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Protein Promiscuity: Drug Resistance and Native Functions—HIV-1 Case

Abstract
The association of a drug with its target protein has the effect of blocking the protein activity and is termed a promiscuous function to distinguish from the protein's native function (Tawfik and associates, Nat. Genet. 37, 73-6, 2005). Obviously, a protein has not evolved naturally for drug association or drug resistance. Promiscuous protein functions exhibit unique traits of evolutionary adaptability, or evolvability, which is dependent on the induction of novel phenotypic traits by a small number of mutations. These mutations might have small effects on native functions, but large effects on promiscuous function; for example, an evolving protein could become increasingly drug resistant while maintaining its original function.

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Protein Promiscuity: Drug Resistance and Native Functions – HIV-1 Case

Opinions & Commentary by

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Dan S. Tawfik;
Ben Berkhout & Rogier Sanders;
Andrzej Kloczkowski,
Taner Sen and Bob Jernigan.

Summary

The association of a drug with its target protein has the effect of blocking the protein activity and is termed a promiscuous function to distinguish from the protein's native function (Tawfik and associates, *Nat. Genet. 37*, 73-6, 2005). Obviously, a protein has not evolved naturally for drug association or drug resistance. Promiscuous protein functions exhibit unique traits of evolutionary adaptability, or evolvability, which is dependent on the induction of novel phenotypic traits by a small number of mutations. These mutations might have small effects on native functions, but large effects on promiscuous function; for example, an evolving protein could become increasingly drug resistant while maintaining its original function.

Ariel Fernández, in his opinion piece, notes that drug-binding "promiscuity" can hardly be dissociated from native functions; a dominant approach to drug discovery is the protein-native-substrate transition-state mimetic strategy. Thus, man-made ligands (*e.g.* drugs) have been successfully crafted to restrain enzymatic activity by focusing on the very same structural features that determine the native function. Using the successful inhibition of HIV-1 protease as an example, Fernández illustrates how drug designers have employed naturally evolved features of the protein to suppress its activity. Based on these arguments, he dismisses the notion that drug binding is quintessentially promiscuous, even though in principle, proteins did not evolve to associate with man made ligands. In short, Fernández argues that there may not be separate protein domains that one could term promiscuous domains.

While acknowledging that drugs may bind promiscuously or in a native-like manner a la Fernández, Tawfik maintains the role of evolutionary adaptation, even when a drug binds native-like. In the case of HIV-1 protease, drugs bind natively, and the initial onset of mutations results in drug resistance in addition to a dramatic decline in enzymatic activity and fitness of the virus. A chain of compensatory mutations follows this, and then the virus becomes fully fit and drug resistant.

Ben Berkhout and Rogier Sanders subscribe to the evolution of new protein functions through gene duplication. With two identical protein domains, one domain can be released from a constraint imposed by the original function and it is thus free to move in sequence space toward a new function without loss of the original function. They emphasize that the forced evolution of drug-resistance differs significantly from the spontaneous evolution of an additional protein function. For instance, the latter process could proceed gradually on an evolutionary time scale, whereas the acquisition of drug-resistance is an all or nothing process for a virus, leading to the failure or success of therapy. They find no evidence to the thesis that resistance-mutations appear more rapidly in promiscuous domains than native domains. Berkhout and Sanders illustrate the genetic plasticity of HIV-1 by citing examples in which well-conserved amino acid residues of catalytic domains are forced to mutate under drug-pressure. HIV drug resistance biology is very complex. Instead of a viral protein, a drug can be targeted at a cellular protein. For example, Berkhout and Sanders claim, a drug targeted at the cellular protein CCR5 inhibits the binding of the viral envelope glycoprotein (Env) to CCR5. However, Env mutates so that it binds to the CCR5-drug complex and develops drug resistance. Interestingly, CCR5 has not evolved to bind to Env, but to a series of chemokines.

Andrzej Kloczkowski, Taner Sen, and Bob Jernigan point out the importance of protein motions for binding. They believe it is likely that different ligands can bind to the diverse protein conformations sampled in the course of normal protein conformational fluctuations. They have been applying simple elastic network models to extract the motions as normal

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Opinions & Commentary

The evolutionary routes, the dynamics of the target protein, and the many other aspects that need to be addressed while designing a drug that may dodge drug resistance, indicate the complexity and multi-disciplinary nature of the issue of drug resistance.

