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## Modulating mobility: a paradigm for protein engineering?

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Please select a section/category for your manuscript.	Biocatalysis for organic synthesis - Novel and significant advances in biocatalysis for organic synthesis, including chemo-, regio- and enantioselective biotransformation, screening and engineering of novel enzymes for biocatalysis, biocatalyst immobilization, and nonaqueous biocatalysis.	
<b>Abstract:</b>	<p>Proteins are highly mobile structures. In addition to gross conformational changes occurring on, for example, ligand binding, they are also subject to constant thermal motion. The mobility of the protein varies through its structure and can be modulated by ligand binding and other events. It is becoming increasingly clear that this mobility plays an important role in key functions of proteins including catalysis, allostery, cooperativity and regulation. Thus, in addition to an optimum structure, proteins most likely also require an optimal dynamic state. Alteration of this dynamic state through protein engineering will affect protein function. A dramatic example of this is seen in some inherited metabolic diseases where alternation of residues distant from the active site affects the mobility of the protein and impairs function. We postulate that using molecular dynamics simulations, experimental data or a combination of the two it should be possible to engineer the mobility of active sites. This may be useful in, for example, increasing the promiscuity of enzymes. Thus, a paradigm for protein engineering is suggested in which the mobility of the active site is rationally modified. This might be combined with more "traditional" approaches such as altering functional groups in the active site.</p>	

**Manuscript #ABAB-D-16-00805**

**Modulating mobility: a paradigm for protein engineering?**

**Margaret McAuley and David J. Timson**

Reviewer: Flexibility of protein plays an important role in protein functions such as activity and stability. Up to now, a number of studies strategized for engineering the protein towards desirable properties are published. The manuscript has a great summary of recent studies based on modulating mobility of protein especially in activity aspect. I recommend its publication after addressing the following issue.

*Response:* We thank the reviewer for his/her support and encouraging remarks.

There are also many reports about the improvement of stability of protein by modulating the flexibility of protein. Such as, "Park.H.J et al, Computational approach for designing thermostable *Candida antarctica* lipase B by molecular dynamics simulation; Zhu, F., et al, Rational substitution of surface acidic residues for enhancing the thermostability of thermolysin; Chen, J et al, Improving stability of nitrile hydratase by bridging the salt-bridges in specific thermal-sensitive regions, and so on". It would be attractive if the authors could incorporate some examples that explain the relationship between activity and stability modulated by flexibility of protein.

*Response:* We thank the reviewer for this suggestion. We have added some material on this topic, including the references suggested.

In addition, we have taken the opportunity to make some small, typographical corrections to the manuscript. All changes are highlighted.

[Click here to view linked References](#)

## Modulating mobility: a paradigm for protein engineering?

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## Abstract

1 Proteins are highly mobile structures. In addition to gross conformational changes occurring on, for  
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3 example, ligand binding, they are also subject to constant thermal motion. The mobility of the  
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5 protein varies through its structure and can be modulated by ligand binding and other events. It is  
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9 including catalysis, allostery, cooperativity and regulation. Thus, in addition to an optimum  
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11 structure, proteins most likely also require an optimal dynamic state. Alteration of this dynamic  
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13 state through protein engineering will affect protein function. A dramatic example of this is seen in  
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15 some inherited metabolic diseases where alternation of residues distant from the active site affects  
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17 the mobility of the protein and impairs function. We postulate that using molecular dynamics  
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19 simulations, experimental data or a combination of the two it should be possible to engineer the  
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21 mobility of active sites. This may be useful in, for example, increasing the promiscuity of enzymes.  
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23 Thus, a paradigm for protein engineering is suggested in which the mobility of the active site is  
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25 rationally modified. This might be combined with more “traditional” approaches such as altering  
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27 functional groups in the active site.  
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37 **Keywords:** enzyme engineering; molecular dynamics; protein flexibility; active site; biocatalysis;  
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## Introduction: protein mobility

