COMMENTARY

REVISED Promiscuous scaffolds in proteins - non-native, non-additive and non-trivial [version 2; peer review: 1 approved with reservations, 2 not approved]

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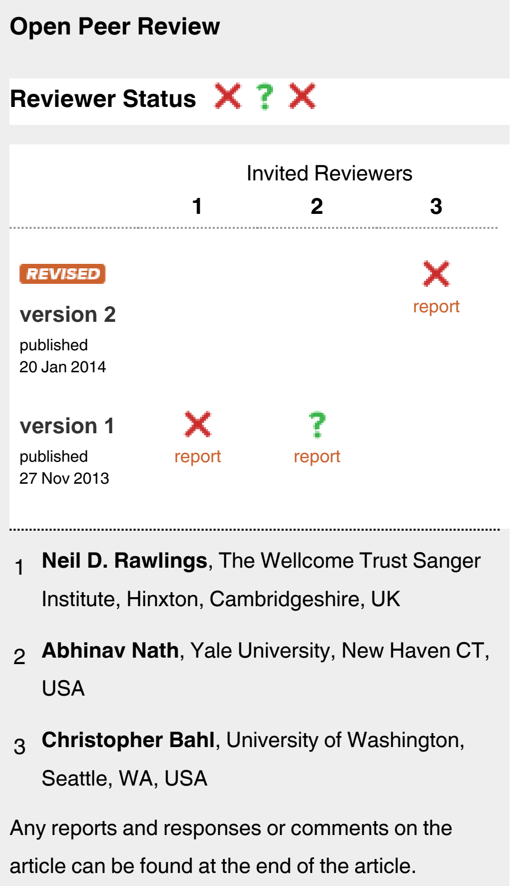
Abstract
Promiscuity, the ability of an enzyme to catalyze diverse activities using the same active site, sets up the stage for the evolution of complex organisms through gene duplication and specialization. The detection of promiscuous motifs is crucial to understand the physiological relevance of a protein, or for any endeavor that intends to rationally modify these latent capabilities to design new proteins under laboratory conditions. We have established a methodology for identifying catalytic residues based on spatial and electrostatic congruence with known active site configurations. Here, we discuss insights gained in several initiatives using our method on different enzymes.



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REVISED **Amendments from Version 1**

We have updated our manuscript based on the comments of the reviewers. The changes, in brief, are:

1) Fixed a missing description in Table 1.

2) Added a description of known promiscuous proteins.

3) Added a small paragraph on the related topic of broad specificity.

4) Minor rephrasing of sentences.

We emphasize once again that this commentary just focuses on results obtained by our group, and is not to be considered as a review.

See referee reports

Introduction

Primitive life presumably had minimal gene content and a minuscule arsenal of enzymes at its disposal. Unfettered from selection pressures by gene duplication, a few select enzymes gained new advantageous functions[1–3]. Nonetheless, the vestiges of secondary activities under neutral drift[4] possess the potential to reemerge under changing selection pressures[5,6]. This ability of an enzyme to catalyze diverse activities using the same active site, termed as promiscuity, is the cornerstone of the evolution of complex organisms from pristine life[7]. In the human context, compound promiscuity plays a major role in drug discovery, and in the therapeutic efficacy of drugs[8]. Databases are a crucial medium of cataloging known aspects of drug promiscuity[9,10].

Ever since Jensen emphasized the role of promiscuity or 'substrate ambiguity' in evolution through 'fortuitous error and gain of multistep pathways', promiscuity in proteins has been the subject of intense and detailed research[7]. It was demonstrated in 1976 that replacing the zinc metal ion by copper in Carboxypeptidase A introduced oxidase catalysis properties[11]. Dioxygenases promiscuously hydrolyse esters[12], while the enolase superfamily is also known to catalyze numerous catalytic reactions[13,14]. Alkaline phosphatases (AP), one of the key proteins in our research, are one of the most widely researched promiscuous enzymes[15]. APs are known to have sulfate monoesterase, phosphate diesterase, and phosphonate monoesterase activities[16–18]. A phosphite-dependent hydrogenase activity was also found in *Escherichia coli* AP (ECAP), but was absent in APs from other organisms[19]. Interestingly, proteins from the AP superfamily show cross activity - *Pseudomonas aeruginosa* arylsulfatase (PAS) which has the primary activity of hydrolyzing sulfate monoesters also catalyzes the hydrolysis of phosphate monoesters[20,21].