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modes, which yield relatively small numbers of conformations that are useful for developing protein mechanisms; while these are typically small motions, for some proteins they can be quite large in scale. One of the major advantages of the approach is that only relatively small numbers of modes are important contributors to the overall motion – so the approach provides a way to systematically map out a protein's motions. These models successfully represent the conformational fluctuations manifested in the crystallographic B-factors, and often suggest motions related to protein functional behaviors, such as those observed for reverse transcriptase, where two dominant hinges clearly relate to the processing steps – one showing anti-correlation between the polymerase and ribonuclease H sites related to the translation and positioning of the nucleic acid chain, and another for opening and closing the polymerase site. Disordered proteins represent a more extreme case where the set of accessible conformations is much larger; thus they could offer up a broader range of possible binding forms. Whether evolution controls the functional motions for proteins remains little studied. Intriguingly, buried in the existing databases of protein-protein interactions may be information that can shed light on the extent of promiscuous binding among proteins themselves. Within these data there are cases where large numbers of diverse proteins have been shown to interact with a single protein; some of these could represent promiscuous protein-protein binding. Uncovering these promiscuous behaviors could be important for comprehending the details of how proteins can bind promiscuously to one another, and can exhibit even greater promiscuity in their binding to small molecules.

Protein function with concurrent promiscuity

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Most researchers assume a clear delineation between native and promiscuous protein functions (1). In contrast with native functions, promiscuous functions are assumed to involve predominantly entropy-driven interactions, to typically exclude pair-wise enthalpic contributions, and to be essentially free from selection pressure, in accord with their purported latency. Thus, a conspicuous illustration of promiscuity is assumed to be enzymatic inhibition by drug association (2-6), a function for which the protein clearly has not evolved naturally. I believe this view needs revision in the light of the following considerations.

There are numerous instances where drug-binding promiscuity can hardly be dissociated from native function, as evidenced by the fact that a dominant approach to drug discovery is the protein-native-substrate transition-state mimetic strategy (2-6). Thus, man-made ligands, for example drug inhibitors, have been successfully made to inhibit enzymatic activity by focusing on the very same structural features that determined the native function.

Furthermore, there are native structural features germane to enzymatic processivity which have been obviously subject to severe selection pressure for a particular role and are utilized in an alternative role in what Aharoni *et al*. (1) would call "promiscuous" functions. Thus, promiscuity may engage highly conserved structural regions of the protein with dual roles, contributing to both a naturally evolved (native) and a promiscuous function. For instance, the flexibility of the β-hairpin flap in HIV-1 protease is required for the processivity of the enzyme (6). Thus, the flap region, must have a highly water-exposed – and hence labile – hydrogen bond, as needed to confer the necessary flexibility associated with the gating mechanism. A naturally evolved and highly conserved glycine-rich loopy region exposes to water a backbone hydrogen bond in the β-hairpin. The over-exposed hydrogen bond is inherently sticky because it can be strengthened and stabilized upon exogenous water removal (6-8). Thus, the lack of protection on the flap backbone hydrogen bond is subservient to a native function of the HIV-1 protease, but becomes also the reason for its stickiness, a property taken advantage of in a promiscuous function. A proper inhibition of the protease then hinges upon the possibility of wrapping of the flap hydrogen bonds with the nonpolar groups of the purported drugs (6).

In this way, we are reporting on an instance of a structural feature – a flexible flap – naturally selected for a purpose and utilized promiscuously for another purpose. Undeniably, the protein has actually evolved to sustain this feature, thus hinting to an apparent inconsistency in the views of Aharoni *et al*. (1), who maintain that promiscuous functions are not naturally evolved.

Furthermore, there are naturally evolved and conserved structural features inherent to catalytic activity that have been used promiscuously by the drug designers aiming at the inhibition of HIV-1 protease activity. Thus, there are intramolecularly under-wrapped or under-dehydrated hydrogen bonds adjacent to the catalytically active site (Asp25) in each monomer of the functionally compentent homodimer (6). These structural features are required to frame an anchoring track for the substrate peptide. This "sticky track" determined by the under-dehydrated hydrogen bonds is required to align the substrate peptide chain across the cavity, as needed for selective nucleophilic attack by the two equivalent catalytic Asp25s. Furthermore, since such bonds promote the removal of surrounding water (6-8), they enhance the electrostatic field generated by the catalytic Asp25, by de-screening its net charge. This is precisely their *raison d'etre*: they foster catalytic activity by exacerbating the nucleophilic potential of Asp25. On the other hand, since these hydrogen bonds are inherently sticky for the reasons mentioned above, they have been targeted by drug designers aiming at inhibiting the protease activity (6). Thus, drug inhibitors provide intermolecular wrapping to these naturally evolved packing defects in the protease

