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Extreme Functional Sensitivity to Conservative Amino Acid Changes on Enzyme Exteriors

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Mutagenesis studies and alignments of homologous sequences have demonstrated that protein function typically is compatible with a variety of amino-acid residues at most exterior non-active-site positions. These observations have led to the current view that functional constraints on sequence are minimal at these positions. Here, it is shown that this inference assumes that the set of acceptable residues at each position is independent of the overall sequence context. Two approaches are used to test this assumption. First, highly conservative replacements of exterior residues, none of which would cause significant functional disruption alone, are combined until roughly one in five have been changed. This is found to cause complete loss of function *in vivo* for two unrelated monomeric enzymes: barnase (a bacterial RNase) and TEM-1 β-lactamase. Second, a set of hybrid sequences is constructed from the 50 %-identical TEM-1 and *Proteus mirabilis* β-lactamases. These hybrids match the TEM-1 sequence except for a region at the C-terminal end, where they are random composites of the two parents. All of these hybrids are biologically inactive. In both experiments, complete loss of activity demonstrates the importance of sequence context in determining whether substitutions are functionally acceptable. Contrary to the prevalent view, then, enzyme function places severe constraints on residue identities at positions showing evolutionary variability, and at exterior non-active-site positions, in particular. Homologues sharing less than about two-thirds sequence identity should probably be viewed as distinct designs with their own sets of optimising features.

Keywords: exposed residues; protein homologues; molecular evolution; neutral theory; sequence space

Introduction

How amino acid sequences encode functional proteins has been a matter of intense interest for decades. Although many aspects of this problem remain unsolved, progress has been made in addressing the basic question of how tightly biological function constrains protein sequences. The currently accepted answer is that the constraints are quite loose, particularly at positions on the exterior of a protein that are not directly involved in binding or catalysis.

The origins of this low-constraint view can be traced to three key developments in the latter half of the 1960s. During this period, the first systematic examination of a family of proteins, the globins, revealed both a high degree of sequence variation overall and a particularly high degree of variation at surface positions (Perutz *et al.*, 1965). A subsequent study of natural haemoglobin variants in humans (Perutz & Lehman, 1968) accounted for the earlier finding by showing that exterior positions readily tolerate substitution while interior ones do not. At about the same time, a hypothesis which became known as the neutral theory of molecular evolution emerged (Kimura, 1968, 1983). This theory proposes that the great majority of amino acid substitutions that become established over the course of evolution do so not as a result of selective benefit, but rather as a result of random drift. In order for this to be true, it must be the case that a sizeable proportion of new substitutions have no significant functional effect. Thus, the globin data

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seem to support the neutral theory, and the neutral theory seems to explain the globin data.

The volume of data relevant to this issue has grown dramatically, but the two main lines of evidence continue to be sequence comparisons among related proteins and functional characterisation of mutant proteins, and the overall conclusions drawn from these evidential lines are very similar to those drawn three decades ago (Matthews, 1995; Sauer, 1996; Chothia *et al*., 1998). When very large collections of related protein sequences are compared (Chothia *et al*., 1998), we continue to find a variety of residues at most positions, with more restrictions evident in the interior. Although strong biases against certain residues are now evident even at surface positions, absolute exclusion is rare (Chothia *et al*., 1998). Likewise, mutagenesis studies where multiple substitutions are tested individually at each position along a protein typically show that most positions tolerate a variety of substitutions, exterior positions (apart from those directly involved in function) usually exhibiting the most tolerance (Reidhaar-Olson & Sauer, 1988; Bowie *et al*., 1990; Rennell *et al*., 1991; Terwilliger *et al*., 1994; Suckow *et al*., 1996; Huang *et al*., 1996; Axe *et al*., 1998). The current view, then, is well summarised by Bowie and co-workers (1990): ''Except for functionally important residues, exterior positions seem to be important chiefly in maintaining a reasonably polar surface. The information contained in buried residues is also degenerate, the main requirement being that these residues remain hydrophobic''.

Results and Discussion

Testing for loose exterior constraints

Although this view seems to follow naturally from the data, it clearly involves an element of extrapolation. It implies that large-scale alterations of protein exteriors should be functionally tolerated if active-site residues and overall hydropathy are preserved, whereas the two principal lines of evidence upon which it is based offer no direct support for this. A direct test of the corresponding extrapolation for protein interiors, where an entire hydrophobic core was randomly replaced by alternative hydrophobic residues, demonstrated that hydrophobicity is indeed the main criterion for a core to support basic enzymatic function *in vivo* (Axe *et al*., 1996).