The evolution of species through sequence mutations leaves a trail via the conservation of fragments or repeats that have been honed to achieve specific functions with remarkable efficiency[22–24]. The sequence-to-structure-to-function paradigm facilitates the functional characterization of new proteins by applying a 'guilt by association' logic, and has essentially revolutionized the field by its easy to use model[25]. However, occasionally nature achieves the same solution to an enzymatic problem through a completely different sequence, arriving at the same spatial conformation required for catalysis. For example, the catalytic Ser-His-Asp triad has virtually the same geometry in the major families of serine proteases (chymotrypsin and subtilisin), which have no sequence or structural homology[26] - a classical example of convergent evolution[27,28]. Such convergently evolved proteins, and those redesigned from chiseled scaffolds through exon shuffling, remain beyond the scope of sequence analysis methods. As such structure-based methods have evolved to detect such relationships[29,30]. The choice of methods for binding site comparisons and methods for binding site detection as well as function prediction has been recently reviewed in detail[31]. Notably, most of these methods are based on structural properties of the binding or the active site. We have demonstrated that such a structural conservation leading to the same function necessitates the conservation of electrostatic properties as well (CLASP - www.sanchak.com/clasp)[32]. The ability of finite difference methods to quickly obtain consistent electrostatic properties from peptide structures provides an invaluable tool for investigating other innate properties of protein structures[33]. Furthermore, using a database of known active sites in proteins (http://www.ebi.ac.uk/thornton-srv/databases/CSA/[34]), we have proposed a methodology to quantify promiscuity in a wide range of proteins[35].

In an endeavor to establish the validity of the computational predictions made by CLASP, we have undertaken several *in vitro* initiatives using different enzymes. The results of these experiments have provided several insights regarding promiscuous functions in proteins. Foremost amongst them is corroboration of the intuitive notion that inhibition is inherently simpler to predict than true catalysis. For example, we detected the presence of the serine protease (SPASE) catalytic triad motif (Ser195, His57, Asp102) in alkaline phosphatases (AP) from various organisms using the spatial and electrostatic congruence, and validated this by inhibition of the native phosphatase activity using inhibitors (AEBSF/PMSF)[32], known to be active on many serine proteases by reaction with the nucleophilic serine[36]. However, true SPASE activity was limited to shrimp AP. Recently, the crown domain in the *E. coli* expressed rat intestinal AP protein was shown to be prone to protease cleavage, which the authors have ascribed to self-cleavage[37]. Another recent review nicely summarizes the various computational approaches applied to the AP superfamily in order to gain insights into the promiscuous functions observed in proteins belonging to the superfamily[15]. The therapeutic potential of AP inhibitors has also seen increased interest from medicinal researchers[38].

In a similar experiment, we detected a SPASE motif in a phosphoinositide-specific phospholipase C (PI-PLC) from *Bacillus cereus* using CLASP[39]. Once again, although we easily established the inhibition of the native activity of PI-PLC using serine protease inhibitors, we struggled to establish proteolysis based on known protease substrates. Fortuitously, we observed protease activity of PI-PLC on UVI31+, a protein under investigation in our group for different reasons[40]. We thus concluded that one should exert caution before ruling out protease activity in an enzyme since theoretically proteases have a large number of possible substrates due to the possible variation in residues flanking the sissile bond, and the corresponding folds that harbor a recognition site for a particular protease[39]. Thus, it is possible that we have not found the ideal

proteolytic substrate for APs[32]. We also tested the proteolytic functions and inhibition using protease inhibitors of the non-toxic *B. cereus* phosphatidylcholine-specific phospholipase C (PC-PLC) and the closely related highly toxic *Clostridium perfringens* α-toxin (CPA) (which possesses an additional C-terminal domain demonstrated to be responsible for its sphingomyelinase, hemolytic, and lethal activities[41,42]). CPA and PC-PLC activities on phospholipids were unaffected by the addition of serine protease inhibitors in concurrence with the CLASP analysis which fails to detect a SPASE scaffold in these proteins[39]. While CPA and PC-PLC did have a metallo-protease motif based on CLASP analysis, and both showed protease activity *in vitro*, the observed proteolytic activity can be attributed as an artifact of a metallo-protease contamination which is difficult to remove in spite of the purification steps. Inhibition of CPA activity using a metallo-protease inhibitor was tried out, but failed to show any results. Such lack of inhibition by a single compound is not sufficient ground to rule out the existence of a metallo-protease scaffold.