Here we find another instance of naturally evolved features compliant with a native catalytic function and used promiscuously for drug-based inhibition. These facts and the very nature of drug discovery seem to disprove the basic tenet that drug binding is quintessentially promiscuous because proteins did not evolve to associate with man-made ligands. In fact, every native function may be turned promiscuous by a sufficiently skillful designer of ligands able to mimic natural substrates and knowledgeable of the mechanisms of protein associations. On the other hand, few native functions may escape promiscuity because exogenous water removal from pre-formed electrostatics – an entropy-related interaction – is a ubiquitous determinant of protein associations (7, 8).

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The evolvability of drug resistance: Response to Fernández

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The article by Fernández (1), and the response that follows, address the following question: To what extent is drug action – namely the association of a drug with its target protein, and blocking of the latter's activity – an inherently promiscuous function; or could drug binding follow the very same characteristics of the protein's native or original activity? The somewhat colloquial term 'promiscuity' is used to describe the ability of an enzyme or protein to perform functions in addition to those for which it is evolutionary maintained. In mechanistic terms, promiscuity can result from active-site features that are the same, overlapping or distinct to those responsible for the native function. Comparison of the two modes – promiscuous *vs*. native – has traditionally focused on the differences in the physico-chemical nature of these interactions. Recently, we have proposed that there are also evolutionary differences between the two modes: Promiscuous protein functions seem to exhibit unique traits of evolutionary adaptability that are distinctly different from those of the native function (2). Evolutionary adaptability, or evolvability, is dependent on the induction of novel phenotypic traits by a small number of mutations. However, the vast majority of mutations have deleterious effects on protein function. How can a protein resist the deleterious effects of mutations yet maintain the ability to adopt new functions and structures? Our results indicated that the evolution of a new function is underlined by mutations that have little effect on the native function, but large

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effects on the promiscuous functions that serve as starting point. Thus, an evolving protein can initially acquire increased fitness towards a new function (*e.g.*, reduced drug binding) while maintaining its original function. This despite the fact that both the promiscuous and the original function take place at the same site (2).

We proposed that the very same mechanism could underline the evolution of drug resistance. The binding of drug to its protein target is, by definition, a promiscuous function that competes with the protein's original or native function – namely the function for which that protein evolved, and has been maintained throughout. That drug resistance is a common, and generally harmful, phenomenon is also beyond dispute. Once an organism has been exposed to a drug that threatens its existence, genetic changes often take place in the form of rapidly acquired mutations that lead to loss of inhibition by the drug, while maintaining the original function of the target protein. We surmise that, mutations that confer drug resistance exhibit characteristics similar to those of mutations that lead to an increase in a promiscuous enzymatic function yet do not alter the enzyme's original function. These mutations are primarily in flexible substrate binding loops, rather then in the catalytic residues or the protein's scaffold (2).

The general view regarding promiscuity (*e.g.*, cross-reaction of antibodies, catalysis of non-native substrates by enzymes, *et cetera*), which is also portrayed in our article, is that, promiscuity is driven primarily by hydrophobic, and other entropy-driven interactions (1). In contrast, binding of the native substrate, or ligand, typically involves several independent, enthalpy-driven interactions (*e.g.*, hydrogen bonds). Our results (2), and numerous records of drug resistance, indicate that, owing to these differences, mutations that abolish drug binding but preserve the original function can rise with astonishing ease. However, as indicated by Fernández, the above view ignores the fact that, in many cases, drug binding and the native function overlap to a large extent (1). In general, the relationship between specific and promiscuous activities are rarely known. For example, the cross-reactivity of antibodies has often been attributed to "hydrophobic stickiness". Yet we could show that, several cross-reactants can bind a single antibody while forming specific hydrogen bonds depending on the particular chemistry of the cross-reactant and the availability of complimentary antibody residues. Consequently, close derivatives of these cross-reactants show very low or no binding, and the cross-reactants exhibit the same degree of specificity as the native ligand (3). Indeed, drug designers often identify highly conserved structural features that are essential parts of the protein's active-site (*e.g.*, an enzyme's catalytic machinery) and make sure that their drug interacts with these structural elements in a highly specific manner. This is the rationale behind the use of transition state analogs as enzyme inhibitors, or the HIV-1 proteinase inhibitors discussed by Fernandez (1). Once a drug interacts with the active-site core and other essential parts of the protein's scaffold, it becomes 'native-like' in its mode of interaction.