The original objective in the present study was to perform a similar direct test on exterior residues, the expectation being that extensive random substitution should be even more readily tolerated. When initial results indicated that this is not the case, a more modest level of tolerance was considered. The revised objective was to determine whether exterior positions known to tolerate substitutions individually could be subjected to highly conservative replacements *en masse* without dramatically impairing function. In order to avoid drawing conclusions that may be peculiar to one protein, two unrelated proteins were examined: barnase, the single-domain monomeric ribonuclease used in the hydrophobic core study (Axe *et al*., 1996), and TEM-1 β-lactamase, a two-domain monomeric enzyme that confers bacterial resistance to penicillin-like antibiotics.

There are 84 exterior non-active-site positions in barnase and 171 in TEM-1 β-lactamase (using 5% solvent-accessible surface area as the lower bound for exterior positions (Miller *et al*., 1987)). Positions where changes are tolerated have been identified in both proteins (Huang *et al*., 1996; Axe *et al*., 1998) by applying biological selection to large populations of mutants carrying one or a few substitutions. Using this information, five groups of five replaceable exterior residues in barnase and four groups of ten replaceable exterior residues in β-lactamase were chosen for simultaneous substitution (Figure 1). Residues in each group were replaced using the following conservative substitution set: Asp ↔ Glu, Lys ↔ Arg, Asn ↔ Gln, Ser ↔ Thr, Ile ↔ Val, Ala → Ser, Leu → Ile, and Met → Leu†. After the mutations were introduced into plasmids carrying the barnase or β-lactamase genes, *Escherichia coli* clones carrying the mutant plasmids were tested for the relevant activity by applying biological screens.

The barnase screen simply indicates whether the cytoplasmic activity is above or below a toxic threshold of about 0.1% wild-type activity (Axe *et al*., 1999). All five single-group mutants of barnase have activities above this threshold (Figure 2). However, when double-group mutants representing all group pairs are constructed, most are found to be inactive. Since the double-group mutants barMG, barMB, and barGB all retain function, the triple-group mutant barMGB was constructed, but this was found to be inactive.

Mutant β-lactamase activities can be quantified by determining maximum tolerable antibiotic concentrations (Table 1). Again, single-group mutants retain significant levels of function. Since the TEM-1 enzyme has two domains, groups were combined so that all substitutions lie in one domain or the other. As with barnase, these double-group mutants are more severely affected. The combination of all four groups in blaMYBG is completely inactivating, but by combining the three less disruptive groups, a mutant with thirty substitutions that shows very weak activity was obtained (blaMYG, Table 1).

The single-group substitutions in blaM, blaY, and blaG affect function only mildly, yet these substitutions result in >99% inactivation when combined. Therefore, although the individual substitutions involved would have minor effects

† Although these substitutions were chosen for chemical similarity, all except Asn ↔ Gln are also the most common in nature (Gonnet *et al*., 1992). The reduced natural frequency of Asn ↔ Gln substitutions is explained by the fact that two DNA base changes are required.