Based on predictions from CLASP, we also demonstrated the inhibition of the native phosphatase activity of a cold active alkaline phosphatase from *Vibrio* strain G15-21 AP (VAP)[43] by a specific β-lactam compound (only imipenem, and not by ertapenem, meropenem, ampicillin or penicillin G)[44]. CLASP analysis detected a spatial and electrostatic congruence of the active site of a Class B2 CphA metallo-β-lactamase (MBL) from *Aeromonas hydrophila*[45] to the active site of VAP. Several β-lactam compounds failed to inhibit *E. coli* or shrimp AP, as was expected by the lower congruence indicated by CLASP as compared to VAP. While all APs contain three metal ion binding sites essential for catalysis[43], MBLs have either one or two metal binding sites[46]. It would be interesting to imagine the existence of a protein (possibly evolved from VAP) that is an MBL and requires three metal binding sites.

Another desired aspect in the search of promiscuous motifs is the ability to search for partial scaffolds, as has been implemented in the DECAAF methodology[47,48]. The search for an elastase-like motif in a plant protein[47] led us to the pathogenesis-related protein P14a[49]. Although the complete motif was missing - stated previously as, '*While Ser195, His57, and Gly193 from the input motif have a highly matching scaffold in P14a, the spatial position of the elastase Asp102 is close to Asn35 and Ser39 in P14a when the proteins are superimposed based on the matching scaffolds*[48]' - the structural similarity of the P14a protein to a snake venom protein with a known elastase function[50] suggested strongly the possibility of pre-existing elastase functionality, or indicated a fair chance of endowing elastase activity through directed evolution techniques.

Another fascinating aspect of enzymes, although strictly not defined as promiscuity, is their ability to catalyze the reaction of a range of similar substrates of the same class[51]. We have hypothesized that duplicate residues, each of which results in slightly modified replicas of the active site scaffold, are responsible for the broad substrate specificity of proteins[52,53].

It might appear that the presence of a motif like a SPASE catalytic triad in a protein structure is trivial, and one could expect any randomly chosen protein with a large number of residues to have such a structural motif. However, the absence of a spatially congruent SPASE catalytic triad in a reasonably large tyrosine phosphatase CD45 (PDBid: 1YGR, sequence length 610) highlights the fact that the SPASE motif is not present ubiquitously (Table 1). Even the presence of a spatially congruent motif, as in the human translation initiation factor (PDBid: 2E9H), does not imply potential congruence (Table 1).

The biggest challenge in detecting promiscuous motifs is to be able to endow the function using rational steps[54–56]. However, the non-additive nature of active site residues makes this a non-trivial task even when a very close partial match exists[57]. For example in a catalytic site consisting of n residues, the existence of a congruent $n - 1$ motif does not imply that it is easy or even possible to add another residue in the structure and obtain the n residue motif. This complexity is best exemplified in the failure to induce β-lactamase activity in a penicillin-binding protein (PBP-5) from *E. coli*[58,59] by generating the L153E mutant of this protein, as proposed by our previous analysis[47] (unpublished results). Although many directed evolution experiments have tried to enhance deacylation in PBPs[60,61], the catalytic step that β-lactamases use to hydrolyze β-lactams[62], very few have

Table 1. Non-triviality of the potential and spatial congruence of the active site residues in proteins from the serine protease motif. The serine protease catalytic triad has been taken from a non-psychrophilic trypsin from a cold-adapted fish species (PDBid: 1A0J). The reasonably large tyrosine phosphatase CD45 (PDBid: 1YGR, sequence length 610) does not contain the spatially congruent catalytic triad. Although, a motif spatially congruent to the catalytic triad is present in the human translation initiation factor (PDBid: 2E9H), it lacks electrostatic potential congruence. D = Pairwise distance in Å. PD = Pairwise potential difference. SLen = sequence length. APBS writes out the electrostatic potential in dimensionless units of kT/e where k is Boltzmann's constant, T is the temperature in K and e is the charge of an electron.

