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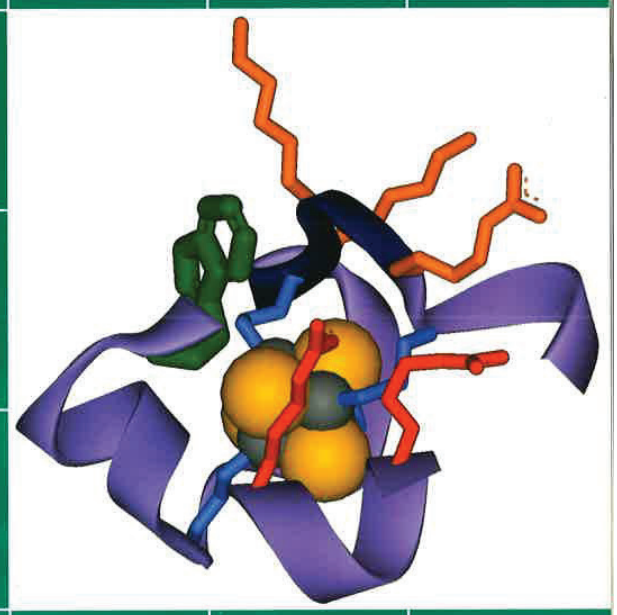

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Bioinorganic chemistry

Edited by Elizabeth C Theil and H Holden Thorp

Biocatalysis and biotransformation

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June 2005 Combinatorial chemistry
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The cover

The cover figure is taken from the review on by Lukianova and David, and is based on the structure of MutY solved by Tainer and co-workers (Guan y *et al.*: **MutY catalytic core, mutant and bound adenine structures define specificity for DNA repair enzyme superfamily.** *Nat Struct Biol* 1998, **5**:1058-1064). This figure illustrates the region around the iron–sulfur cluster of MutY that is involved in recognition of damaged DNA.

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Predicting enzyme function from protein sequence

Jeremy Minshull, Jon E Ness, Claes Gustafsson and Sridhar Govindarajan

There are two main reasons to try to predict an enzyme's function from its sequence. The first is to identify the components and thus the functional capabilities of an organism, the second is to create enzymes with specific properties. Genomics, expression analysis, proteomics and metabonomics are largely directed towards understanding how information flows from DNA sequence to protein functions within an organism. This review focuses on information flow in the opposite direction: the applicability of what is being learned from natural enzymes to improve methods for catalyst design.

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Introduction

The sequencing of the human genome is probably more significant as a portent of the future than as the source of cures for human ills. Its three billion sequenced base pairs are a sign of the glut of information available and an incentive to interpret that information.

Hard on the heels of the genomics revolution came structural genomics and proteomics. As with the human genome sequencing project, the primary motivation behind these endeavors is to decipher the physiological functions of naturally occurring sequences. A major short-term goal for both genomics and proteomics is to identify protein drug targets and thus candidate small-molecule pharmaceuticals to either modulate the function of human proteins [1], or to selectively inhibit essential functions in pathogens [2,3].

Two new fields of study are emerging to take advantage of our accumulating understanding of how organisms are put together. The first of these is systems biology, which further extends the information-gathering philosophy and is driven by the belief that it will ultimately be possible to predict the behavior of large networks of biological elements. Complementing these research efforts that translate physical systems into information is the field of synthetic biology, which seeks to create modified or new genes, proteins and organisms, using our expanding knowledge of natural systems as a jumping-off point (Figure 1). How can synthetic biology take advantage of the information that is being gathered and processed from natural biological systems and the tools used to do so? This review aims to address ways in which studies of nature can benefit protein engineers.

Protein engineering and re-engineering

Increasing computational power is improving the ability of synthetic biologists to predict the structures into which amino acid sequences will fold [4]. Nevertheless, it is still a substantial step from predicting a protein fold to accurately designing a catalyst *de novo*. This difficulty is exemplified by studies of $(\beta/\alpha)_8$-barrel proteins: the fold is found in 21 homologous superfamilies. The common structure appears to confer some conserved properties, but there is a great diversity of function within the 76 different $(\beta/\alpha)_8$-barrel sequence families [5–7]. This difficulty for assignors of protein function is also a barrier to designers of enzymes.