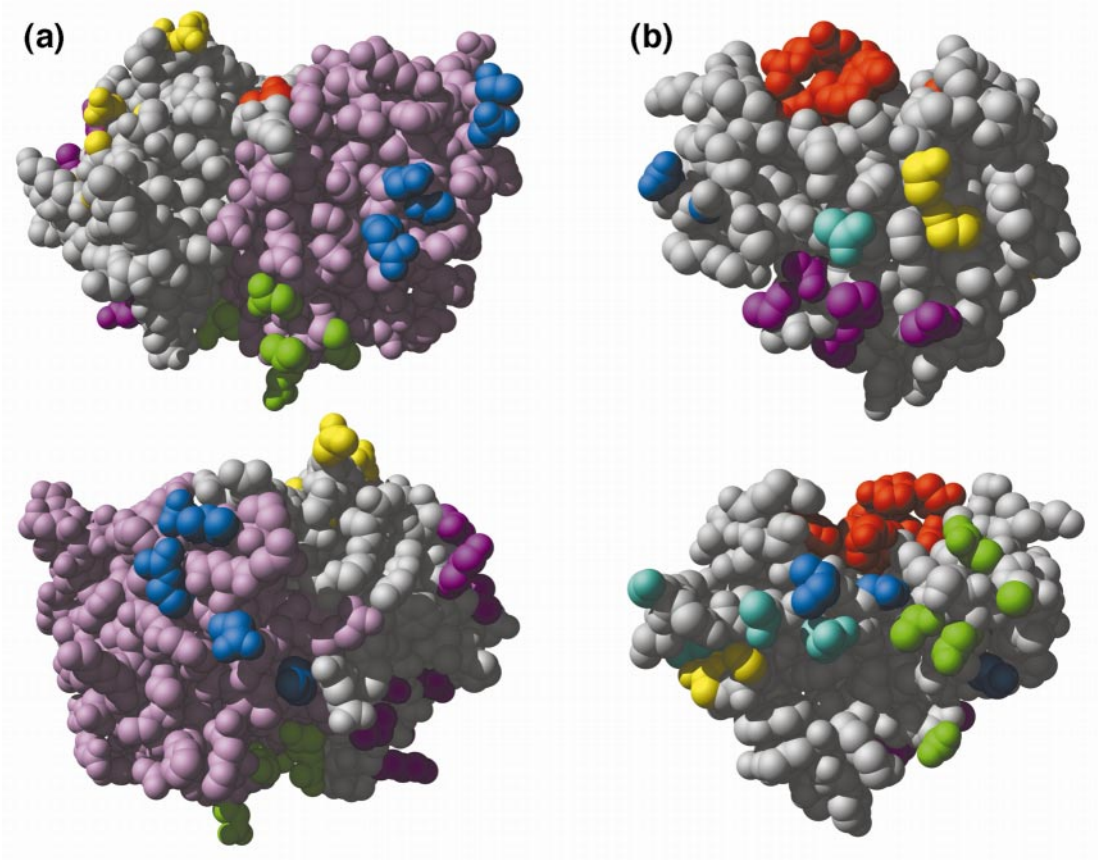

Figure 1. Wild-type residues replaced in group-substitution experiments on TEM-1 β-lactamase and barnase. All chosen residues substantially exceed the criterion of 5 % accessibility (relative to the extended Gly-X-Gly conformation (Miller *et al.*, 1987)), with mean accessibilities being 45 % for those in β-lactamase and 48 % for those in barnase. Mutant names are of the form blaX or barX, the lower case prefixes designating TEM-1 β-lactamase or barnase and the upper case letters designating (by colour codes given below) the groups of residues that have been replaced. Energy-minimised model structures (Guex & Peitsch, 1997) of the fully substituted mutants, blaMYBG and barMGBCY, show no significant backbone changes relative to the wild-type structures (data not shown). Images were rendered with POV-ray (http://www.povray.org). (a) TEM-1 β-lactamase (PDB entry 1TEM (Maveyraud *et al.*, 1996)) viewed from opposite sides. A substrate analogue (red) shows the active site location at the top. Light pink and grey shades differentiate the two domains of the enzyme. In the gray domain, the magenta (M) group includes residues V31, K32, K34, D35, D38, K55, I56, L57, E58, S59, and the yellow (Y) group includes Q269, T271, D273, E274, R275, A280, E281, A284, S285, I287. In the pink domain, the blue (B) group includes T114, D115, T118, R120, E121, E171, I173, E177, R178, M182, and the green (G) group includes T188, R191, K192, T195, E197, L198, T200, L201, A202, Q205. (b) Barnase (PDB entry 1BRN (Buckle & Fersht, 1994)), complexed with a substrate analogue (red), viewed from opposite sides. The magenta (M) group includes residues V3, I4, N5, T6, D8; the green (G) group includes D22, T26, E29, Q31, A32; the blue (B) group includes A43, D44, K49, D54, I55; the cyan (C) group includes S67, R69, T70, R72, N77; the yellow (Y) group includes S92, D93, L95, K98, T100.

in isolation, they have a catastrophic effect in combination. The fact that conservative replacement of only a fifth of the exterior residues causes near total loss of activity in both enzymes shows that function actually places very tight constraints on amino acid identities at exterior positions (a quantitative estimate follows).

The assumption of context independence

Although this result contradicts the accepted interpretation of previous studies, we should consider whether it may be consistent with the actual data from those studies. Given the sequences of a set of similar proteins that perform the same function, we typically seek a generic account of the sequence-function relationship that applies equally to all members of the set. To this end, it is often assumed that the functional importance of each position can be assessed in a generic way, without considering the various sequence contexts in which that position finds itself. Thus, when a variety of amino acid residues are found at a particular position, the usual inference is that function places little constraint on sequence at that position. This inference has become so prevalent and fundamental, both in studies of mutant proteins and in analyses of related proteins, that it is taught in introductory textbooks: ‘‘Comparisons of the primary structures of homologous proteins... indicate

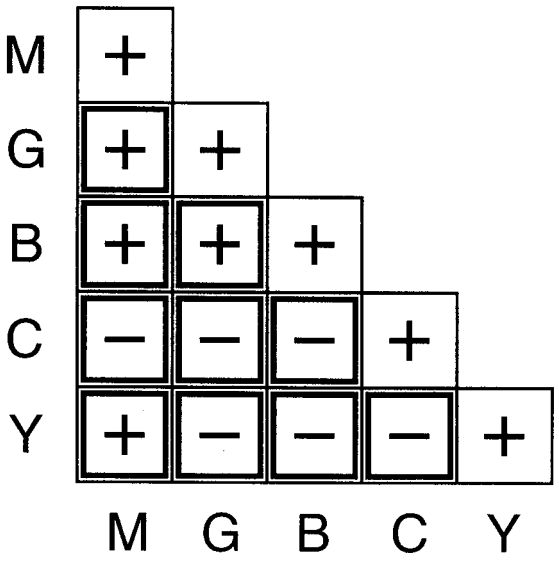

Figure 2. *In vivo* activities of barnase single and double-group mutants. Activities of mutants with group substitutions indicated by row and column labels (see Figure 1 legend) are shown as either + (above threshold) or − (below threshold). Double-group mutants indicated by bold boxes.

which of the proteins' residues are essential to its function, which are of lesser significance, and which have little specific function. ... [M]any different residues may be tolerated at a particular amino acid position, which indicates that the functional requirements of that position are rather nonspecific'' (Voet & Voet, 1995).