The differences between drugs that are 'promiscuous' in their mode of binding, in oppose to 'native-like', can be demonstrated by the completely different patterns of acquisition of drug resistance observed in response to HIV-1 protease inhibitors *versus* reverse transcriptase (RT) inhibitors. As insightfully described by Berkhout (4), drug resistance to RT inhibitors follows a simple route whereby one, or a few, mutations result in an RT variant that resists inhibition but largely maintains enzymatic function. This is precisely the mode of evolutionary adaptation we described (2). In contrast, the appearance of drug resistant mutations in the protease follow the expected negative tradeoff (5), and is, therefore, accompanied by a dramatic decline in enzymatic activity and in the fitness of the virus. A long chain of compensatory mutations follows that restores viral fitness. Only some of the mutations actually increase the protease's activity (and scarcely to a wild-type level). Most mutations act indirectly, *e.g.*, through modification of the Gag-protein cleavage sites that comprise the protease's substrate, so that virus fitness is

regained while maintaining drug resistance. In a simplified manner, the differences in the way drug resistance evolves can be ascribed to the differences in the mode of drug binding. The protease inhibitors generally bind in a 'native-like' mode while interacting with the core of the enzyme's active-site. But the RT inhibitors act in a promiscuous mode; they bind to flexible and external parts of the enzyme that are distant from the core of its active site (4).

In conclusion, in my view, regardless of the mode and strength of interaction, no protein has evolved to sustain drug binding. Rather, drug binding may be maintained 'parasitically', using conserved features of the protein, but not by contributing to their evolutionary preservation. Drug designers can make use of these conserved features, make their drug 'go native', and thereby avoid the rapid emergence of drug resistant mutants. Foremost, because a promiscuous function can make use of an active site conformation that is fundamentally different from the conformation conferring the original function (6), a drug should make use of the same active-site conformation used by the native ligand. In addition, a drug should ideally interact with all the key active site residues that are used by the protein to perform its original function. In both these respects, the identification of lead molecules from combinatorial libraries is not, in our view, a promising avenue. This protocol that may generate potent drugs is likely to lead to a promiscuous rather than native-like mode of binding. In contrast, structure-based drug design could ensure that the above requirements are met, and thereby maximize the overlap between the native function and drug association, and guarantee that any mutation affecting drug binding is likely to impair the original function. In other words, once a drug has been 'naturalized', expedition through a mutation that would otherwise abolish 'illegitimate' or promiscuous binding, becomes much more difficult. To conclude, I would like to note that, the above conclusions are based on an evolutionary model that only takes into account the target protein, and ignores a whole variety of other issues, including the pathogenic organism and its host. The accompanying commentaries, and works on drug resistant mutants identified in the clinic (7), reflect the complexity of this problem and its many facets.

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An accepted scenario for the evolution of new protein functions is through gene duplication (8). With two identical protein domains, one domain can be released from constraint imposed by the original function and it is thus free to move in sequence space toward a new function, without loss of the original function. Aharoni *et al*. presented an alternative scenario for the evolution of multi-functional proteins through the acquisition of mutations in a so called promiscuous domain that leads to a new function, without a negative effect on the original protein function that is executed by another native domain (1). The commentary by Ariel Fernández (12) and the response by Danny Tawfik (13) move this discussion toward the field of evolution of drug-resistance, *e.g*., as a response to antiviral therapy of HIV-AIDS patients. It should be emphasized that this forced evolution of drug-resistance differs significantly from the spontaneous evolution of an addi-

The evolvability of drug resistance: The HIV-1 case

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tional protein function. For instance, the latter process could proceed gradually on an evolutionary time scale, whereas the acquisition of drug-resistance is an all or none process for a virus, leading to failure or success of therapy (4).