Because of the large gap that still exists between designing a protein fold and specifying a catalytic function, protein engineers have largely focused on the more modest but more tractable goal of re-engineering catalytic activity. In the most dramatic success of this approach to date, Dwyer *et al.* recently succeeded in converting a periplasmic binding protein into a triose phosphate isomerase. The authors computationally 'stripped out' the protein's ribose-binding site, and used dead-end elimination based algorithms to select amino acid mutations to produce target ligand docking and design an active site [8•]. This protein re-design required a high-resolution three-dimensional structure as a starting point. However, production of high-resolution structures still lags the rate at which sequence information is being generated. It would thus be useful if sequence information alone could guide choices of amino acid changes that might lead to desired changes in function.

Using homologous sequences for protein engineering

On microscopic timescales, the sequence of a protein determines the fold that it will adopt and thus the other molecules that it will interact with or transform. However, on macroscopic timescales it is the folding and function of proteins altered by random mutation that determines the

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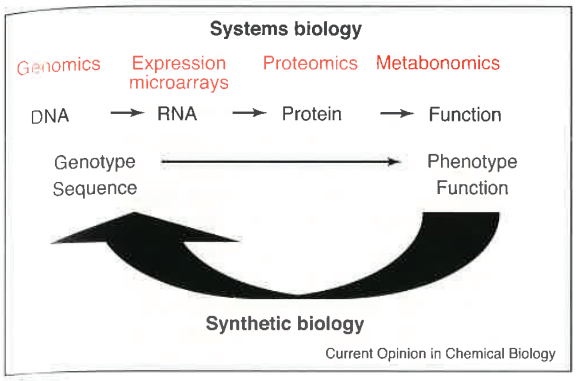
Figure 1



Systems biology, synthetic biology and information flow. Large amounts of information from genomics, proteomics and metabonomics are analyzed to create models describing the functions of individual genes and proteins. Systems biologists are now developing methods for predicting the behavior of networks of these elements. Synthetic biology closes the loop by reversing the direction of information flow, creating novel genes, proteins and organisms based on data about naturally occurring systems, allowing tests to be made of the predictive algorithms, as well as creating new and useful compositions of matter.

elements of protein sequence that are retained (Figure 2). The sequences of orthologous proteins are thus a potentially rich source of information regarding sequence changes that are acceptable without drastically disrupting protein function, while paralogs may provide additional information about protein sequence elements responsible for functional change.

One example of the use of homologous sequence information for protein re-engineering is the approach of DNA shuffling. Homologous sequences are recombined randomly with one another, and the resultant library screened for target properties. Here, the sequence information is contained within the homologous genes. The physical PCR process used to produce recombinants ensures that recombination occurs at regions where there is substantial sequence identity [9]. Because the boundaries of recombination events are confined to DNA sequences that are reasonably conserved, the exchange sites themselves are biased against those likely to drastically disrupt protein structure or function. Nevertheless libraries of 10 000 or more recombinants must typically be screened to identify those with useful improvements.

High-throughput screening of protein function itself presents some problems, primary amongst which is the frequent lack of good correlation between a screen that is simple and one that accurately predicts function under application conditions. It would therefore be useful to develop methods for reducing the numbers of variants that must be tested.

Two methods have recently been proposed for identifying regions of proteins that may be productively transposed to a homolog. The first of these invokes the use of structural information to assess intra-protein amino acid interactions. Segments of homologous proteins are judged to be more transferable the fewer amino acid interactions are disrupted [10,11]. Although this method requires structural information, it is less demanding than the docking approach; even predicted structures can be acceptable [12•].

The second method uses only sequence information. Castle *et al.* recently created an enzyme capable of acetylating the widely used herbicide glyphosate by shuffling selected amino acids from four homologous proteins into a parental backbone. Neither the structure nor function of these donor enzymes were known. However, amino acids selected from these sequences increased the k_{cat}/K_m of the acetyl transferase from around 100 $min^{-1}mM^{-1}$ to over 8000 $min^{-1}mM^{-1}$ [13••].