The potential pitfalls of assuming context independence are well illustrated by a linguistic analogy (Figure 3). Starting with a functional (i.e. meaningful) string of characters, we can produce a large set of ''mutant'' strings containing single character replacements. Although some of these mutant strings would fail to perform the function of the original, a great many would adequately convey the original message. This collection of adequate mutant strings is therefore analogous to a collection of functional mutants of a wild-type protein. By assuming context independence, we would conclude that acceptable replacements remain acceptable in the context of any number of other acceptable replacements. The example of Figure 3 demonstrates how wrong this conclusion can be and shows that the corresponding conclusion pertaining to functionally equivalent homologous sequences can be equally wrong.

Testing for context independence

To find out whether context independence holds for proteins, the experimental equivalents of the linguistic exercises in Figure 3 were performed. TEM-1 β-lactamase is well suited to these experiments because, unlike barnase, its reported functional variants are very highly functional (Huang *et al.*, 1996), and it belongs to a large class of homologous enzymes, the Class A β-lactamases (Ambler *et al.*, 1991).

Of the 40 substitutions carried by mutant bla-MYBG, 33 are among those represented in a large collection of highly active triple mutants (Huang *et al.*, 1996). It is possible that the remaining seven substitutions are equally acceptable, their absence from the reported set being a consequence of incomplete sampling. As a strict test of context independence, though, the amino acid residues at each of these seven positions were changed to alternatives that, although less similar to the wild-type, were shown to allow very high activity *in vivo* (Huang *et al.*, 1996). The resulting single-domain mutants, bla{MY} and bla{BG}, and the combined mutant, bla{MYBG}, all remain dramatically impaired (Table 1). Like the left-hand charac-

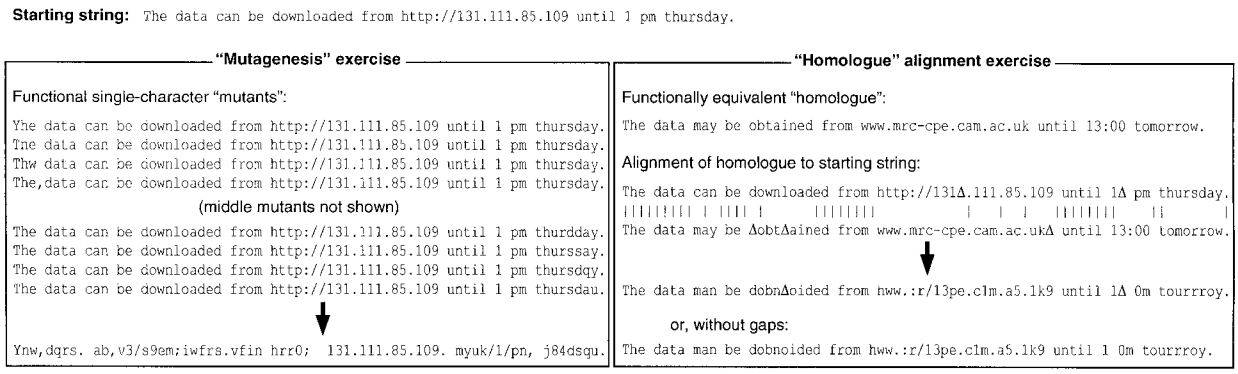

Figure 3. Linguistic illustration of the potential consequences of assuming context independence. The two boxed exercises parallel mutagenesis studies and homologue-alignment studies on proteins. Δ represents gaps (i.e. the absence of a corresponding character position). By presuming context independence, we are led to conclude (bold arrows) that any composite of functional sequences must itself be a functional sequence. Since the bottom strings clearly are not functional, this presumption is invalid in these cases.

Table 1. *In vivo* activities of TEM-1 group mutants

	Maximum permissive concentration[a] (MPC; μg/ml) of		Relative hydrolysis activity[b] toward	
	Ampicillin	Penicillin G	Ampicillin	Penicillin G
Plasmid-free	4	30	0	0
w.t. TEM-1	4000	12,000	1	1
Single-group mutants				
blaM	2500	4200	0.62	0.35
blaY	1500	4000	0.37	0.33
blaB	200	250	0.049 (0.50)	0.018 (0.20)
blaG	2500	5000	0.62	0.42
Domain mutants				
blaMY	250	300	0.062 (0.30)	0.023 (0.097)
blaBG	30	60	0.0065 (0.099)	0.0025 (0.022)
bla{MY}[c]	100	200	0.024 (0.20)	0.014 (0.064)
bla{BG}[c]	5	<40	0.00025 (0.011)	<0.00084 (0.0017)
Both domains affected				
blaMYG	30	50	0.0065 (0.049)	0.0017 (0.013)
blaMYBG	<5	<40	<0.00025	<0.00084
bla{MYBG}[c]	<5	<40	<0.00025	<0.00084

[a] See Materials and Methods for protocol.