PDB	Active site atoms(a,b,c)		ab	ac	bc	SLen
1A0J	SER195OG,HIS57NE2,ASP102OD1	D PD	3.3 183.7	7.8 153.2	5.6 -30.4	223
1YGR	SER1101OG,HIS1041NE2,ASP1043OD1	D PD	6.7 -385.2	13.1 -341.0	7.1 44.1	610
2E9H	SER128OG,HIS117NE2,ASP115OD1	D PD	2.9 -44.3	7.1 191.6	6.8 235.9	197

been successful. Even the successful attempts have reported low gains in β-lactamase activity (110-fold in[60] and 90-fold in[61]).

In spite of the inherent difficultly in rationally designing proteins, we believe that the fast maturing field of protein structure prediction might soon allow us to quickly iterate over *in silico* mutations[63]. A method like CLASP may be used to discriminate the predicted structures in order to select the mutations that achieve the desired congruence with a reference scaffold - setting up the flow to mimic the natural 'evolutionary walk' *in vitro*, and accelerate this 'random walk' into a 'resolute sprint'.

Author contributions

All authors contributed equally to the writing, and subsequent refinement, of this commentary.

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Christopher Bahl
Department of Biochemistry, University of Washington, Seattle, WA, USA

In this commentary, the authors discuss enzyme promiscuity and the difficulties inherent in predicting and experimentally validating enzymatic activity. As stated by the authors, this commentary is intended to be a discussion of their own research. As such, the authors focus primarily on experimental testing of predictions made using their CLASP algorithm.

I have the following major points:

1. By focusing only on their own research, the utility of this commentary for the greater scientific community becomes unclear. Furthermore, the authors do not provide sufficient background on their own research and methods. The authors discuss the CLASP algorithm, but provide no details about how it functions or how its output is evaluated.

2. The authors make several unsubstantiated assumptions. One such example is their claim that inhibition is simpler to predict than catalysis. Readers are left to assume they intend that inhibition by a small molecule is a simpler computational prediction problem than identifying a specific substrate (or class of substrates) for a particular protein. Why is this? How can one predict and test the efficacy of an inhibitor for a protein with an unknown substrate profile and catalytic activity?

3. Other sections are unclear and confusing. What is meant by "*'guilt by association' logic*"? How has this been revolutionary, and for which field? How is this modeled?

4. It is unclear how Table 1 should be interpreted. The authors provide pairwise distances and pairwise potential distances for three crystal structures. How and why were these three structures selected? What do these values represent, how were they determined, and how should readers evaluate and interpret these values? For example, the authors do not discuss how the sequence length should be compared to the nuclephile-histidine pairwise potential difference. Is this a meaningful comparison, why or why not?

Enzyme promiscuity is a well known phenomenon, and has been the subject of many comprehensive reviews. The opinion expressed by the authors in this commentary seems to be that predicting and experimentally evaluating enzyme activity is difficult. Unfortunately, by focusing only on their own

research, this opinion is not sufficiently substantiated. Furthermore, this commentary lacks the breadth and background required to serve as a general discussion of the authors own work. Significant re-writing is required for this article to be of utility to the general scientific community.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Version 1

Reviewer Report 07 January 2014

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? **Abhinav Nath**
Department of Molecular Biophysics & Biochemistry, Yale University, New Haven CT, USA

The commentary by Chakraborty *et al.* focuses on the important topics of understanding and modeling enzyme promiscuity, and raises some intriguing points about the importance of local electrostatic effects (beyond structure alone) on enzyme activity. However, the commentary is quite tightly focused on protease-type activity in different scaffolds, and even more so on the authors' own work. This manuscript would be improved by providing more context on how their previous work relates to other studies of AP promiscuity/polyspecificity (including in particular the work of the Herschlag and Hollfelder groups) and electrostatic approaches to enzyme mechanism (Warshel, Houk, and many others). This would broaden the potential audience for this commentary, which as it stands is rather narrow.