Reducing the screening burden

Impressive though the glyphosate acetyl transferase example is, it required screening over 50 000 variants to achieve the necessary increase in specific activity. Preserving the successful philosophy of using substitutions found in homologs, while reducing the screening burden, requires the answers to three questions.

1. How can functional homology best be determined to identify suitable donor enzymes?
2. What is the best way to choose amino acid substitutions from these donors?
3. How can maximal information about the substitutions be derived from screening data?

Genomics, structural biology and proteomics provide useful tools to address the first two questions. In seeking to assign function to predicted proteins, phylogenetic and structural information are frequently compared between sequences of unknown function and those whose activities have been experimentally determined. The third question may be answered by looking to traditional engineering methods, including structure–activity relationships that have found application in small-molecule drug design.

Finding homologs

Prediction of protein function from sequence and structure is a difficult problem, because homologous proteins often have different functions. Despite this difficulty, the increasing availability of sequences, structures and known activities is facilitating the task. Comparative genomics permits many approaches to function prediction when the data are available. These include the use of protein-protein interaction patterns, and correlations between occurrences of related proteins in different organisms

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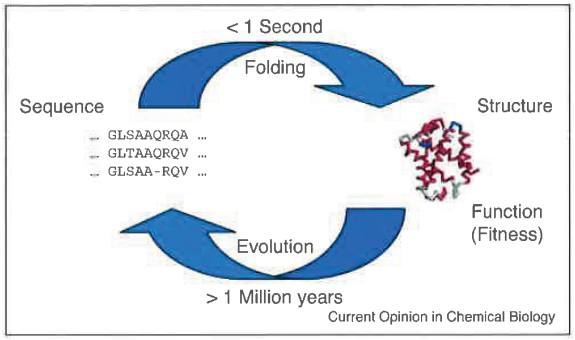
Figure 2



Sequence determines function, function constrains sequence. The sequence of a protein's amino acids, together with environmental factors such as other proteins within a cell determines its fold, typically in less than a second. The three-dimensional structure of the protein in turn governs its function. Random changes to the protein sequence occur over evolutionary timescales; changes are retained if they are functionally equivalent or confer a selective advantage.

[14–17]. For example it is now known that members of mechanistically diverse superfamilies share the ability to catalyze a common partial reaction [18]. Single amino acid changes near the active site of L-Ala-D/L-Glu epimerase and muconate lactonizing enzyme converted these proteins into catalysts for a third reaction catalyzed by another superfamily member, *o*-succinyl benzoate synthase [19••]. A better understanding of the catalytic promiscuity of natural enzymes would undoubtedly aid in protein re-engineering [20].

The conservation of structure and partial function between superfamily members makes them appear attractive substitution donors and acceptors. A new structure–function linkage database (http://sfld.rbvi.ucsf.edu) aims to store sequence–structure–function information that can be interrogated with desired chemical reactions [21]. This will likely be a valuable resource once it is sufficiently populated.

Analysis of residue conservation and variation among homologous sequences that have evolved under similar constraints also provides information about residues that confer desired properties. For example, in one recent study, the consensus amino acid sequence of 13 fungal phytases was synthesized and found to be thermostable at temperatures between 13 and 26 °C higher than any of the 13 wild-type phytases used to create the consensus. Addition of more phytase sequences resulted in a new consensus sequence with further improved thermostability [22•].

Superfamily members often share relatively low sequence identities. In the absence of reliable functional assignments, probing public databases for proteins with reasonable amino acid identity is the simplest method for identifying potential donors. When the functional similarity of 486 000 homologous protein pairs were compared, very little variation in EC number was observed in sequences sharing more than 40% identity [23]. In the glyphosate acetyl transferase experiments, enzymes were selected purely on the basis of sequence identity with the acceptor gene [13••]. The amino acid sequence identity of these donors ranged from 49% to as low as 28%, the two most homologous of these donors catalyzed the transferase reaction less well than the acceptor, the two least homologous were not even tested for function. Despite this apparent functional unsuitability, the two more homologous donors contributed 13 amino acid substitutions and the two least homologous 5 substitutions to an 800-fold improvement of k_{cat}/K_m.