[b] Wild-type strains of *E. coli* constitutively produce AmpC (Bennet & Chopra, 1993), a protein shown recently to have a role in cell wall synthesis (Henderson *et al.*, 1997). Like other enzymes with this function, AmpC can hydrolyse some β-lactam antibiotics. Plasmid-free cells consequently show low-level penicillin resistance but no significant resistance to ampicillin. Innate resistance is taken into account by calculating relative activities as $(MPC_{mutant} - MPC_{plasmid\text{-}free})/(MPC_{w.t.\ TEM} - MPC_{plasmid\text{-}free})$. This value is proportional to the velocity of the hydrolysis reaction at the maximum permissive antibiotic concentration for each mutant. Values in parentheses correspond to a separate set of experiments (see below) where plates were incubated at 25 °C for 36 hours (concentration data not shown).

[c] Brackets denote mutants that have been modified such that only substitutions shown by Huang *et al.* (1996) to be tolerated are used (see Testing for context independence). The following substitutions in these mutants deviate from the conservative substitution set: in the {M} group, V31K, D35S, D38L; in the {Y} group, A280M; in the {B} group E177P, R178N; in the {G} group L198S.

ter string at the bottom of Figure 3, bla{MYBG} is a functionless composite of functional sequences. As such, it demonstrates that the acceptability of mutant substitutions cannot be judged in a context-independent manner.

A Class A β-lactamase from *Proteus mirabilis* (Sakurai *et al.*, 1991) has a high degree of sequence similarity to the TEM-1 enzyme (50 % amino acid identity; 71 % similarity by pam250S groupings). Both enzymes are highly active against penicillin G and ampicillin. Although the structure of the *P. mirabilis* enzyme has not been determined, the close structural resemblance between the TEM-1 enzyme (Maveyraud *et al.*, 1996) and a less similar homologue (that from *Bacillus licheniformis* (Knox & Moews, 1991), showing 0.9 Å backbone rmsd from TEM-1 over 90 % of the structures, with 38 % sequence identity) implies a very high degree of structural similarity between the TEM-1 and *P. mirabilis* enzymes. To determine whether differing residues at aligned positions in these enzymes are comparably suitable, irrespective of context, a set of plasmids encoding 20 hybrid proteins was produced. The hybrids are identical to the TEM-1 enzyme except for a stretch of 78 residues at the C terminus, where the sequences are random composites of the TEM-1 and *P. mirabilis* sequences (Figure 4). Because of the similarity of these parent sequences, the hybrids are about 90 % identical to the TEM-1 sequence, three-fourths of the differences from TEM-1 occurring at exterior positions. Screening for *in vivo* activity (as before) revealed no detectable plasmid-mediated antibiotic resistance, meaning that activities are <0.025 % and <0.084 % of wild-type TEM-1 activities toward ampicillin and penicillin G, respectively.

Although proteins showing 50 % sequence identity are expected to align unambiguously, it is important in this case to consider whether the obvious alignment might be incorrect. In the standard alignment, the pattern PXXKP appears in both proteins, one position out of register. Figure 4 shows an alternative alignment where this pattern is matched, albeit at the cost of introducing two gaps. This alternative appears unlikely when the whole set of Class A sequences is examined, and, consistent with this, molecular modelling (Guex & Peitsh, 1997) of the *P. mirabilis* enzyme fails to produce a structure where the PXXKP regions align spatially (data not shown). Still, to rule out the possibility of misalignment, a set of plasmids encoding ten alternative hybrid proteins was produced (Figure 4). Again, no activity is detected in these hybrids. The assumption of context independence thus appears to be as problematic for proteins as it is for the linguistic character strings of Figure 3.

Severity of exterior constraints

In addition to active sites, enzymes depend upon protein scaffolds with suitable structural properties. If an appropriate pattern of hydrophobic and polar residues were the only sequence

Figure 4. Sequence alignment of TEM-1 and *P. mirabilis* β-lactamases with hybrid sequences shown below. Shading indicates residues that differ from TEM-1. The mature TEM-1 sequence runs from position 26 to position 290 by the conventional numbering scheme (Ambler *et al.*, 1991). Boxes indicate two possible alignments for a single region comprising eight residues in both parent proteins (standard alignment on left, alternative on right). Ten hybrids have been constructed based upon the alternative alignment. Except for the boxed regions, these alternative hybrids are each identical in sequence to one of the original 20 hybrids. Hybrids were constructed by overlapping PCR. Where parent sequences differ, primers contained base mixtures (where possible) allowing them to specify either parent residue. Where this was not possible (because codons differ by more than one base or because of the need to have non-variable priming regions) a pseudo-random number generator was used to determine which parent residue would be incorporated. Alternative hybrids were constructed by using a single pair of primers to modify one of the original hybrid constructs.