Moving on to specifics, I am unconvinced by the use of protease inhibitors as a test of *bona fide* protease activity in several of the works discussed. The fact that a particular compound inhibits an enzyme does not necessarily mean that it resembles a substrate, or that the enzyme's mode of action resembles that of the compound's other targets. On the other hand, as the authors acknowledge, the absence of inhibition by a particular compound is not enough to demonstrate the lack of a particular activity or functional motif. These assays thus have significant risks of both false positives and false negatives, and these should be explicitly discussed. What we are left with are apparently observations of protease activity both by enzymes predicted to have SPASE activity (shrimp and *E. coli* APs, and PI-PLC) and by those predicted to lack it (PC-PLC and CPA) – unfortunately, not very strong evidence for the power of their approach. The authors should discuss these issues and their potential solutions.

In Table 1, it is not clear what 1A0J is. Also, it is not clear what the criteria are for either spatial or potential congruence. More broadly, it is difficult to assess the frequency of SPASE-compatible triads in proteins in general from just 3 examples. Is it feasible for the authors to estimate the frequency of spatial and potential congruence in a larger set of protein structures (or even the entire PDB)?

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 16 Jan 2014

Sandeep Chakraborty, Tata Institute of Fundamental Research, Mumbai, India

Dear Dr Nath,

We would like to thank you for taking the time to review our manuscript. We have responded in detail to another review by Dr Rawlings, and an updated version of our manuscript has now been published. We hope our dialogue with Dr Rawlings will provide more clarity on our work. For the sake of brevity, we have not repeated several aspects that we have pointed out there. Please find our detailed responses to your comments below.

The commentary by Chakraborty *et al.* focuses on the important topics of understanding and modeling enzyme promiscuity, and raises some intriguing points about the importance of local electrostatic effects (beyond structure alone) on enzyme activity. However, the commentary is quite tightly focused on protease-type activity in different scaffolds, and even more so on the authors own work. This manuscript would be improved by providing more context on how their previous work relates to other studies of AP promiscuity/polyspecificity (including in particular the work of the Herschlag and Hollfelder groups) and electrostatic approaches to enzyme mechanism (Warshel, Houk, and many others). This would broaden the potential audience for this commentary, which as it stands is rather narrow.
We appreciate your encouraging comments on the importance of the field of our work. We would like to emphasize that we have also worked on ß-lactamases apart from proteases - and are currently working on proteins with other enzymatic activities as well (lipases, sulfatases, etc.).

We have mentioned that we intend this commentary to focus exclusively on our work (and any novel aspects we might have unraveled), and not the work of previous researchers on whose results we have built our research - thus, we have kept this as a commentary, and not a review. We have discussed previous studies in each of our papers separately - for example, the field of quantum, classical, and statistical techniques that have evolved in the last few decades (involving Karplus, Levitt, Warshel, Herschlag and many others) has been discussed in detail in Chakraborty S (2013) A Quantitative Measure of Electrostatic Perturbation in Holo and Apo Enzymes Induced by Structural Changes. PLoS ONE 8(3): e59352. doi:10.1371/journal.pone.0059352.

Moving on to specifics, I am unconvinced by the use of protease inhibitors as a test of bona fide protease activity in several of the works discussed. The fact that a particular compound inhibits an enzyme does not necessarily mean that it resembles a substrate, or that the enzymes mode of action resembles that of the compounds other targets.
We agree that inhibition does not "*not necessarily mean that it resembles a substrate, or that the enzymes mode of action resembles that of the compounds other targets*". However, given the *in silico* demonstration of the congruence of the new scaffold to existing ones with known function, it does add credence to the hypothesis. Further, we have confirmation from other groups (unknown

to us) that a recombinant AP does have self proteolytic function - they cite our work in order to rationalize this result [37].

On the other hand, as the authors acknowledge, the absence of inhibition by a particular compound is not enough to demonstrate the lack of a particular activity or functional motif. These assays thus have significant risks of both false positives and false negatives, and these should be explicitly discussed.
This is something we should touch upon. We thank you for pointing this out.

What we are left with are apparently observations of protease activity both by enzymes predicted to have SPASE activity (shrimp and E. coli APs, and PI-PLC) and by those predicted to lack it (PC-PLC and CPA) unfortunately, not very strong evidence for the power of their approach. The authors should discuss these issues and their potential solutions.
We do see protease activity in PC-PLC and CPA - however, it is impossible to remove protease contamination, and thus we cannot ascribe it to the protein. This is not a negative result for us - it is just a result we cannot confirm under current constraints of protein purification, and thus we cannot (and have not) used it to forward our hypothesis.

In Table 1, it is not clear what 1A0J is.
We have fixed this in version 2 (the other reviewer has pointed this out too).

