with known sequences, restricting our data set to yeast proteins with $> 80\%$ similarity to a sequence in the PDB limits the size of our data set. However, we felt it was important to set this relatively stringent criterium for sequence identity to ensure that the PDB structures accurately represented the actual folded conformations of the yeast proteins with which they were matched.

For each yeast protein with a match, we recorded the aligned residues, the secondary structure for each aligned residue, and the percent solvent-accessible area for each aligned residue. The latter number was computed using only atoms within that protein chain in the PDB structure (that is, we did not consider surface area buried by atoms of other protein chains in the PDB structure). We first calculated the exposed surface area using the program given by McConkey, Sobolev, and Edelman (2002), and then normalized these values by the reference surface areas of an extended Gly-X-Gly peptide, as given on page 142, Table 4.4 of Creighton (1992). We counted a residue as buried if it had less than 25% solvent accessibility, and exposed otherwise.

We calculated contact maps of all the 275 PDB structures. We considered two residues in contact if any of their two heavy (non-hydrogen) atoms were within a distance of 4.5Å from each other, and if the two residues were not immediate sequence neighbors (that is, we excluded trivial contacts). We then determined the contact density for each structure by calculating the average number of contacts per residue.

Protein chains were assigned to the Structural Classification of Proteins (SCOP) database (Murzin et al. 1995) classes given in version 1.69 available at http://scop.mrc-lmb.cam.ac.uk/scop/parse/inc
For each protein chain, we first searched for the PDB structure ID in the downloadable dir.cla.scop.txt_1.69 file. If we found the ID, we then searched for the mmCIF file chain ID in the file. In cases where a structure had multiple chains with different SCOP classes, we took care to make sure the mmCIF chain ID was matched appropriately with the correct SCOP class (the chain ID used in the mmCIF file is not always the same as the one used in the PDB file for the same structure, and the SCOP classifications are made according to the PDB chain ID). Some chains have different regions assigned to distinct SCOP classes. If we found multiple entries for different regions of the chain, we recorded the SCOP class only if all regions of the chain were assigned to the same class.

We calculated evolutionary rates (dN) using the reciprocal-shortest-distance method (Wall, Fraser, Hirsh 2003). All ORFs in *S. cerevisiae* were BLASTed against those in *Saccharomyces bayanus*, and vice versa. Pairwise hits with an E-value of $< 10^{-20}$ were retained and aligned with CLUSTAL W, using the aligned protein sequences to align the nucleotide sequences. Evolutionary rates, the numbers of non-synonymous substitutions per non-synonymous site (dN) and synonymous substitutions per synonymous site (dS), were computed for these hits using the PAML (Yang 1997) program codeml operating on codons with a 9-free-parameter model for codon frequencies. Pairs with less than 80% aligned residues were discarded, since there are no well established methods for dealing with gaps when calculating evolutionary distances. Remaining aligned gene pairs having each other as the shortest-distance (smallest dN) hit were designated orthologs and used in our analysis. We retained all ORFs among the 275 with structural information for which we found a *S. bayanus* ortholog, and found 203 such ORFs.

We calculated evolutionary rates at buried/exposed sites and sites of specific secondary structure by discarding all but the relevant portions of the ortholog alignments generated to compute overall evolutionary rates. For example, to compute the evolutionary rates at buried sites, we considered only buried residues (identified as described above) and assembled the corresponding codons into a reduced pair of ortholog sequences, from which evolutionary rates were calculated exactly as described above. This procedure was carried out for buried and exposed residues and for residues corresponding to the four secondary structure types of helix (DSSP class H), sheet (DSSP class E), turn (DSSP classes S, T) and coil (DSSP classes B, G, I, '.'). (DSSP is the "Dictionary of Protein Secondary Structure", Kabsch and Sander 1983.)

We calculated codon adaptation indices (CAI) exactly as described by Sharp and Li (1987), using tabulated codon relative adaptiveness values for *S. cerevisiae* (Sharp and Li 1987). We used expression data from Holstege et al. (1998). After discarding all ORFs for which we did not have expression data, we arrived at our final data set of 194 ORFs. This data set is given as online supplementary table.

All statistical analyses were carried out with the statistics software R, version 2.1.1 (R Development Core Team 2005). Principal component regression was done using the R package "pls". Unless mentioned otherwise, all analyses were carried out on ranks, rather than on the actual values of the quantities. In particular, all correlations are Spearman correlations, and all principal component regression analyses were carried out on rank-transformed data.

Results

Differences in substitution rates of different classes of residues

For each ORF, we determined the evolutionary rate (dN) of the buried and of the exposed residues, and also of residues belonging to each of the four secondary structure classes of helix, sheet, turn, and coil. We found that exposed sites evolve substantially faster than buried sites, while secondary structure has little effect on evolutionary rate (Fig. 2). For all residue types, the distribution of evolutionary rates was highly skewed. For example, the fastest evolving buried sites evolve much faster than the median exposed site. Overall, our findings for type-specific evolutionary rates confirmed the consensus in the literature that solvent accessibility has a strong effect on the conservation of individual residues, while secondary structure type has at most a weak effect (Goldman, Thorne, and Jones 1998; Bustamante, Townsend, and Hartl 2000; Dean et al. 2002).

Effect of contact density on evolutionary rate

Because buried sites are more conserved, we might expect that proteins with a larger fraction of buried sites should evolve slower. On the other hand, the prediction of England and Shakhnovich (2003) is that proteins with a higher contact density are more designable, and thus would be expected to evolve faster. To find out which of the two views is correct, we correlated the overall evolutionary rate dN with contact density and the fraction of residues that are buried. The former is the average of the number of contacts per residue, and is the quantity treated theoretically by England and Shakhnovich (2003) (the first term of Eq. 1). The latter does not explicitly count contacts, but is strongly correlated with contact density (Table 1). Throughout this paper, we usually report results which hold for both measures for only one measure, the contact density. An alternative method for estimating the designability as given by Eq. 1 is to use the maximum eigenvalue of the contact matrix (England and Shakhnovich 2003); doing so yields results that are highly similar to those for contact density for all correlations shown in Table 1 (the Spearman correlation between the maximum eigenvalue and dN is $\rho = 0.25$).