requirement for such scaffolds (the binary-code hypothesis, Kamtekar *et al.*, 1993), then we would expect roughly half of the 20 amino acid residues to be suitable at every position. This would mean that there are 10^{171} suitable amino acid specifications for the exterior of TEM-1 β-lactamase. A much lower figure can be obtained by applying the assumption of context independence to the collection of acceptable substitutions reported by Huang and co-workers (henceforth, the Huang set; Huang *et al.*, 1996). Thus, multiplying the number of residues in the Huang set (including wild-type) at all 171 exterior non-active-site positions gives 10^{110} acceptable specifications. By way of illustration, the ratio of these two numbers (10^{110}:10^{171}) is similar to the ratio of the volumes of an iodine nucleus and the moon. It is clear that even when context independence is presumed, the data indicate that the binary-code hypothesis greatly underestimates the extent to which function constrains sequence.

The assumption of context independence also leads to a vast underestimate, though. Comparison of blaMY and blaBG to bla{MY} and bla{BG} (Table 1) shows that substitutions from the conservative set used here are significantly less disruptive than many substitutions in the Huang set. As a consequence, we would expect function typically to be lost well before 30 exterior substitutions from the Huang set could be combined. Inspection of the Huang set shows the average (geometric mean) number of acceptable substitutions at exterior positions to be 3.4. If the TEM-1 enzyme could tolerate 15 such substitutions in any combination, this would amount to about 10^{29} acceptable exterior specifications $[3.4^{15} \times 171!/(15!\ \ 156!) = 10^{29}]$. A different calculation, where it is assumed there is a single alternative to the wild-type residue at each exterior position such that up to 30 of these alternatives can be introduced in any combination, gives a higher estimate of 10^{33} acceptable specifications $[171!/(30!\ 141!) = 10^{33}]$. This higher figure compares to the figure obtained assuming context independence (10^{110}) as the volume of an electron compares to the volume of a sphere with a radius equal to the mean Earth-Sun distance.

With exterior constraints being so severe, how is it that mutant studies often find greater tolerance at exterior positions? The catastrophic effect referred to above suggests an answer. When TEM-

1 substitution groups are combined (Table 1), the resulting relative hydrolysis activities are always lower than the product of the relative activities for the various groups. One likely explanation for this co-operative functional disruption is that it results from progressive loss of stability of the native fold. If most individual substitutions are mildly destabilising, several may be introduced without catastrophic loss of stability and function. As a consequence, studies using small numbers of substitutions typically would not detect the corresponding minor disruption.

To see whether the TEM-1 group mutants show evidence of destabilisation, antibiotic resistance tests were repeated at 25 °C for all mutants that showed substantially reduced but measurable activities at 37 °C. Relative activities increased significantly in all cases (Table 1), indicating that loss of structural stability does indeed contribute to functional disruption. If loss of function were due exclusively to destabilisation, however, we would expect to find the same relative activities against penicillin G that we find for ampicillin. The fact that relative activities show significant substrate dependence (Table 1) indicates that distortion of the active site is also occurring and may contribute to functional disruption. The important and unexpected finding, of course, is not that destabilisation or distortion can be inactivating, but that stability and active-site conformation place such extreme constraints on the acceptable residues at exterior positions.

Implications

Protein classification

If context independence is assumed when analysing a group of homologous proteins having similar sequences and equivalent functions, all differences among these proteins are seen as functionally inconsequential. The proteins have become distinguishable, by this viewpoint, in the same way that identical manufactured things become distinguishable as they acquire nicks and scratches over time, but like these manufactured things, they exhibit part-for-part equivalence. Each part of one has a clear corresponding part in all others, and there are no functionally important differences between these corresponding parts. If we represent the relationship between protein sequence and function as a seascape (Figure 5), this understanding of the group of homologues implies that they would all be represented by points near the summit of a single dry mountain.

The results reported here indicate that the true picture is very different. In the hybrid experiment, a set of sequences that are direct intermediates between the two parent β-lactamase sequences was produced. All of these hybrid sequences, in other words, lie on conceptual paths by which one parent sequence is transformed into the other with a minimum number of substitutions. The fact that they all lack biologically significant function means that the points representing these intermediate sequences in a seascape picture are below sea level. Since all of the direct paths from the TEM-1 enzyme to the *P. mirabilis* enzyme that were sampled dip below sea level, it is reasonable to conclude that a substantial majority of the possible direct paths do likewise. These two natural enzymes would therefore be best pictured as points on different quasi-islands (dry peaks largely surrounded by water). There must be a dry path connecting these quasi-islands *via* others if they are descendants of a single enzyme, and there may also be direct connecting paths, but from an aerial view, much more water than land separates them. The two enzymes are different designs in the important sense that their smallest corresponding parts, their aligned residues, are not freely interchangeable.