1 It is a truth universally acknowledged that protein structure determines function. Indeed this  
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3 postulate is a key feature of introductory, undergraduate biochemistry programmes. This fact also  
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5 underpins structure-based drug design and many aspects of enzyme engineering. It is often clear  
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7 from looking at protein structures that their structure fits them for binding to small molecule  
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9 ligands, macromolecular binding partners (e.g. other proteins, nucleic acids) and for catalytic or  
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11 energy transducing applications. Consequently, structural biology has been highly successful in  
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13 furthering our understanding of how proteins work (46, 5, 43).  
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20 However, it is also clear that proteins are not static structures. That they must undergo  
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22 conformational changes in order to function as catalysts, molecular motors and signalling molecules  
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24 is well-established. Furthermore, like all molecules, they are subject to fluctuations and vibrations  
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26 arising from the thermal energy of the protein and surrounding solvent molecules. It is becoming  
27  
28 increasingly clear that the various motions that proteins undergo are vital to their functions. Indeed  
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30 it is likely that these motions are at least as important as the overall, three-dimensional structures in  
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32 mediating these functions (16, 47). It is important to be clear about the scale of molecular motions  
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34 being considered. In general, the term “flexibility” is used to refer to larger scale motions occurring  
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36 over a timescale of  $10^{-7}$  s to 10 s and includes the motions required for protein folding, allosteric  
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38 regulation and ligand binding. In contrast “dynamics” refers to faster motions ( $10^{-12}$  s to  $10^{-8}$  s)  
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40 such as side chain rotations (47). Understanding the links between these levels of mobility remains  
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42 a considerable challenge in protein science.  
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52 Within an individual protein molecule there is considerable variation in the magnitude of the  
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54 molecular motions present. While some parts may show very limited motions, others may be  
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56 highly mobile. There are various measures of these motions. The B-factor or the Debye-Waller  
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58 factor is a measure of thermal motion usually determined during X-ray crystallography (10, 52).  
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1 Root mean square deviation (RMSD) and root mean square fluctuation (RMSF) are measures of  
2 deviation from a reference structure. RMSD is usually measure of deviation from the initial  
3 structure whereas RMSF is a measure of deviation from an average structure (4). Normal mode  
4 analysis (NMA) and principal component analysis (PCA) have emerged as useful tools for the study  
5 of conformational changes in proteins. NMA utilises a harmonic model to determine possible  
6 molecular deformations in a particular equilibrated system (27, 29, 44). PCA extracts the  
7 contribution of fluctuations from a large selection of conformations (usually from molecular  
8 dynamic simulations) to the overall dynamics of the molecule (44, 23, 26). These approaches are  
9 reviewed in more detail elsewhere (44, 4).

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23 There are a variety of motions which proteins can undergo. These include large scale domain  
24 movements where domains move relative to each other over a scale of tens of angstroms. Such  
25 motions dramatically re-arrange the overall structure of the protein and have been observed in  
26 enzymes such as hexokinase (EC 2.7.1.1) (1-3, 32) and molecular motors such as myosin (25).  
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32 More subtle changes include the local rearrangement of residues, for example in induced fit around  
33 enzyme substrates or other ligands. For example, the interaction between phosphodiesterase 5  
34 (PDE5; EC 3.1.4.35) and sildenafil (Viagra, Revatio) requires mutual conformational changes in the  
35 protein and the ligand to create a complementary binding site. Other PDE isoforms are unable (or  
36 less able) to undergo this conformational change, hence the selectivity of the drug for this isoform  
37 despite high levels of similarity between PDE isoforms (53). There are also other subtle changes  
38 such rotations of side chains. Surface-exposed side chains which are not bonded to other molecules  
39 can usually rotate freely and rapidly. This can be constrained by binding to other parts of the  
40 protein (or other ligands) or by steric hindrance. The activation energy barriers between the  
41 different conformers is often small, hence the rapid rates. Therefore, in addition to flipping between  
42 two (or more) conformational states, we also need to consider changes that result in side chains  
43 altering the fraction of time spent in various states. These changes can have profound influences on  
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ligand binding and protein function (e.g. (41, 28)).

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3 Recent studies have suggested that the natural vibrational properties of protein molecules can be  
4 important in mediating catalysis and intra-molecular communication (16). Thus, these vibrational  
5 motions can be “tuned” at a local level by events such as ligand binding or changes in the external  
6 environment of the protein and may be critical for enzyme catalysis. However, this hypothesis is  
7 not accepted by all authors. For example, some studies consider that there is no experimental proof  
8 linking molecular motions with catalysis (22). These authors propose that electrostatic pre-  
9 organisation and consequent alterations in free energies of activations provide a better explanation  
10 (with greater experimental evidence) of the catalytic power of many enzymes (22, 15).  
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25 One key group of proteins which are postulated to use coupled molecular motions, are those which  
26 demonstrate cooperativity in ligand binding. Typically these proteins have at least two ligand  
27 binding sites. While these sites may appear structurally similar in x-ray or NMR studies, they do  
28 not function independently. The binding of a ligand at one site, will affect the affinity at the other  
29 site. Two forms of cooperativity are recognised – positive (in which binding at the first site  
30 increases the affinity at the second site) and negative (in which binding at the first site makes  
31 interaction at the second one less likely (13, 48)). Both forms of cooperativity generally require  
32 some form of allosteric communication between the active sites. Most obviously this can be  
33 achieved through conformational changes which ramify through the structure linking one active site  
34 to the other (eg haemoglobin (34)). However, it is also possible to achieve this communication  
35 through modulation of the mobility of the protein, e.g. in glutamate dehydrogenase (51). Recent  
36 models of allosteric communication suggest that there are many different structural forms of the  
37 enzyme present (possibly including some intrinsically disordered states) (33).  
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60 That protein motions can affect functions of enzymes has been well established experimentally. In  
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1 dihydrofolate reductase (DHFR; EC 1.5.1.3), alterations in the dynamic behaviour have been  
2 mapped to various stages of the catalytic cycle (42). In the prolyl *cis-trans* isomerase cyclophilin A  
3 (CypA; EC 5.2.1.8), NMR measurements demonstrated that motions required for catalysis also  
4 occur in the free enzyme. This suggested that the enzyme's intrinsic motions are evolved for  
5 catalysis (12). Similar observations were made with adenylate kinase (EC 2.7.4.3) (17). In  
6 ribonuclease A (EC 3.1.27.5) motions in the RNA binding site and the catalytic site are coupled (9).  
7 In addition, the motions of a surface loop distant from the active site are also correlated:  
8 substitution of the this loop with a shorter sequence from a structurally similar protein resulted in  
9 slower loop motions, reduced active site dynamics and a lower turnover number (11).  
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### 23 **Towards an alternative paradigm for protein engineering**