Also, it is not clear what the criteria are for either spatial or potential congruence. More broadly, it is difficult to assess the frequency of SPASE-compatible triads in proteins in general from just 3 examples. Is it feasible for the authors to estimate the frequency of spatial and potential congruence in a larger set of protein structures (or even the entire PDB)?
We have done something like this in a recently published work where we show the inhibition of PI-PLC using dpp4 inhibitors that are used in type 2 diabetes therapy - http://f1000research.com/articles/2-286. A comprehensive set of 5000 human proteins has been queried using SPASE like motifs, and the significant results have been seen to be small in comparison to the number of proteins queried. For example, using a four residue motif from trypsin we find only 1% of proteins (about 50 proteins) that have significantly congruent active site motifs (considering both spatial and electrostatic properties).

We appreciate the reservations you have, and we share some of the concerns as we strive to establish our work in a collaborative effort. We hope you will find our arguments compelling enough to accept our manuscript.

Best regards,

Sandeep Chakraborty

Competing Interests: No competing interests were disclosed.

Reviewer Report 20 December 2013

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Neil D. Rawlings
Wellcome Trust Genome Campus, The Wellcome Trust Sanger Institute, Hinxton, Cambridgeshire, UK

This commentary reads more like a review and only describes work previously performed by the researchers. I have a number of reservations about this previously published work. The authors should present more detail from at least one example where dual activity has been proven.

Some of the claims made by the authors are extraordinary. Can the authors supply any explanation for the presence of a serine peptidase activity in alkaline phosphatase from a shrimp, but not *Escherichia coli*? If there was any physiological relevance for the peptidase activity for an alkaline phosphatase then surely it would be conserved. It seems extraordinarily serendipitous that the authors are working on UVI31+ and that this is the only substrate for the peptidase activity of shrimp alkaline phosphatase the authors could detect. This is unlikely to be a physiological substrate, which implies that the serine peptidase activity of alkaline phosphatase is very general, and it is behaving as if its' role were protein degradation rather than limited processing.

Given that there are so many other peptidases for recycling proteins - the proteasome in the cytoplasm, lysosomal peptidases, digestive enzymes of the stomach and gut, for example - what would be the evolutionary pressure to maintain such a generalized function for alkaline phosphatase? Have the alleged serine peptidase activity of shrimp alkaline phosphatase, and the alleged inhibition of alkaline phosphatase activity by a serine peptidase inhibitor been confirmed by other researchers? I can understand that a small molecule like PMSF might block any active site serine, regardless of the type of enzyme, but the authors claim that the protein ovoinhibitor reduces alkaline phosphatase activity, which would require not only an active site but also at least one substrate binding site for the reactive site in the inhibitor. Perhaps this is merely some irrelevant, non-specific binding rather than true inhibition.

Why were synthetic substrates not used instead of proteins? Then proper kinetics could be measured, rather than presenting bands on a gel. Clearly the authors are aware of the dangers of contamination, because they admit to having had problems with a metallopeptidase contaminating phospholipase C, for which their CLASP analysis predicts a metallopeptidase-like active site. The controls that the authors use in their experiments, namely to denature all proteins with SDS, would not address this problem, because any contaminant would be denatured as well as the enzyme under experimentation. Use of peptidase inhibitors would be equally useless if the catalytic type of the contaminating peptidase and the enzyme in question were the same. Perhaps the authors should abandon commercial preparations and use recombinant enzymes where it would be possible to mutate active site residues to show that a knockout abrogates activity?

The authors state that their CLASP analysis is unable to find a serine peptidase-like active site in phospholipase C, and that this is confirmed by failure to inhibit phospholipase C with a serine peptidase inhibitor. This is an example of a negative result proving a negative hypothesis, which really doesn't prove anything.

In Table 1, the structure 1A0J should be described in the legend.

Promiscuity in enzyme active sites is a known phenomenon. An example that comes to mind is leukotriene A4 hydrolase, which not only hydrolyses the epoxide but also acts as an aminopeptidase using the same active site. There are also numerous examples where an active site performs different functions in different organisms (or even in the same organism for some protein products of paralogous genes): for example proteins with the alpha/beta hydrolase fold, where some are peptidases and some are lipases. It would strengthen the authors' case considerably to include examples such as these before discussing their own results.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 16 Jan 2014

Sandeep Chakraborty, Tata Institute of Fundamental Research, Mumbai, India

Dear Dr Rawlings,

We would like to thank you for taking the time to review our manuscript. We appreciate several incisive and relevant points raised by you, and hope that our arguments are convincing enough for you to change your opinion about our work. There are two aspects of our work that need to be dissected separately.