As predicted by the hypothesis that more designable proteins should evolve faster (Fig. 1), we found that contact density and the fraction of buried sites correlated significantly with dN (Table 1, Fig. 3). However, this correlation does not imply *per se* that the increased rate of evolution is caused by contact density. It is well known that more highly expressed proteins evolve more slowly in yeast (Pal, Papp, and Hurst 2001), and therefore we always

have to control for expression level before we can conclude that any quantity has an effect on dN (Pal, Papp, and Hurst 2003; Bloom and Adami 2003; Drummond, Raval, and Wilke 2006). We calculated the correlations between contact density and both expression level as measured by DNA microarrays (Holstege et al. 1998) and codon adaptation index CAI, another proxy for gene expression (Table 1). Expression level is not significantly correlated with contact density. There was a weak correlation between CAI and contact density, but this correlation had the opposite sign from what we would expect if a correlation between CAI and contact density were to cause the correlation between contact density and dN. These results indicate that the correlation between contact density and evolutionary rate is not caused by an underlying correlation between contact density and expression level.

The results from the analysis of evolutionary rate at buried and exposed sites (Fig. 2) seem to be at odds with the results from the analysis of the overall evolutionary rate of the proteins (Fig. 3). Buried sites tend to be more conserved than exposed sites, but the overall evolutionary rate increases with increasing contact density (and also fraction of buried sites). However, this apparent paradox can be understood if we consider the effect of high contact density on buried and exposed sites separately (Fig. 4). Whereas the dN at buried sites shows a moderate increase with increasing contact density, the dN at exposed sites grows dramatically. Even though proteins with high contact density have a reduced fraction of exposed residues, the residues that are exposed in these proteins evolve very rapidly. Therefore, the reduction in the fraction of exposed residues is more than compensated for by the increased variability of exposed residues in proteins with high contact density.

Effect of protein length on evolutionary rate

We found a significant correlation between protein length and dN, and a strong correlation between length and contact density (Table 1). This observation prompted us to investigate the relationship between contact density and protein length. We found that the correlation between these two quantities stems primarily from short proteins (Fig. 5). For very short proteins, there is a large variation in contact density. In this regime, contact density can be as low as 4 or as high as 7. As the protein length increases, there is an overall increase in contact density, but at the same time the variability in contact density decreases. Eventually, contact density levels off and remains in a range between approximately 6 and 8. Therefore, we next calculated the correlations between contact density, fraction of buried sites, protein length, and dN separately for short ($<$250 residues) and for long ($\geq$250 residues) proteins (Table 2). For short proteins, both the fraction of buried sites and the protein length showed a significant positive correlation with dN. For long proteins,

on the other hand, only the correlation between the fraction of buried sites and dN was positive and significant; the correlation between length and dN turned negative and lost significance. The correlation between contact density and dN was similar to but weaker than the correlation of the fraction of buried sites and dN, but was not significant at the 5% level. Finally, we noted that the correlation between length and either contact density or fraction of buried sites was stronger for short proteins (Table 2).

We then wanted to know whether the correlation between dN and protein length we observed was potentially caused by a biased selection of protein structures, because our data set was biased towards short proteins (median length is 263.5 residues for the 194 proteins with structural information and a *S. bayanus* ortholog, versus 440 residues in all 4532 *S. cerevisiae* proteins with a *S. bayanus* ortholog). Therefore, we calculated the correlation between length and dN for all ORFs, including those without structural information. We found that the overall correlation between length and dN was significant but weak (Table 3), as previously reported (Drummond, Raval, and Wilke 2006). When we considered long and short proteins separately, we found a similar picture as before. Length correlates much more strongly with dN for short proteins than for long proteins. In fact, the amount of variance in dN explained by length alone is approximately 10 times larger for short proteins than for long proteins.

Since longer proteins are known to be expressed at lower levels (Coghlan and Wolfe 2000; Munoz, Bogarad, and Deem 2004), a positive correlation between length and dN could also be caused indirectly by expression level differences. To ascertain whether differences in expression level could explain the difference in correlation between length and dN for short and long proteins, we also calculated the correlation between expression and length for short and long proteins separately. For all ORFs with evolutionary rate data, this correlation was almost identical for short and long proteins (Table 3). For the 194 ORFs with structural information, neither length nor contact density correlated significantly with expression level when we considered short and long proteins separately (not shown). Therefore, it is unlikely that the increased correlation between length and dN for short proteins is an artifact of expression level differences in these proteins.

Effect of secondary structure composition on evolutionary rate

We also asked whether a protein's composition of secondary structure types has an influence on evolutionary rate. For example, do proteins that are composed primarily of helices evolve faster or slower than other proteins? To this end, we correlated dN with the fraction

of helix sites f_{H}, fraction of sheet sites f_{E}, fraction of turn sites f_{T}, and fraction of coil sites f_{C} (Table 1). We found that none of these quantities correlated significantly with dN, and neither did they correlate with expression level or CAI (apart from a marginally significant correlation between CAI and the fraction of turn sites). However, not surprisingly, we found several strong and significant correlations among the different secondary structure measures (Table 1).

Principal component regression

The correlation analysis presented in the previous subsections is useful to get an initial understanding of the data and to find broad trends, but cannot detect more subtle interactions between the various predictor variables or quantify the amount of variance in dN these predictors explain independently of each other. Therefore, we carried out a principal component regression (Mandel 1982; Drummond, Raval, and Wilke 2006) of dN against the nine predictor variables of expression level, CAI, fraction of buried sites, contact density, protein length, and the fractions of the four secondary structure types. Table 4 summarizes the results from the principal component regression. We found five components that made a significant contribution to the regression, and the total amount of variance explained was 43.34%. The component composition is given in Fig. 6. Among the components that contributed significantly to the regression, Component 1 measures primarily the contact density of a protein. Component 2 measures primarily aspects of secondary structure. Component 3 represents a protein's expression level. Component 6 measures primarily the difference between contact density and length. Finally, Component 7 measures the difference between expression level and CAI. The components that did not contribute significantly to the regression represent secondary structure (components 4, 5, and 9, not shown) or differences between contact density and the fraction of buried sites (Component 8). Thus, the component measuring expression level explained approximately 34% of the variation in dN, while all other components together explained approximately 10% of the variation in dN.