Fernández (12) argues that there may not be separate protein domains that one could term promiscuous domains. Indeed, protein domains without any function will be lost in evolution, and this will occur surprisingly rapid if we stick to the HIV-1 example, which is known for its enormous evolutionary speed. Nevertheless, it is obvious that some domains are more critical than others, and this is usually reflected in more or less sequence variation when comparing different virus isolates. Inhibitors of the HIV-1 Reverse Transcriptase (RT) that are currently used in the clinic represent drugs that target either a promiscuous/more variable domain (non-nucleoside RT-inhibitor or NNRTI) or the catalytic domain that executes the native polymerase function (nucleoside RT inhibitor or NRTI). Although it could *a priori* have been predicted that resistance-mutations appear more rapidly in promiscuous domains than native domains, there is no evidence for this. For instance, the potent 3TC antiviral is a dNTP-mimic that is incorporated in the catalytic core, but resistance develops in patients within weeks (10). More strikingly, the resistance mutation occurs in the absolutely conserved YMDD motif within the catalytic core, which mutates to YVDD or YIDD (7). Mutation of this important motif illustrates the genetic plasticity of HIV-1 and argues that even well-conserved amino acid residues of catalytic domains can be forced to mutate under drug-pressure.

As argued by Tawfik (13), mutation of a residue within a native domain will more likely result in a loss of function, and reduced fitness is indeed what is observed for 3TC-resistant viruses (2). However, reduced viral fitness has also been described for resistance mutations in less conserved promiscuous domains (6). There could be differences in the frequency and magnitude of the fitness loss for drugs that target native versus promiscuous domains, but such a broad survey has not been performed thus far. HIV-1 studies also demonstrate the enormous possibilities that are created by evolution. Although the initial drug-resistance mutations may reduce enzyme function, compensatory changes will appear quickly to improve enzyme function, and the end result may be an enzyme/virus that is more active/fit than the wild-type (5). There are more exotic scenario's, *e.g.*, the appearance of a defective HIV-1 variant that replicates exclusively in the presence of the antiviral drug (3).

An interesting evolutionary feature of HIV-1 is the switch of the viral envelope glycoprotein (Env) from usage of CCR5 to CXCR4 as a second receptor in addition to CD4. This switch, which requires multiple amino-acid changes, occurs in about half of the HIV-infected individuals, broadens the host cell range to naïve CD4+ T cells and is correlated with a more rapid progression to AIDS. Although this phenomenon may be related more to cross-reactivity by molecular mimicry than to promiscuous binding, it follows the path from a highly specialized CCR5-using Env, via a dual-tropic generalized Env with a relatively low affinity for either receptor, to another specialized CXCR4-using Env (9). What is the native function in such a case? From a cellular perspective, the native function is binding of the receptors to their natural ligands; the chemokines RANTES, MIP1-α, MIP1-β (CCR5) and SDF-1 (CXCR4). Binding of CCR5 or CXCR4 to Env would then be parasitic and promiscuous, since these receptors did not evolve to bind HIV-1 Env. From the viral perspective, the latter would be the native function since Env did evolve to use CCR5 or CXCR4. This CCR5 to CXCR4 switch also has important implications for drug design and viral resistance. Several novel drugs that target the viral entry process are currently under investigation in clinical trials. Caution is justified with respect to the use of CCR5 inhibitors in the clinic because they may induce the switch to the more pathogenic CXCR4-using virus variants. However, recent evidence indicates that HIV-1 takes another path of drug resistance, in which Env changes its mode of CCR5-binding (11). Thus,

promiscuous binding of the drugs induces a conformational change in CCR5 such that Env can no longer bind. Resistance is thought to occur through mutations in Env that alter the mode of native binding, such that Env is able to bind to the CCR5-drug complex. This is a rather unique example because the drug does not target a viral protein, but a cellular protein.

Finally, Tawfik (13) argues that drug development should preferentially use structure-based design to yield compounds that bind in the active site of the native domain, such that escape mutations will impair the original function. He also argues that screening of combinational libraries will mostly yield compounds that bind to promiscuous protein domains, which allows more rapid and easier escape. We feel that it is not opportune to close a drug discovery route. When the target is a virus like HIV-1, we think that any potent antiviral, no matter how it was developed and what protein domain it targets, will be a welcome addition to the current arsenal of antivirals. In fact, several potent antiretrovirals of the first generation of NNRTI drugs that are still used successfully in the clinic have been developed by random screening programs. Moreover, several of the new and promising inhibitors that are under investigation in phase II and phase III clinical trials, including some that target CCR5 or CXCR4, are derived from the screening of combinatorial libraries.