STAGE1: The first stage establishes the presence of active site scaffolds (for example, the serine protease catalytic triad) through:

(a) spatial congruence (a method devised previously [29, 30]).

(b) electrostatic congruence of cognate pairs of active site residues in proteins with the same function - our original contribution.

STAGE2: In the next stage, we have attempted to correlate our *in silico* findings through *in vitro* experiments. It is salient to note that the results of STAGE1 are not nullified irrespective of the results in these experiments. However, the results have indeed corroborated our hypothesis in several experiments. Although, as you so aptly point out, there is still work that needs to be done.

Point (b) in STAGE1 might be subject to scrutiny for several reasons:

(i) The validity and accuracy of finite difference Poisson Boltzmann methods.
(ii) The non-trivial nature of the electrostatic potential difference.

We have argued in favor of both these points throughout our work. "*This invariance in the electrostatic features (measured in structures that have been solved independently over many years) is an innate property required for the enzymatic activity. This also speaks highly of the reliability of the APBS/PDB2PQR implementation" [51]*. Assuming that (a) and (b) are valid; surely one cannot deny the existence of a scaffold resembling the catalytic triad from subtilisin and trypsin in alkaline phosphatases (Table 3 in [32])?

Please find our detailed responses to your comments below:

This commentary reads more like a review and only describes work previously performed by the researchers. I have a number of reservations about this previously published work. The authors should present more detail from at least one example where dual activity has been proven.
We have explicitly stated in the abstract that we intend to '*discuss insights gained in several initiatives using our method on different enzymes*'. Thus, this would not qualify as a review since we do not consider the work of those researchers whose work we have built upon.

Can the authors supply any explanation for the presence of a serine peptidase activity in alkaline phosphatase from a shrimp, but not *Escherichia coli*? If there was any physiological relevance for the peptidase activity for an alkaline phosphatase then surely it would be con- served. It seems extraordinarily serendipitous that the authors are working on UVI31+ and that this is the only substrate for the peptidase activity of shrimp alkaline phosphatase the authors could detect. This is unlikely to be a physiological substrate, which implies that the serine peptidase activity of alkaline phosphatase is very general, and it is behaving as if its role was protein degradation rather than limited processing. Given that there are so many other peptidases for recycling proteins - the proteasome in the cytoplasm, lysosomal pepti-dases, digestive enzymes of the stomach and gut, for example - what would be the evolutionary pressure to maintain such a generalized function for alkaline phosphatase? Have the alleged serine peptidase activity of shrimp alkaline phosphatase, and the alleged inhibition of alkaline phosphatase activity by a serine peptidase inhibitor been confirmed by other researchers?
It is precisely these questions that our findings raise - and it would be unfair to put the onus of answering these fundamental questions on the method itself. Fortunately for us, the recent findings of a group working on alkaline phosphatase (and not personally known to us), have shown the presence of auto cleavage in these alkaline phosphatases - "*the loss of crown domain due to protease cleavage could result from self-cleavage of the protein, specifically when metal sites are not fully occupied*" [36]. Citing our work, they have hypothesized on the physiological relevance of the peptidase activity. Since they use recombinant protein, the danger of contamination is minimal - a point that you have raised below.

It is to be said that we agree that it seems unlikely that there is an evolutionary pressure to maintain a peptidase function in APs, given the plethora of such enzymes that you have pointed out. However, promiscuity often allows functional units (partial or complete) to serendipitously piggyback on the 'real' function being maintained and honed by evolution. It is not the existence of peptidase activity, but the distinct possibility that APs may have some peptidase activity that is of interest. The peptidase-like scaffold in APs might be one such latent functionality - possibly a remnant of a primeval parent protein that duplicated to result into the current serine peptidases and APs (both hydrolases).

Finally, there is precedence of a promiscuous activity that is present in only one of the APs (ECAP) - "*A phosphite-dependent hydrogenase activity was also found in ECAP, but was absent in APs from other organisms* [*19*]". Another example is the inhibition of VAP using imipenem, which does not inhibit ECAP.