We also regressed dN separately against expression level and CAI and against the seven structural variables, to determine how much variance these two groups of variables explained individually. Our results were very much in agreement with those of the joint regression against all nine predictor variables. The regression of dN against expression level and CAI explained 34.03% of the variance, while regression of dN against the seven structural variables explained 11.97% of the variance. Thus, the total amount of variance explained in the regression against all nine variables is approximately the sum of the amounts of variance

explained from the two individual regressions, and therefore the regression of dN against structural variables is unlikely to be confounded by expression level effects.

Does structure classification determine contact density or evolutionary rate?

We investigated the relationship between protein structure classification and both contact density and evolutionary rate for 137 proteins (out of our final data set of 194) for which we could determine the class of the structure according to the Structural Classification of Proteins (SCOP, Murzin et al. 1995). Fig. 7 shows that there are some differences in both contact density and evolutionary rate among proteins with different structure classes, but that these differences are relatively minor. We found all-α proteins to have the lowest median contact density and the highest variability in contact density, while α/β proteins had the highest median contact density and the lowest variability. The median contact density was significantly higher for α/β proteins than for all-α proteins ($p = 0.0001$) or for $\alpha+\beta$ proteins ($p = 0.0008$), but not for all-β proteins ($p = 0.070$) [Wilcoxon rank sum tests with false-discovery-rate correction (Benjamini and Hochberg 1995) for multiple testing]. The median evolutionary rate was the highest for all-β proteins, but the individually largest evolutionary rates were observed for α/β and $\alpha+\beta$ proteins. However, neither all-β proteins nor any other class of proteins had a significantly elevated evolutionary rate after correction for multiple testing (Wilcoxon rank sum tests with false-discovery-rate correction).

Discussion

Our results show that protein structure has a moderate effect on protein evolutionary rate. Furthermore, this effect is consistent with the idea that proteins with more designable structures, as indicated by higher contact densities (England and Shakhnovich 2003), tend to evolve more rapidly. Specifically, contact density, the fraction of buried sites, and protein length each showed a significant correlation with dN, with R^2 values between 4% and 8%. These correlations were not caused by confounding effects due to co-correlations with protein expression level, which is the dominant determinant of evolutionary rate in yeast (Pal, Papp, and Hurst 2001; Drummond, Raval, and Wilke 2006). The contact density and the fraction of buried sites were strongly correlated with each other, and had roughly comparable predictive power for the evolutionary rate, although the fraction of buried sites tended to be a slightly better predictor than contact density. We found that secondary structure composition and protein fold classification had almost no effect on evolutionary rate. Protein

length was significantly correlated with both dN and contact density, making it difficult to fully elucidate the separate contributions of length and contact density to evolutionary rate. However, the fact that length is positively correlated with dN only for short proteins, but that higher contact density leads to faster evolutionary rates for both long and short proteins, supports the notion that higher contact density increases evolutionary rate independent of length effects.

We corroborated the correlations we observed with a principal component regression of dN against all structural predictor variables plus protein expression level and CAI. We found that the principal components that measured primarily aspects of contact density or fraction of buried residues explained 6.35% of the variation in dN. In comparison, the principal component related to secondary structure explained only 1.32% of the variation in dN. Overall, the principal component regressions indicated that the structural characteristics we considered explained between 10 and 12% of the variation in evolutionary rate, and that the important variables in this explanation were contact density (or the highly correlated variable of fraction of buried residues) and protein length.

We had to restrict our analysis to those yeast proteins to which we could confidently assign structures. Although there are tools that use homology modeling or other computational methods to predict the structure adopted by a protein sequence, we chose to consider only those yeast proteins that matched with at least 80% identity to an experimentally determined structure. This choice eliminated the possibility of introducing biases due to inaccuracies of the structure prediction tools, but it also substantially reduced the size of our data set since the number of proteins with experimentally solved structures is relatively low. Overall, we were able to match structures with only 194 of the 4223 yeast proteins for which we had expression and evolutionary information. The proteins that we matched with structures tended to be both more highly expressed and slower evolving than all yeast proteins for which we had expression and evolutionary information (the median transcript per cell levels were 2.7 and 0.8, respectively; the median dN values were 0.03 and 0.08, respectively). One clear consequence of limiting ourselves to proteins with experimentally solved structures is that we are excluding those (usually faster evolving) proteins that contain large regions that are intrinsically disordered (Brown et al. 2002). It is possible that the subset of yeast proteins with structures also contains other biases that affect the correlation between contact density and evolutionary rate. However, it is impossible to assess any such effects with the currently available protein structures, and so further analysis of this question will have to await the experimental determination of more protein structures.

At first glance, our findings that proteins with higher contact densities (and therefore a higher fraction of buried residues) evolve more slowly seems at odds with the tendency for buried residues to be more conserved (Koshi and Goldstein 1995; Goldman, Thorne, and Jones 1998; Mirny and Shakhnovich 1999; Bustamante, Townsend, and Hartl 2000; Dean et al. 2002). The key point is to realize that while buried residues are generally more conserved than exposed ones, increasing the fraction of buried residues leads to an overall increase in the evolutionary rate of all residues in the protein, primarily via a dramatic increase in dN for the exposed residues. We suggest that the increase in designability that accompanies high contact density enhances the mutational tolerance of exposed residues enough to more than offset the higher fraction of slower-evolving buried sites. A potential reason for the elevated evolutionary rate at exposed sites is increased protein stability. Highly designable proteins tend to be more stable (Wingreen, Li, and Tang 2004), and stability promotes mutational tolerance (Bloom et al. 2005, 2006). A larger fraction of buried residues suggests a more robust protein core whose stability may thus allow loops and other surface features to mutate more freely. In other words, regions of high contact density form stabilizing cores of conserved highly interacting amino acids that allow other exposed regions of the sequence to mutate more freely (Shakhnovich et al. 2005).

Overall, our results support the notion that proteins with more designable structures tend to evolve their sequences more rapidly. These findings suggest that the structures of real proteins may differ substantially in their mutational tolerances, and that this effect is manifested in the rates of sequence evolution across the yeast proteome. However, the overall contribution of protein structure to evolutionary rate that we detect is still much smaller than that made by protein expression level: the principal component representing protein expression explains three times more variance in dN than all of the structural components. Earlier work has shown that highly expressed proteins are more likely to adopt mixed α-helix and β-sheet folds (Jansen and Gerstein 2000). However, this tendency did not lead to a net relationship between contact density and expression level in our data set, since we found these two variables to be uncorrelated. Therefore, protein structure appears to make an independent contribution to evolutionary rate, although expression level remains the more dominant force in determining the rate of sequence evolution.