It appears that the same could be said of an even more similar pair of enzymes. The β-lactamase hybrids described here are inactive despite being shuffled along only a third of the total chain length. This implies that most hybrids shuffled along their entire lengths would lack activity even if the parent sequences shared somewhat more than 50 % sequence identity. An analysis of intermediate sequences between two 85 %-identical ribonucleases (Serrano *et al.*, 1993) suggests that all hybrids are active at this level of similarity. Somewhere between these levels then, in the neighbourhood of two-thirds identity, lies a boundary below which differences between homologues can be expected to have functional importance. A group of homologues like the Class A β-lactamases is therefore viewed more accurately as a quasi-archipelago (a group of quasi-islands interconnected by narrow land bridges) than as a summit (Figure 5).

Although many previous studies show that functional hybrids can be constructed from natural homologues (Schneider *et al.*, 1981; Mas *et al.*, 1986; Houghton *et al.*, 1989; Malcolm *et al.*, 1990; Zülli *et al.*, 1990; Olsen *et al.*, 1991; Vos *et al.*, 1991; Guez-Ivanier *et al.*, 1993; Serrano *et al.*, 1993; Vuilleumier & Fersht, 1994; Jermann *et al.*, 1995; Singh & Hayashi, 1995; Hosseini-Mazinani *et al.*, 1996; Nixon *et al.*, 1997; Crameri *et al.*, 1998; Ostermeier *et al.*, 1999; Lehmann *et al.*, 2000), none suggest residue-for-residue interchangeability among sequences that differ at a third or more of their positions. One or more of three factors can account for the ability to produce active hybrids in each case. First, many functional hybrids are so similar to a wild-type enzyme that they would be expected to retain function on this basis alone (Schneider *et al.*, 1981; Malcolm *et al.*, 1990; Zülli *et al.*, 1990; Vos *et al.*, 1991; Serrano *et al.*, 1993; Vuilleumier & Fersht, 1994). This appears to account for the functionality of hybrid enzymes designed to replicate ancestral sequences (Jermann *et al.*, 1995) or to match a consensus sequence (Lehmann *et al.*, 2000) since these hybrids share 80-90 % identity with extant wild-type sequences

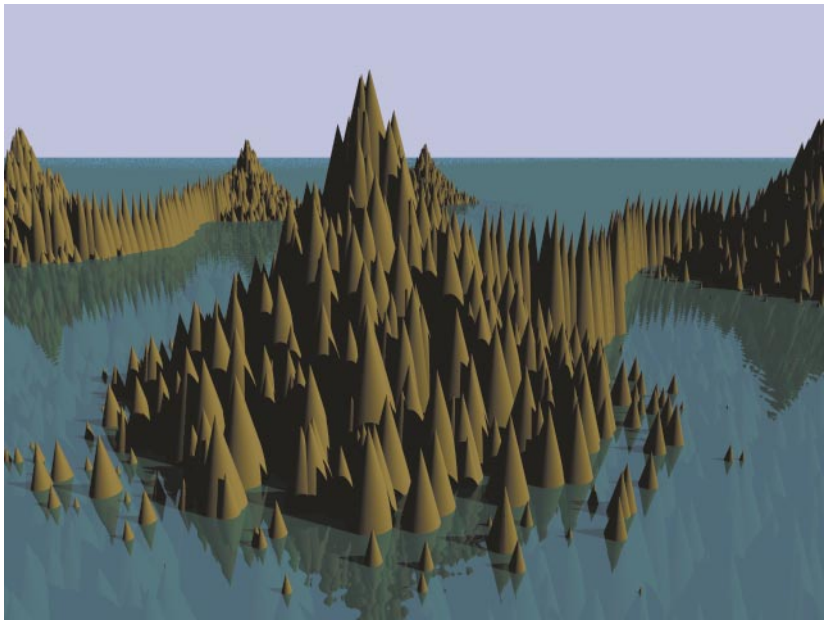

Figure 5. Seascape representation of the relationship between protein sequence and function. Horizontal dimensions represent sequence space with land elevation representing proficiency in performing a particular biological function. Sea level represents the minimum level of function that has biological significance. Rendered with POV-ray (http://www.povray.org).

and probably significantly higher identity with ancestral sequences. Second, in most cases hybrids are constructed by splicing contiguous stretches from parent sequences (Schneider *et al.*, 1981; Mas *et al.*, 1986; Houghton *et al.*, 1989; Zülli *et al.*, 1990; Olsen *et al.*, 1991; Vos *et al.*, 1991; Guez-Ivanier *et al.*, 1993; Vuilleumier & Fersht, 1994; Singh & Hayashi, 1995; Hosseini-Mazinani *et al.*, 1996; Nixon *et al.*, 1997; Crameri *et al.*, 1998; Ostermeier *et al.*, 1999). If, as proposed here, divergent wild-type sequences become distinct designs with their own sets of optimising features, one would clearly have improved chances of building a functional hybrid if contiguous stretches of co-optimised residues were retained. Finally, some hybrids have been subjected to random mutagenesis and selection to produce functional variants and weed out non-functional ones (Hosseini-Mazinani *et al.*, 1996; Crameri *et al.*, 1998).