I can understand that a small molecule like PMSF might block any active site

serine, regardless of the type of enzyme, but the authors claim that the protein ovoinhibitor reduces alkaline phosphatase activity, which would require not only an active site but also at least one substrate binding site for the reactive site in the inhibitor. Perhaps this is merely some irrelevant, non-specific binding rather than true inhibition.
We have shown the binding of AEBSF to shrimp alkaline phosphatase through MALDI-mass spectrometry (Fig 4d in [32]).

Why were synthetic substrates not used instead of proteins? Then proper kinetics could be measured, rather than presenting bands on a gel.
We did try out many synthetic substrates, but we could not find any protease activity. However we have not yet tried the Pro-Xxx that would duplicate the bond in UVI31+ cleaved by PI-PLC. We believe that '*one should exert caution before ruling out protease activity in an enzyme since theoretically proteases have an innumerable number of possible substrates due to the infinite possible DNA sequences that can result in proteins and their corresponding infinite folds*', as mentioned in the current commentary. However, I do think we ought to try out prolyl peptidases substrates (and will in the near future).

Clearly the authors are aware of the dangers of contamination, because they admit to having had problems with a metallopeptidase contaminating phospholipase C, for which their CLASP analysis predicts a metallopeptidase-like active site.
Precisely - it would be improper to consider that the metallophospholipase has protease activity as it is difficult to remove the protease contamination, and we have not.

The controls that the authors use in their experiments, namely to denature all proteins with SDS, would not address this problem, because any contaminant would be denatured as well as the enzyme under experimentation. Use of peptidase inhibitors would be equally useless if the catalytic type of the contaminating peptidase and the enzyme in question were the same.
We have not tried to rule out contamination by denaturing the proteins or using inhibitors for the reasons mentioned by you. Could you kindly point out where this might have been mistakenly specified by us?

Perhaps the authors should abandon commercial preparations and use recombinant enzymes where it would be possible to mutate active site residues to show that a knockout abrogates activity?
We agree that recombinant enzymes with a knock out mutation would clinch our argument. However, financial and logistic restrictions make this currently unfeasible (for me on a personal note). Fortunately, the protease activity in a recombinant alkaline phosphatase has been shown by a different group [37].

The authors state that their CLASP analysis is unable to find a serine peptidase-like active site in phospholipase C, and that this is confirmed by failure to inhibit phospholipase C with a serine peptidase inhibitor. This is an example of a negative result proving a negative hypothesis, which really doesn't prove anything.
There has been some misunderstanding - we mention '*CPA and PC-PLC activities on phospholipids were unaffected by the addition of serine protease inhibitors in concurrence with the CLASP analysis which fails to detect a SPASE scaffold in these proteins*'. This is expected, since they are metallo proteins. CLASP does find the serine peptidase-like active site in PI-PLC, which

we go on to confirm through in vitro experiments [39].

In Table 1, the structure 1A0J should be described in the legend.
We have made the correction, thank you.

Promiscuity in enzyme active sites is a known phenomenon. An example that comes to mind is leukotriene A4 hydrolase, which not only hydrolyses the epoxide but also acts as an aminopeptidase using the same active site. There are also numerous examples where an active site performs different functions in different organisms (or even in the same organism for some protein products of paralogous genes): for example proteins with the alpha/beta hydrolase fold, where some are peptidases and some are lipases. It would strengthen the authors' case considerably to include examples such as these before discussing their own results.
We acknowledge that promiscuity in enzymes has been a phenomenon that has been established over the last couple of decades by several researchers. In accordance with your suggestion, we have added a brief discussion of promiscuous enzymes and related work. Our contribution, we think, has been to formalize computational methods that facilitate the search for latent promiscuous scaffolds in the active site of proteins, and increase the specificity of matches by introducing a filtering in the form of electrostatic potential congruence. In this endeavor, we believe, we have further strengthened the previously hypothesized key role that promiscuity plays in the evolution of proteins [7].

We hope you will find our arguments compelling enough to accept our manuscript. Once again, we thank you for your valuable comments as we strive to establish our work in a collaborative effort.

Best regards,

Sandeep Chakraborty

Competing Interests: No competing interests were disclosed.

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