However, it is possible that our analysis underestimates the contribution of protein structure to evolutionary rate. In attempting to apply the designability theory of England and Shakhnovich (2003), our ignorance of the a_n coefficients in Eq. 1 has forced us to make the severe approximation of truncating all higher order powers of the contact matrix, and esti-

mating designability solely from contact density. The effect of this truncation is unknown, but contact density is surely less informative than the full series of Eq. 1. Furthermore, the derivation of Eq. 1 by England and Shakhnovich (2003) makes the twin assumptions that proteins are stabilized only by pairwise contacts and that designability is simply equal to the number of sequences that fold to a structure with an energy below some cutoff — both of these assumptions are unlikely to be completely true for real proteins. For these reasons, contact density is clearly an imperfect proxy for designability, and our inability to more accurately quantify designability probably causes us to underestimate its true contribution to protein evolutionary rate.

Despite these caveats, our work makes an important contribution by providing the some of the first evidence about how a protein's structure influences its evolutionary rate. Structure is clearly only one of many factors that determines the extent of constraint on a protein's sequence, but its effect appears to be significant. Future progress in developing theoretical treatments of structural designability and in better characterizing the other factors that constrain sequence evolution should eventually allow for improved measurements of the net effect of structure on protein evolution. For now, we simply add a structure's designability to the pantheon of factors that shape the rate of protein sequence evolution.

Supplementary Material

The data set of 194 *S. cerevisiae* ORFs analyzed in this work is available as supplementary material at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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Table 1: Spearman correlations between variables considered in this study.

	dN	x	c	$f_{\rm bur}$	d	L	$f_{\rm H}$	$f_{\rm E}$	$f_{\rm T}$
x	-0.58^{***}								
c	-0.40^{***}	0.71^{***}							
$f_{\rm bur}$	0.29^{***}	-0.09	$0.20^{*(*)}$						
d	$0.24^{**(*)}$	-0.01	$0.20^{*(*)}$	0.80^{***}					
L	$0.19^{*(*)}$	$-0.14^{(*)}$	0.13	0.79^{***}	0.57^{***}				
$f_{\rm H}$	0.03	-0.01	0.05	-0.05	0.13	0.07			
$f_{\rm E}$	-0.01	0.06	-0.05	0.10	0.06	-0.04	-0.80^{***}		
$f_{\rm T}$	-0.10	0.10	0.17^{*}	$0.19^{*(*)}$	$0.19^{*(*)}$	0.06	-0.46^{***}	0.28^{***}	
$f_{\rm C}$	0.02	-0.10	0.02	-0.02	-0.33^{***}	-0.01	-0.40^{***}	-0.06	0.11

Significance levels: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$

NOTE.—dN: nonsynonymous evolutionary rate; x: gene expression level; c: codon adaptation index; $f_{\rm bur}$: fraction of buried sites; d: contact density; L: protein length; $f_{\rm H}$, $f_{\rm E}$, $f_{\rm T}$, $f_{\rm C}$: fraction of sites with secondary structure helix, sheet, turn, coil, respectively. Significance levels in parentheses disappear after correction for multiple testing (Benjamini and Hochberg 1995).

Table 2: Spearman correlations ρ for all ORFs with structural information, calculated separately for short and long proteins.

	$L < 250$ ($n = 85$)	$L \geq 250$ ($n = 109$)
$\rho(d, dN)$	0.20^{+}	0.14
$\rho(f_{\rm bur}, dN)$	0.27^{*}	0.21^{*}
$\rho(L, dN)$	0.22^{*}	-0.14
$\rho(d, L)$	0.46^{***}	0.22^{*}
$\rho(f_{\rm bur}, L)$	0.61^{***}	0.47^{***}

Significance levels: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; $^{+}P < 0.1$

NOTE.—n is the number of ORFs in each group, and other symbols are defined as in Table 1.

Table 3: Spearman correlations ρ for all ORFs with evolutionary rate data, calculated separately for short and long proteins.

	all lengths ($n = 4523$)	$L < 250$ ($n = 935$)	$L \geq 250$ ($n = 3597$)
$\rho(L, dN)$	0.07^{***}	0.13^{***}	0.04^{*}
$\rho(L, x)$	-0.24^{***}	-0.17^{***}	-0.15^{***}

Significance levels: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$

NOTE.—n is the number of ORFs in each group, and other symbols are defined as in Table 1.

Table 4: Percent variance explained in dN and in the predictor variables, as found by a principal component (PC) regression of dN against 9 predictor variables.

	PC 1	**PC 2**	**PC 3**	PC 4	PC 5	**PC 6**	**PC 7**	PC 8	PC 9	total
dN	**2.81**	**1.32**	**33.59**	0.00	0.00	**3.54**	**1.27**	0.65	0.15	43.34
predictor space	**28.83**	**23.86**	**19.10**	12.47	7.94	**3.63**	**1.27**	0.65	0.15	100.00

NOTE.—Predictor variables are x, c, f_{bur}, d, L, f_{H}, f_{E}, f_{T}, and f_{C}, with symbols defined as in Table 1. Principal components that make a statistically significant contribution to the variation in dN are shown in boldface ($P < 0.05$ for all components in boldface). The percent values for the individual components do not sum exactly to the numbers given under total because of rounding errors. See Fig. 6 for component composition.

Figure 1: A model for how the designability of a protein's structure might affect the rate at which its sequence evolves. If many sequences fold into a given structure (highly designable), then many mutations preserve the structure, i.e., the structure is mutationally tolerant. As a consequence, we observe rapid evolutionary divergence. Conversely, if few sequences fold into a given structure, then the structure is mutationally brittle, and evolutionary divergence is slow.

Figure 2: Distributions of evolutionary rates dN for different residue types, shown as modified box plots. (Boxes enclose all data between the first and third quartile, and are divided at the median. Whiskers at top extend to the maximum observation or 1.5 times the box height from the top of the box, whichever is smaller. Similarly, whiskers at bottom extend to the minimum observation or 1.5 times the box height from the bottom of the box, whichever is larger. All data points outside the range of the whiskers are drawn individually. See e.g. Sokal and Rohlf 1994.) For all residue types, the distribution of dN is heavily skewed, and thus not well characterized by its mean and standard deviation. For the two residue types "exposed" and "helix", one outlier each falls outside the top boundary of the graph. The evolutionary rates in all groups are significantly different from each other with $P < 0.01$ (Wilcoxon signed rank test), apart from helix and coil ($P = 0.01$), and coil and turn ($P = 0.37$, not significant).