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Promiscuous vs. native protein function. Insights from studying collective motions in proteins with elastic network models

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The functional dynamics of proteins can be manifested in different ways; in particular some proteins can bind to multiple ligands in ways that are facilitated by the intrinsic motions of the protein. Unstructured proteins could offer some broad range of structures as alternative binding sites. Specific binding, in contrast to non-specific binding, has long been discussed for binding to DNA, but has rarely been considered for proteins. Domain swapping offers a particularly interesting example of multiple binding states – internally within one monomer, or between two monomers. In domain swapping, monomers undergo some internal dissociation, and form multimeric structures by binding between monomers, often in nearly identical ways as within the monomeric structure. Such large scale motions are seen often in proteins and have recently been shown to be well represented by protein motion models using simple elastic network models (1). When the motions are sufficiently large in scale there are opportunities for different ligands to find favorable binding sites at different locations along the motion pathways. These elastic models are proving their abilities to represent the important motions in proteins, and presumably can also offer other opportunities for drug design by targeting accessible flexible structural sub-states, or interfering with essential hinge motions.

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In a recent paper (2) published in *Nature Genetics* the research group of Dan Tawfik has shown that proteins that have evolved to perform a given function often have the ability to adapt to other, completely unrelated functions as well. They have created a speeded-up version of evolution in the lab by introducing random mutations into the genes encoding various proteins and simulating evolutionary pressure by selecting mutants with higher levels of activity for one of the promiscuous traits. They were able to significantly increase the activity for which they were selecting, and found that the levels of the other promiscuous activities in most cases, dropped dramatically, but in some there was also a significant increase. Thus, an evolving protein can acquire increased fitness for a new function without losing its original native function. Gene duplication and the divergence of a completely new protein may then follow. This is a surprising result, since both activities take place at the exact same site on the enzyme. According to the authors maintaining protein native function as well as their responsiveness to mutations for promiscuous functions are both extremely important for living organisms. Natural evolution, leading to native protein functions, takes an extremely long time to develop. Promiscuous functions may not have been under selection pressure and evolution may not have provided for rapid adaptation. This creates one possible mechanism for the phenomenon of rapidly developing drug resistance. A better understanding of the problem of promiscuity of protein function is extremely important for progress with drug discovery. In particular, two problems need to be resolved – just how 'promiscuous' can a protein be in accepting other drug molecules? And how can drugs be designed to become more specific? It is likely that some relevant information might be uncovered from studies of multiple interactions for proteins as collected in the various protein-protein interactions for organisms such as yeast, fruit fly, and worm (3-6). In other words, functionally important promiscuity of binding partners could occur naturally for some proteins.

Ariel Fernández (3) has noted that promiscuous functions cannot be dissociated from native functions and that structurally conserved binding sites in proteins contribute to both native and promiscuous functions. Our studies on proteins dynamics lend support to this point of view by suggesting that the motions intrinsic to a protein could be requisite for the multiple bindings.

Our group and others (1, 7-17) have been studying the effects of collective motions in proteins. The main rationale behind this approach is that protein structure determines its dynamics, and protein dynamics is a key factor in the determination of protein function. In the past we have studied for example the relationships between the structure, functional mechanism and collective motions of the HIV-1 reverse transcriptase (RT) by using the Gaussian network model (GNM) of proteins (10, 11, 18). This model is particularly suitable for elucidating the global dynamic characteristics of large proteins such as the heterodimeric RT comprising a total of 982 residues. By treating the macromolecule as a coarse-grained uniform block of material, utilizing only the C^{α} positions, the normal modes of motion can be obtained. Local packing density and coordination order of amino acid residues was found to determine the type and range of motions, both at the residue level and on a global scale, such as the correlated movements of entire subdomains. Of the two subunits, p66 and p51, forming the RT, only p66 has a DNA-binding cleft and a functional polymerase active site. This difference in the structure of the two subunits is reflected in their dynamic characteristics: only p66 has the potential to undergo its large-scale cooperative motions in the heterodimer, while p51 is essentially rigid. Taken together, the global motion of the RT heterodimer is comprised of movements of the p66 thumb subdomain perpendicular to those of the p66 fingers, accompanied by anti-correlated fluctuations of the RNase H domain and p51 thumb, thus providing details of the processivity mechanism. A few clusters of residues, generally distant in sequence but close in space, are identified in the p66 palm and connection subdomains to form the hinge-bending regions that control the highly concerted motion of the subdomains. These regions include the catalytically active site and the non-