Choosing substitutions

Many computational methods are now being tested for their ability to identify functionally important residues. These residues can then be used to help functional assignment of proteins, or to predict regions or residues involved in protein–protein interactions or catalysis. Functional residue predicting methods typically use one or more of the following strategies:

1. Use of phylogenetic analysis to define evolutionary distances between homologs [24••] and cluster them based on evolutionarily related sub-families. Evolutionary trace, sequence motif and conserved functional group analysis of homologous proteins to identify putative active sites [25–28].
2. Degree of amino acid conservation at positions in related proteins [24••,29•].
3. Use of principal component analysis of sequences [24••,30].
4. Use of structural information such as proximity of observed changes to active site [29•].
5. Use of structural information such as conservation of specific residues in specific three-dimensional positions [31•,32,33].
6. Use of substitution matrices that describe the relative likelihood of amino acid conversions [31•,34,35].
7. Analysis of rates of synonymous and non-synonymous substitutions in the genes and identifying nature of selection (adaptive or neutral) undergone by the gene or specific sites in the protein [36,37].
8. Identification of functional domains using sequence- and structure-based methods [16,38].

In general, these methods are tuned to recognize amino acids whose alteration would be deleterious to enzyme function. This information can also assist in achieving the balance required to alter amino acids that will affect protein function but not destroy it, important in designing a new enzyme. For example, changes at key active site

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residues or other highly conserved residues are generally deleterious [29•]. Changes that are found in highly related proteins are likely to be acceptable, even if close to an active site [29•]. The effect of amino acid substitutions on protein function can also be inferred from substitution matrices derived from natural proteins. Substitution matrices are formal representations of the probability that one amino acid may be replaced by another within a functional protein. Such tables can be derived from sources ranging from very large sets of proteins [39,40] to smaller and functionally related families [41], to specific regions of proteins [35,42], including those that are most conserved [34]. They can also be extended to incorporate the frequency with which dipeptides occur [43], or be based on the physicochemical properties of residues [44].

Evolutionary conservation seems to have been a consideration in the choice of substitutions to use in the shuffling of glyphosate acetyl transferase [13••]. The authors appear to have made substitutions in the target gene that were present in one of the homologs (28–49% amino acid identity). The majority of these substitutions are also those that substitution matrices indicate have a high generic likelihood of being able to functionally replace one another, as shown in Table 1. For example, in a large number of closely related proteins, isoleucine is often replaced by leucine and vice versa. Likewise, other amino acid pairs that share chemical similarities are often exchanged in highly homologous proteins: aspartate and glutamate are both acidic, arginine and lysine both basic, serine and threonine both have hydroxyl groups and tyrosine and phenylalanine are both aromatic. By constraining substitution choice in this way, the number of candidate substitutions is drastically reduced: only a dozen or so of the possible 380 changes (19 possible changes for each of 20 amino acids) found in homologs are considered. This constraint increases the likelihood of selecting changes that would have been found in closer homologs than those actually available. Substitutions that are evolutionarily closer are more likely to retain structure and function when placed in the context of a homolog [45]. It is also clear from the glyphosate example that conservative substitutions still provide sufficient variation to achieve very considerable increases in activity (see column 3 in Table 1). From this success, it seems that the types of combined calculation methods to identify regions of conservation that are being developed for functional assignment of proteins [24••,31•] will also be useful for the choice of substitutions for protein engineering projects.

Table 1

Evolutionary conservation as a guide for functional compatibility.