Figure 3: Evolutionary rate dN as a function of a protein's contact density.

Figure 4: Evolutionary rate dN for buried sites (left panel) or exposed sites (right panel) only, as a function of the protein's contact density.

Figure 5: Contact density as a function of protein length.

Figure 6: Composition of the first eight principal components (PCs). Symbols representing predictor variables are as defined in Table 1. Each dot represents the contribution of the corresponding predictor variable to the principal components. For example, in the top-left panel we see that L makes a strong, positive contribution to PC 1, and a weak, negative contribution to PC 2.

Figure 7: Modified boxplots of the distributions of contact density (left) and evolutionary rate (right) for different SCOP classes. Not shown are 3 proteins classified as multi domain (α and β), 2 classified as membrane and cell surface proteins and peptides, 2 for which the classification was ambiguous, and 55 for which no classification could be determined.

Figure 1

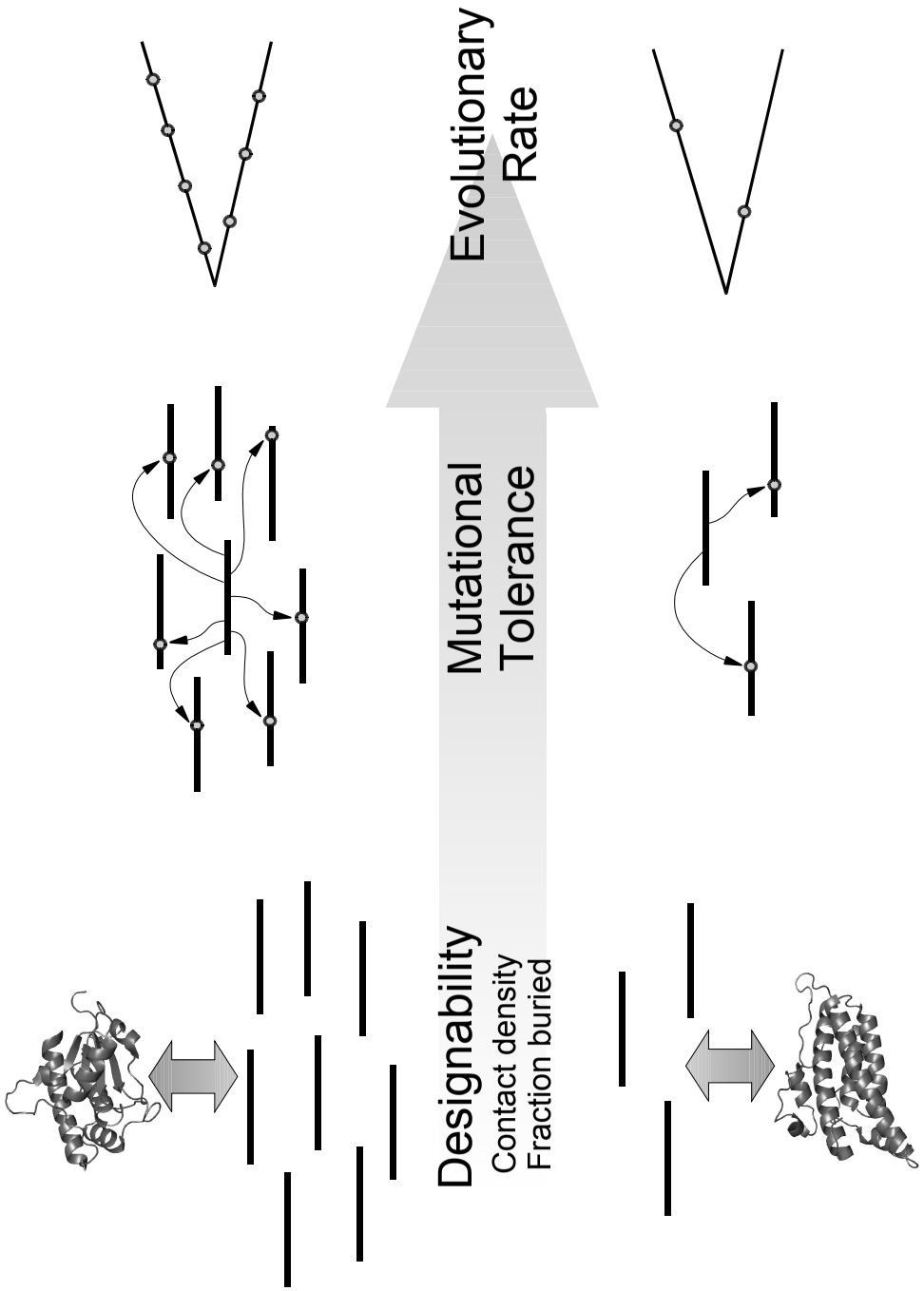

Evolutionary Rate
Mutational Tolerance
Designability
Contact density
Fraction buried