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25 The engineering of proteins to enhance or alter their activity is now well-established (39, 6). The  
26 aspiration of such studies is to adopt an engineering approach in which a well-understood enzyme is  
27 redesigned for a specific role. For example, for an enzyme with a structurally characterised active  
28 site and well defined catalytic mechanism, the aim would be to alter the side chains in order to  
29 provide altered chemical functionality and, consequently, altered function. In theory, this appears  
30 quite straightforward especially if combined with molecular modelling approaches to predict the  
31 architecture of the redesigned active site. Using such approaches it ought to be possible to redesign  
32 active sites around specific, novel substrates and produce enzymes capable of catalysing non-  
33 physiological reactions with activities sufficient to be commercially viable. While there have been  
34 some considerable successes in the design of novel biocatalysts, practical considerations have  
35 limited the application of enzyme redesign. Such considerations include our current limited ability  
36 to predict the structural consequences for the whole protein of even single amino acids changes.  
37 Current modelling techniques also struggle to account for entropic changes (e.g. resulting from  
38 solvation/desolvation in the active site) of such alterations. Linked to both of these factors, is the  
39 need to consider not just alterations to the mean structure of the protein but also to the mobility of  
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1 the molecule, especially around the active site. It is clear from some studies on inherited metabolic  
2 diseases that single point mutations which alter one amino acid (normally away from the active site)  
3 can have profound effects on structure, folding and consequently activity. In some cases, these  
4 changes have been shown to result in increased or decreased flexibility of the protein, e.g. galactose  
5 1-phosphate uridylyltransferase (EC 2.7.7.12), UDP-galactose 4'-epimerase (EC 5.1.3.2),  
6 phosphoglycerate kinase 1 (EC 2.7.2.3), cystathionine  $\beta$ -synthase (EC 4.2.1.22) and mevalonate  
7 kinase (EC 2.7.1.36) (31, 49, 38, 37, 18, 7). Such studies strongly suggest the an optimum amount  
8 of flexibility is required for maximum activity, Indeed, it seems likely that, in addition to optimal  
9 three-dimensional structures, proteins also require optimal dynamics to fit them for their functions.  
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23 Recognition of the need for optimal dynamics as well as structure suggests a paradigm in protein  
24 engineering. Instead of (or as well as) attempting to alter the chemical functionality of active sites,  
25 it should be possible to alter their flexibility. In one early study, the hinge regions of various  
26 immunoglobulins were engineered with consequent effects on the flexibility and function of the  
27 molecules (40). Modulation of the flexibility of a hinge region in triose phosphate isomerase (TPI;  
28 EC 5.3.1.1) markedly affected the steady-state kinetic parameters (45). [There has also been](#)  
29 [considerable success in the engineering the mobility of proteins such that their overall stability is](#)  
30 [enhanced. In the case of nitrile hydratase \(EC 4.2.1.84\), molecular dynamics \(MD\) simulations](#)  
31 [were used to identify regions responsible for initial thermal unfolding. Residues in these regions](#)  
32 [were then altered in order to introduce additional salt bridges into the protein. When additional salt](#)  
33 [bridges were introduced into the C-terminal region of the  \$\beta\$ -subunit, there was a substantial increase](#)  
34 [in stability without loss of activity \(8\). MD simulations were also used to investigate the role of](#)  
35 [surface-exposed glutamate and aspartate residues in thermolysin \(EC 3.4.24.27\). Substitution of two](#)  
36 [of these \(Asp37 and Glu119\) resulted in a more thermally stable protein \(58\). Lipase B \(EC](#)  
37 [3.1.1.3\) was redesigned following MD simulations which identified highly mobile regions. One](#)  
38 [variant \(A251E\) was approximately 2.5 times more stable than the wild-type protein \(36\). In a](#)  
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1 separate approach, rigidification of active site residues in this enzyme also resulted in a more  
2 thermally stable protein (54). MD simulations have also been carried out to investigate the effects  
3 of organic solvents on lipase B. These solvents are predicted to reduce the flexibility of the enzyme  
4 (50). A site on the surface of the enzyme which is affected by methanol was identified by MD  
5 simulations and then altered resulting in a protein with higher stability in this solvent (35). In a  
6 number of these cases, greater stability was initially obtained at a cost to the enzyme's activity.  
7 Further rounds of simulations and/or experiments were often required to generate a more stable  
8 protein with comparable activity to wild-type.  
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20 It might be expected that an active site with increased flexibility compared to the wild-type may  
21 accommodate a wider range of substrates. This has been suggested in the case of galactokinase (EC  
22 2.7.1.6), where alterations to the sequence of a  $\beta$ -sheet structure distant from the active site resulted  
23 in a broader range of substrates being accepted and processed by the enzyme (19, 20, 55-57).  
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29 Preliminary molecular modelling studies suggested that this may be due to increased mobility of  
30 two loop structures in the active site. This prediction is consistent with the kinetic data which  
31 demonstrated widely increased range of monosaccharides which were recognised by the enzyme,  
32 but also reduced activity compared to the wild-type (24). The increased mobility of the active site  
33 will result in a greater range of conformations adopted by this part of the structure. In turn, this will  
34 enable a greater range of complementary interactions with sugar molecules, but also decrease the  
35 time spent by the active site in a catalytically competent conformation. We have previously noted  
36 that combining this approach with methods to increase the overall stability of the enzyme may  
37 result in galactokinases with broader specificity and higher activities (30).  
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54 Engineering the dynamics of proteins requires a detailed knowledge of their motions. While this  
55 can sometimes be obtained experimentally for smaller proteins (typically < 25 kDa) by NMR  
56 spectroscopy it is generally only possible to obtain limited information experimentally. However, *in*  
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*silico* approaches to model the dynamics of proteins are well-established. These can provide a model of the overall dynamic behaviour of a protein combined with estimates of the numerical measures of mobility described above. Extending these methods permits the prediction of the effects of alteration of amino acid residues on local and global mobility. However, [molecular dynamics methods](#) are computationally expensive and time-consuming. The generation of 100 ns of simulation typically requires weeks of computer time. Therefore, it is not currently realistic to undertake computational studies in which the alteration of large numbers of residues are evaluated for their effects on active site flexibility. Intelligent analysis of MD data can, however, enable sensible predictions to be made about which residues might be altered. For example, the use of MD data to identify regions of high or low mobility which correlate with that of the active site may suggest regions to target for alteration. The existence of tables of mean residue flexibility (e.g. (21)) facilitate the alteration of residues to ones which impart more or less mobility than the wild-type. At the extremes of flexibility are glycine (generally the most flexible) and proline (normally highly conformationally constraining).