Interconverted pair	Occurrences in library	Occurrences in 'winner'	Dayhoff PAM 100 matrix scores (rank/190)
I and L	9	5	2.61 (8)
D and E	6	1	3.37 (4)
R and K	7	4	3.54 (3)
S and T	4	2	2.60 (9)
Y and F	6	3	5.25 (1)
V and I	6	4	4.21 (2)
L and M	5	1	3.30 (5)
E and Q	2	1	2.46 (10)
D and N	1	0	2.41 (12)
M and I	1	0	2.76 (7)
M and V	1	1	0.64 (24)
Q and K	0	1	1.92 (14)
L and T	0	1	−4.02 (110)
G and S	0	1	−0.99 (41)
R and G	0	2	−3.55 (99)
V and A	0	1	−0.23 (28)
T and I	0	1	−2.16 (120)

Thirty independent changes were incorporated into the 146 amino acid glyphosate acetyl transferase protein, resulting in a 10 000-fold improvement in k_{cat}/K_m. The table shows the frequency with which specific amino acid interconversions were represented in the initial libraries and in a sequenced highly active shufflant. A change of I to L and a change of L to I are both included as interconverted pair I and L.

Predicting function from sequence

One of the most exciting advances in elucidating the relationship between sequence and function is the use of dimension-compressing techniques such as principal component analysis to identify key sequence elements that correlate with specific activities. These techniques were pioneered to assist in functional assignments for naturally occurring proteins [30]. However, they are also capable of identifying the specific amino acids that appear to be involved in those functions [46]. In this way they are similar to the combined phylogenetic and structural approaches mentioned above [24••,31•].

Unlike combined phylogeny/structural methods, however, dimension-compressing sequence methods also allow construction of quantitative relationships between sequence and activity. This is extremely useful for sequence variants that have been synthesized and tested in the laboratory. Sequence–activity relationships may be derived in three steps. First, amino acid sequences are encoded, for example as a matrix as shown in Table 2 and Table 3. Second, an equation is produced for each sequence, an example is the form shown in Equation 1. Third, values of c_i are chosen such that the experimentally measured values of y are as close as possible to those predicted using Equation 1.

$$y = c_1x_1 + c_2x_2 + c_3x_3 + c_4x_4 + \ldots c_ix_i \quad (1)$$

y: the experimentally measured activity

x_i: the amino acid variation at position i, as shown in Table 2 and 3

c_i: a weighting factor

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Table 2

Encoding sequences for sequence-activity determination.

Sequence #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	A	G	R	W	G	I	G	A	Y	H	K	L	I	M	A
2	A	G	R	T	G	V	G	V	Y	H	K	L	I	M	A
3	A	G	R	W	G	V	G	V	Y	H	R	L	I	M	A
4	A	G	R	T	G	M	G	A	Y	H	R	L	I	M	A

Four 15 amino acid sequences contain differences at 4 positions. (See also Table 3.)

The most widely used method for minimizing differences between experimentation and prediction is partial least squares projection onto latent variables, although there are many other possible methods. Once the best values for c_i have been calculated, validation of the relationship can be visualized graphically (Figure 3). The substitutions with the highest weighting factors are those that are predicted to be the most important for the measured function. The simplest optimization method is thus to combine all of the substitutions that are determined to be favorable [47••].

Equation 1 is linear in form; it does not take into account possible interactions between amino acids. In reality, it is reasonable to expect that an amino acid change at one position may affect the amino acids that are acceptable at one or more other positions. By selecting changes that are found in homologs, the chance of choosing a change that is incompatible with overall protein structure and function is greatly reduced. Even changes that appear to occur together, that is, both are found in some homologs and neither appear in others, may reflect the relative paucity of sequence information rather than a true functional constraint. In a recent study 52 amino acid changes found within a 180 amino acid region of 15 naturally occurring subtilisin variants were randomly recombined. Of the 100 amino acid pairs that appeared to co-vary in the parental sequences, only 7 of these appeared to result from functional constraints [48•]. In another study, of 14 substitutions that improved thermostability of prolyl endopeptidase, in only one amino acid pair (out of a total of 91 pairs) did the substitution at one position affect the choice of substitution at one other position [49•]. Such a low level of functional interaction allows relatively simple modifications of Equation 1, for example the addition of a single interactive term, to accurately model sequence–activity relationships while still using only small datasets. With or without interactive terms, sequence–activity models have been used successfully to identify and combine functionally beneficial residues to optimize peptides and proteins [47••,49•,50–52].