Figure 2

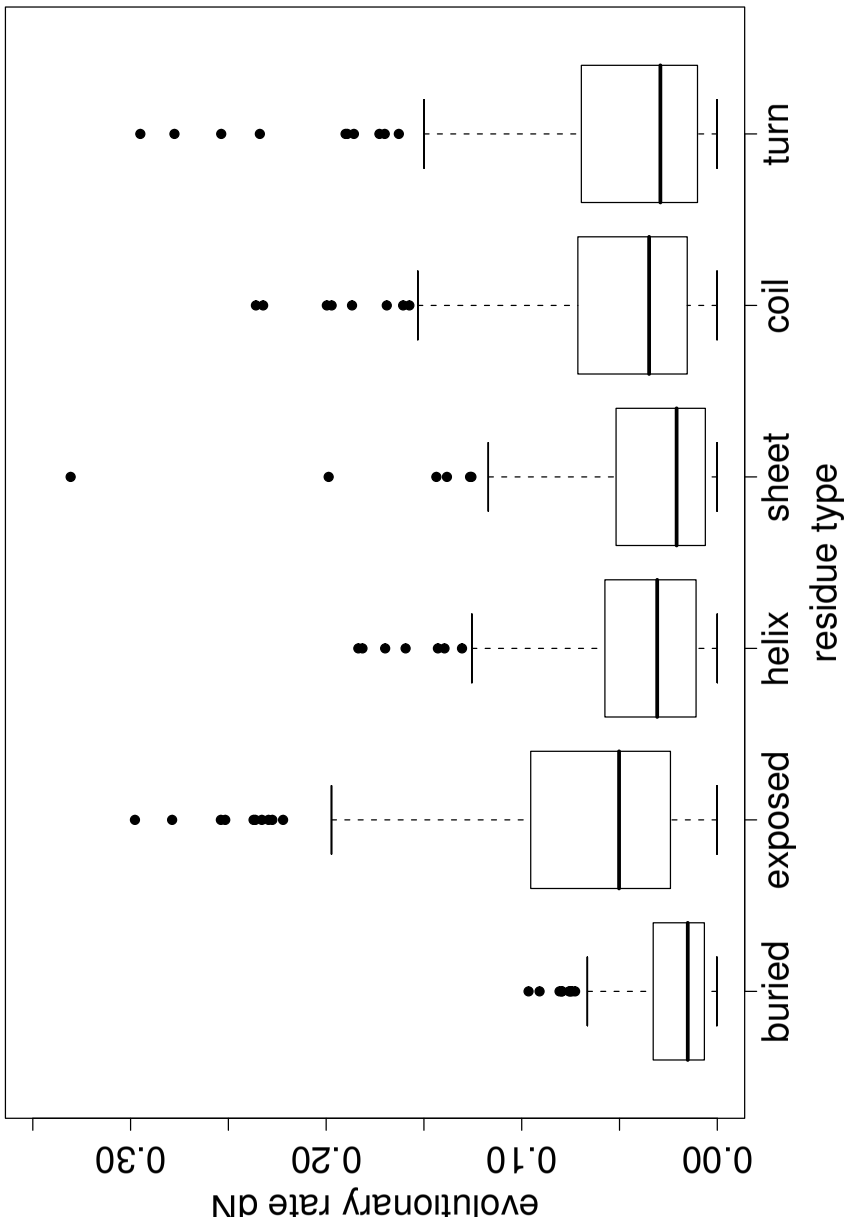

evolutionary rate dN
0.00
0.10
0.20
0.30
buried
exposed
helix
sheet
coil
turn
residue type