nucleoside inhibitor binding pocket of p66 polymerase, as well as sites whose mutations have been shown to impair enzyme activity. It is easily conceivable that this hinge region, indicated by GNM analysis to play a critical role in modulating the global motion, is locked into an inactive conformation upon binding of an inhibitor. Bahar *et al*. have shown that some inhibitors can change the directions of the intrinsic motions (18). Comparative analyses of the dynamic characteristics of the unliganded and liganded dimers indicate severe repression of the mobility of the p66 thumb in RT's global mode, upon binding of non-nucleotide inhibitors.

Our most recent results obtained for transmembrane proteins further support the hypothesis of the strong interconnection between structure, dynamics and protein function (19). *E. coli* requires an efficient signaling and transport system to successfully sequester iron from its environment. FecA, a transmembrane protein, serves a dual role. It binds iron in the form of ferric citrate and initiates a signaling pathway which results in the transcription of several iron transporter genes. FecA interacts with several intracellular membrane proteins to perform these tasks. We combined the predicted structure of the NH2-terminal domain (with A. Kolinski) with the plug and the barrel domains and applied elastic network models to derive global modes of motion of the FecA protein (Fig. 1) and obtained high correlation with the experimental B-factors. The FecA global motions derived from normal modes show various motions in the NH_2-terminal domain relative to the plug and the barrel domains, which are anchored in the membrane. These motions are illustrated in Figure 1 with arrows. In the slowest mode, the NH_2-terminal domain swings towards the right side, approaching the periplasmic loops. In the second slowest mode, the NH_2-terminal domain swings to the right, interacting with the loops located on that side. Our analysis of the motion in the plug and barrels shows that these periplasmic loops are also highly mobile, and that the NH_2-terminal domain may interact with these loops. In the third slowest motion, the NH_2-terminal domain pulls out from the barrel. In the fourth slowest motion, the NH_2 domain twists sideways. And, in the fifth slowest mode, the NH_2-terminal domain rotates slightly to the right, a motion hindered by the coil region connecting the NH_2-terminal domain to the plug domain. The last three motions may be useful to ensure flexibility when FecA is interacting in a complex or to reduce energy barriers during protein-protein binding. Since the slowest modes play a dominant role in determining a protein's functional mechanism, FecA may employ a single slow mode, or a combination of modes to activate diverse pathways in the signaling and transporting of iron in the form of ferric citrate. This is a remarkable example that can explain how information is transferred from the extracellular to the periplasmic side and how ferric citrate binding to the extracellular loops can activate different motion patterns in the periplasmic NH_2-terminal domain. Binding does not alter the normal motions of the NH_2-terminal domain, but influences the combination of these modes affecting FecA's coupled activation/deactivation signaling ability. Evolution could exploit the large number of combinations of such motions to redefine a protein's function. From this perspective, the native and promiscuous functions are just different combinations of the same group of motions as, differentially favored during evolution.

Elastic network models have proven themselves in characterizing the important pathways dictated by a protein's structure, which means that they will also be useful for deriving structures deviating from the known native structure, and consequently can be an important tools for characterizing promiscuous binding, including those variable sites important for drug design.

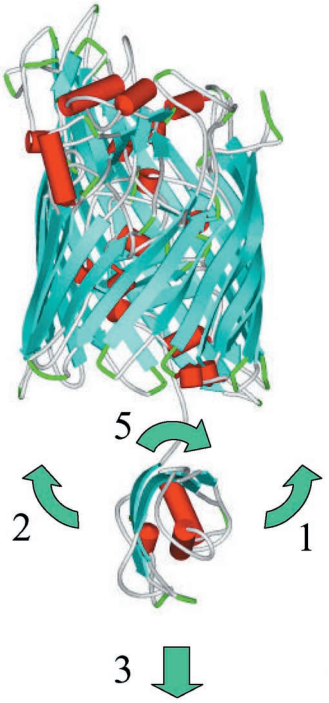

Figure 1: Representation of FecA protein and its major motions. The NH_2-terminal domain is modeled using *ab initio* and is located in the periplasm under the plug and the barrel domains. The arrows illustrate the predicted global motion directions in the slowest modes. 1 represents the slowest mode, 2 the second slowest mode etc. The directional information of the 4th slowest mode is provided in the text.

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