Over 20 years ago, Alan Fersht proposed “rules” for enzyme engineering (14). In essence these rules suggested that small changes may be more likely to result in successful outcomes. For example, alteration of a glutamate residue to aspartate (to create more space) or to a glutamine (to abolish the charge) may be more successful than alteration to lysine (to reverse the charge) or phenylalanine (to abolish the charge, increase size and generate a more hydrophobic environment). We suggest that similar “rules” may apply when modulating mobility. Alteration of glycine to proline is likely to result in greatly enhanced rigidity (and the imposition of a very limited range of conformations). This will have effects beyond the local region and may well result in misfolded, non-functional protein. In this case, alteration to the next most flexible residue (serine) may be more appropriate. Further theoretical and experimental studies to understand the links between local and global changes in mobility will be required before more sophisticated “rules” can be

proposed.

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3 The approaches suggested above have an element of trial and error. They involve the prediction of  
4 residues likely to affect flexibility on the basis of MD simulations (or experimental data) followed  
5 by further MD work and, ultimately, experimental testing. For a more design-orientated approach,  
6 it would be desirable to decide where more mobility was required (e.g. in the active site) and then  
7 use computational methods to predict where changes should be made to best achieve this. This may  
8 require the development of novel MD approaches and analyses. Doing so would open up new  
9 possibilities of protein engineering and should increase the success rate.  
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23 Overall, we consider that the dynamic behaviour of proteins should be considered to be at least as  
24 important as their three-dimensional structures. We further emphasise that this dynamic behaviour  
25 is critical in the redesign of proteins and is an important factor in the success or failure of protein  
26 engineering projects. Finally, we postulate a paradigm for protein engineering in which the  
27 mobility of active sites (and other critical parts of proteins) are redesigned based on MD simulations  
28 and/or experimental data.  
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43 PhD studentship.  
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