Figure 3

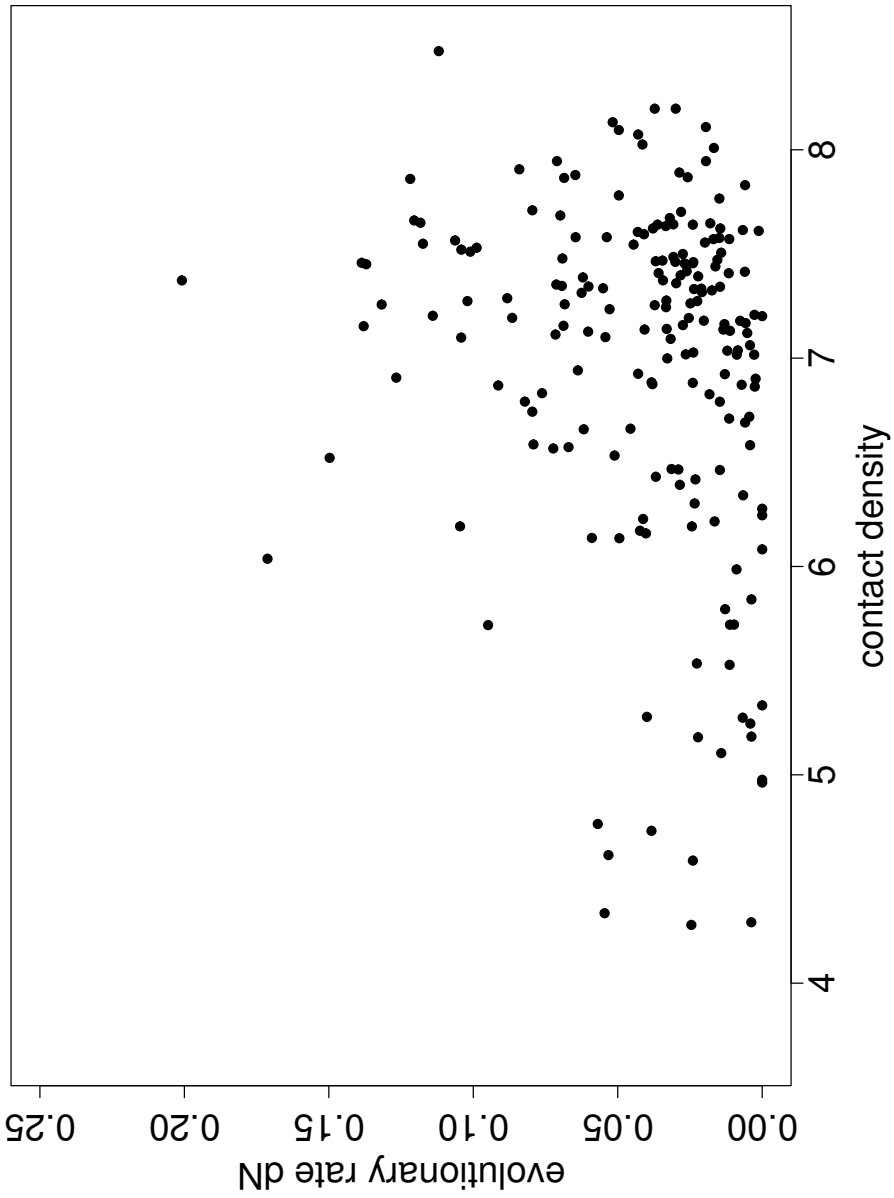

evolutionary rate dN
0.00
0.05
0.10
0.15
0.20
0.25
contact density
4
5
6
7
8

Figure 4

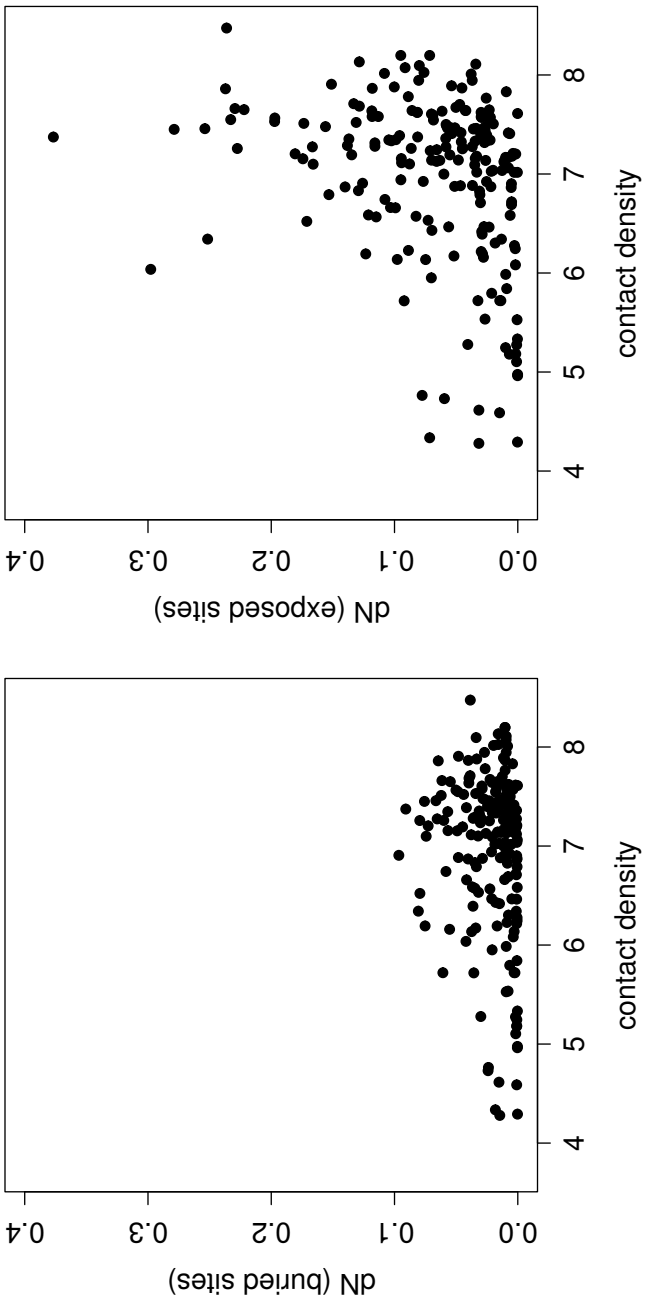

dN (exposed sites)
0.0
0.1
0.2
0.3
0.4
contact density
4
5
6
7
8
dN (buried sites)
0.0
0.1
0.2
0.3
0.4
contact density
4
5
6
7
8

Figure 5

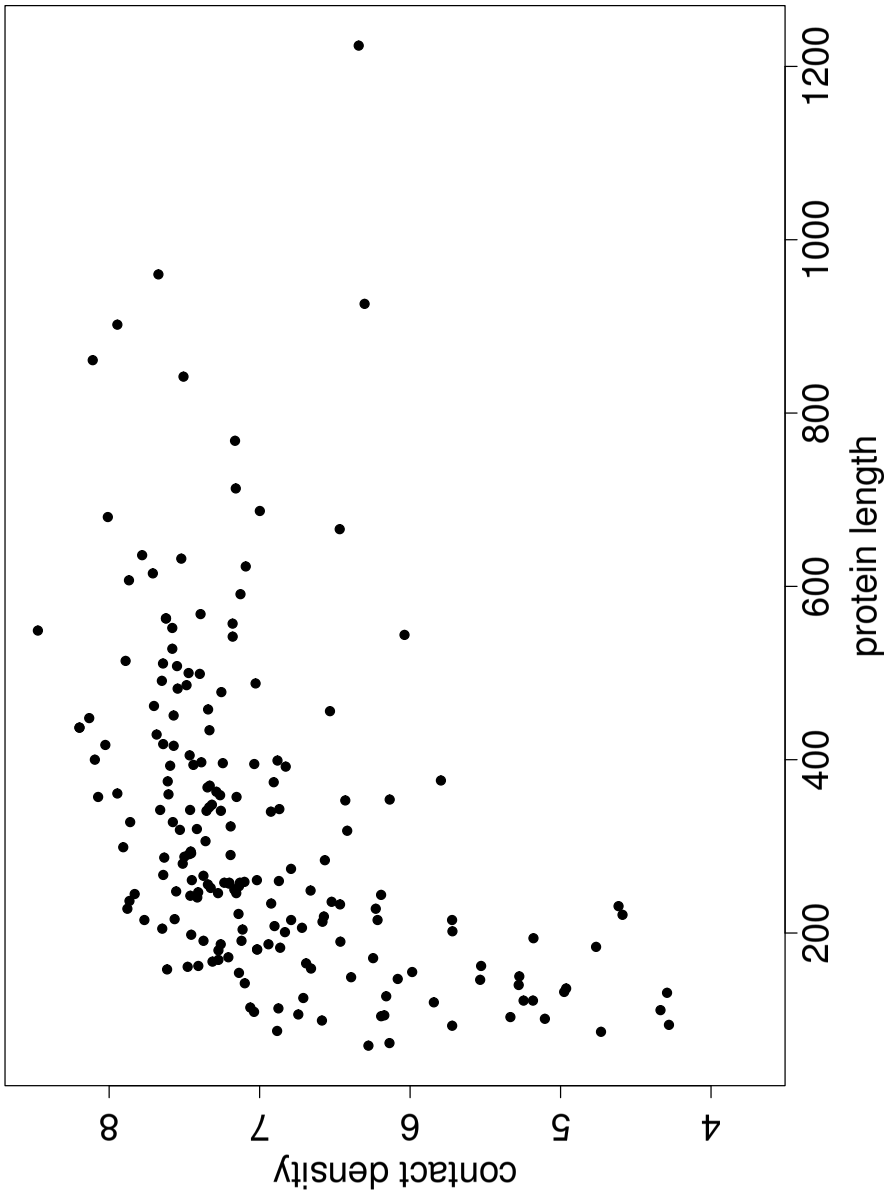
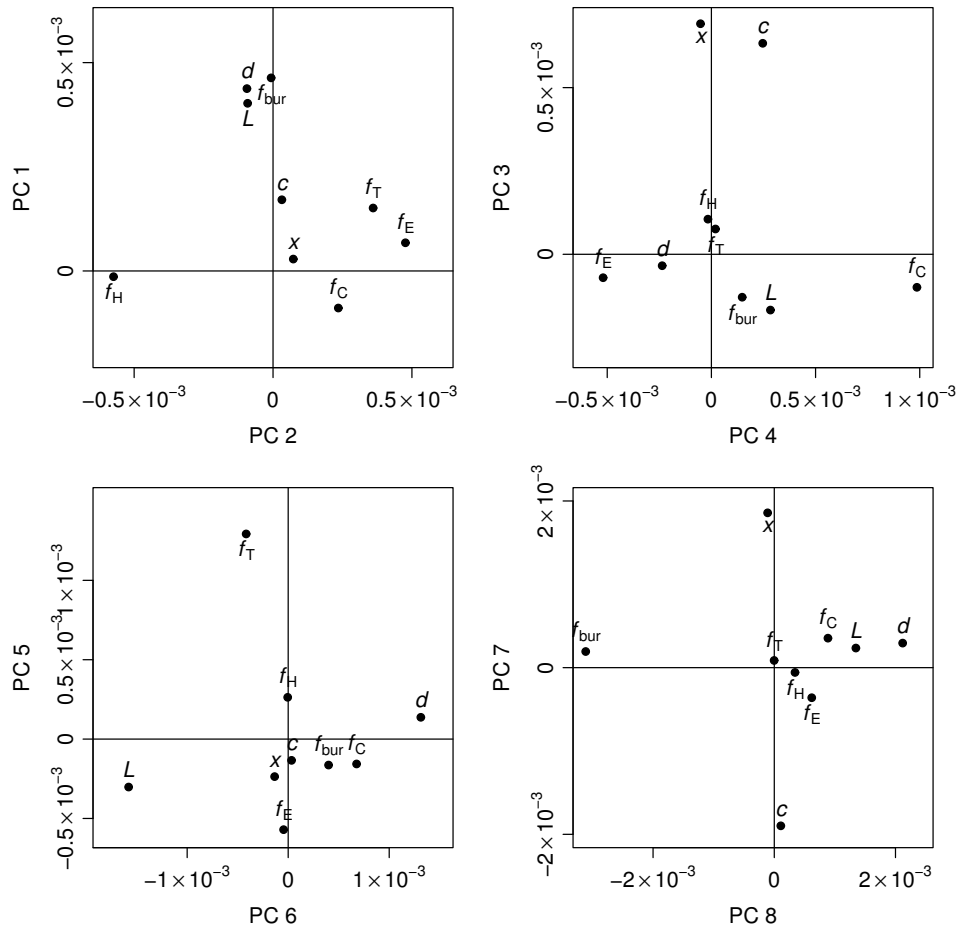