The neutral theory

The fact that the amino acid differences between two closely related β-lactamase enzymes clearly are not functionally inconsequential might seem to contradict the notion that homologues are connected by a path of neutral substitutions. However, the neutral theory claims neither that most possible substitutions nor that most possible paths between close homologues are selectively neutral. It claims, rather, that the actual historical paths consist primarily of substitutions that each were neutral in their own context.

The findings of this work can be accommodated within the framework of the neutral theory by postulating that substitutions initially having no significant effect become invested with functional importance as further substitutions accumulate. The catastrophic disruption observed in the hybrids demonstrates a high degree of co-optimisation among the residues unique to either parent. Whether we view evolution as the accumulation of substitutions with zero effect or alternating slightly positive and slightly negative effects, we cannot explain consistent catastrophic disruption upon shuffling unless most of the side-chains introduced by evolution acquire significant positive roles.

An important question arises regarding the prevalence of neutral substitutions. The neutral theory implies that for any protein, numerous possibilities for neutral change must exist. What sorts of substitutions are likely candidates? Previous answers, which now appear unsatisfactory, have been that exterior positions (Kimura, 1983) and positions showing variation among homologues (King & Jukes, 1969; Kimura, 1991) are under little functional constraint and therefore are able to accommodate substitutions readily. Since accumulation of substitutions of these types results in catastrophic disruption, the real candidates, whatever their nature, must constitute a smaller, more special set.

Simplified amino acid sets

The idea that the requirements for producing native-like proteins may be far simpler than is

suggested by the complexity of a 20 amino acid code has attracted much attention in recent years (for reviews, see Sauer, 1996; Cordes *et al*., 1996; Plaxco *et al*., 1998). Two interesting results that have been cited in connection with this (Plaxco *et al*., 1998) are that co-operatively folded (but not fully native-like) structures can be formed by random sequences composed of three amino acid residues (Davidson *et al*., 1995), and that proteins with native-like structure can be designed from a set of seven amino acid residues (Schafmeister *et al*., 1997). Comparison of these two results suggests that the demands on sequence increase considerably as structural demands are increased. Since the necessity of function is what requires natural proteins to possess ''native-like'' structure in the first place, one might argue that the most relevant structural demands are those imposed by function. An attempt to reduce the set of amino acids used in a small peptide-binding domain indicates that even this relatively simple biological function requires considerable residue diversity (Riddle *et al*., 1997). The more complex function of enzymatic catalysis is shown here to entail severe sequence constraints, even apart from consideration of the active site. Because fewer amino acid residues means fewer possibilities for satisfying these constraints, it may prove very difficult to build native-like enzymes from a substantially reduced set.

Materials and Methods

Mutagenesis

Group-substitution mutants and hybrid mutants were prepared by using pairs of outwardly directed primers to amplify the entire plasmid. Amplification, ligation, and transformation protocols have been described previously (Axe *et al*., 1996). In some cases (e.g. construction of plasmids carrying hybrid β-lactamase genes) multiple rounds of PCR amplification were performed using different primer pairs. After using the final products to transform *E. coli*, individual transformant colonies were used to prepare plasmid DNA for sequence analysis. Sequence data (obtained using an automated sequencer) covered the entire barnase or β-lactamase genes. Mutants containing errors (usually small deletions at the point of ligation) were rejected.

In vivo activity screens

Barnase group-mutants were tested for activity by the described method (Axe *et al*., 1999).

Activities of β-lactamase group mutants and hybrid mutants were determined by diluting overnight cultures (grown with chloramphenicol plasmid selection) to a concentration of about 10^4 cells per ml. Portions of these dilutions (40 μl) were spread on LB agar plates containing various amounts of ampicillin or penicillin G (benzylpenicillin) but no chloramphenicol. As a control, an equal portion was spread on an LB agar plate containing chloramphenicol alone. Plates were incubated at 37°C for 20 hours before visual inspection (except as indicated in footnote b of Table 1). In all cases, hundreds of colonies were present on the chloramphenicol plate. Maximum permissive concentrations for either β-lactam antibiotic were taken to be the highest concentration at which visible growth (typically, numerous extremely small colonies) was present. Where no growth was visible, incubation was continued for an additional 24 hours at room temperature. In no case did this extended incubation result in visible growth.

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