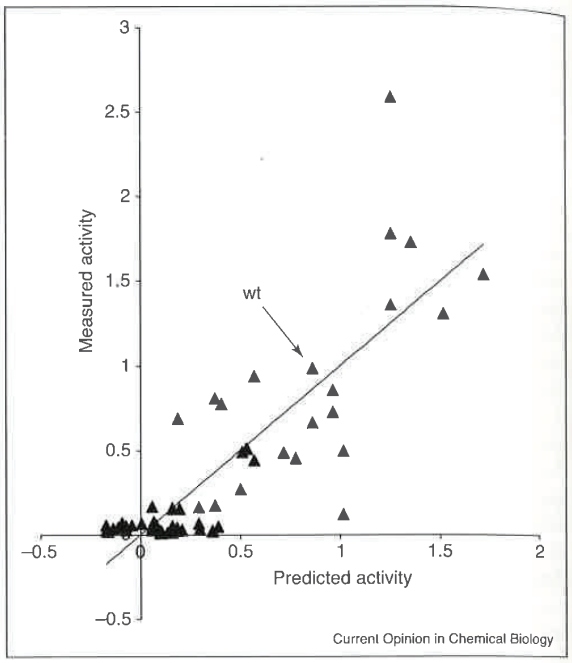
Table 3

Encoding sequences for sequence–activity determination, continued.

	4T	4W	6I	6V	6M	8A	8V	11K	11R
Sequence #	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8	x_9
1	0	1	1	0	0	1	0	1	0
2	1	0	0	1	0	0	1	1	0
3	0	1	0	1	0	0	1	0	1
4	1	0	0	0	1	1	0	0	1

These differences can be encoded as a matrix, where each possible amino acid at each variable position occupies a column. In more complex encoding strategies, columns may represent physicochemical properties of the amino acids [54].

Figure 3



Validating a sequence–activity relationship. Predicted values for a set of sequences (*x*-axis) are plotted against those experimentally determined (*y*-axis). The data are taken from [47••]. The better the correlation, the more closely the data lies on a straight line. Activity is expressed as relative to wild type (wt).

The small number of variants that must be tested to derive a predictive sequence–activity model has the tremendous advantage that these variants can be tested under conditions that are much more faithful to the final application than is generally possible in a high-throughput screen.

There are currently few published examples in which sequence–activity relationships have been used for protein optimization. The major reason for this is probably that the approach has been limited by molecular biology: it has been far easier to synthesize libraries, for example by mutagenic PCR or DNA shuffling, than it has been to

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create even a modest set of 50 designed genes. Indeed, it has been proposed that sequence–activity relationships could be used to analyze active variants in such libraries, enabling much smaller focused libraries to be synthesized [53•]. One strong caveat to such an approach is the frequent lack of correlation between activities measured by high-throughput screens and those actually required in an application.

In contrast to the library-screening approach, Ness *et al.* recently constructed a partial least squares regression model correlating the sequences of 48 individually designed and synthesized proteinase K variants with three different but related proteolytic activities. One of these sequence–activity models was experimentally validated: a variant that combined all of the amino acid changes that the model identified as beneficial was found to be more active than the test set, as predicted by the model [47••]. Models for the other two proteolytic activities predicted quite different beneficial amino acid changes. Consistent with the experiences of many high-throughput screeners, this suggests that extreme caution is needed when optimizing protein performance using a surrogate screen. Since sequence–activity relationships require the testing of orders of magnitude fewer variants than traditional empirical approaches [47••,53•], the use of such predictive algorithms will permit researchers to design lower throughput screens that more accurately reflect the properties they are seeking.

Conclusions

Most tools for predicting enzyme function from amino acid sequence have been developed as part of the ongoing effort to decode the genomes of humans and pathogens for health-related goals. With relatively small adaptations, these tools may now be used to aid in enzyme redesign: identifying suitable proteins as catalytic starting points, choosing changes to make, and assessing the effects of those changes in a powerfully predictive way. In particular, the use of sequence–activity relationships should free protein engineers from the expenses and inaccuracies of high-throughput screens, and allow them to directly measure the protein properties that they really care about.

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