protein length
200
400
600
800
1000
1200
contact density
4
5
6
7
8

Figure 6


PC 1
PC 2
PC 3
PC 4
PC 5
PC 6
PC 7
PC 8
d
f_bur
L
c
f_T
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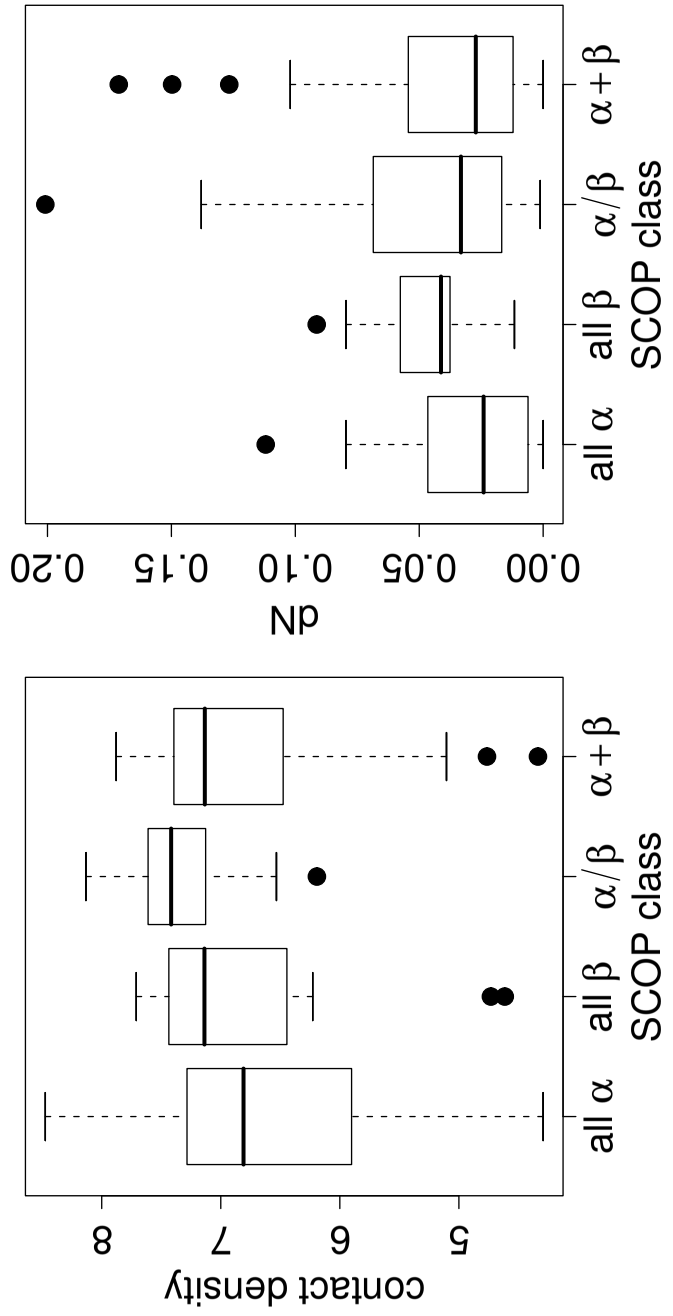
x
f_H
f_C
0.5×10^−3
−0.5×10^−3
0
1×10^−3
−1×10^−3
2×10^−3
−2×10^−3

Figure 7


dN
0.00
0.05
0.10
0.15
0.20
all α
all β
α/β
α+β
SCOP class
contact density
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6
7
8
all α
all β
α/β
α+β
SCOP class