



Prediction of protein binding sites and hot spots

Juan Fernández-Recio*

Protein–protein interactions are involved in the majority of cell processes, and their detailed structural and functional characterization has become one of the most important challenges in current structural biology. The first ideal goal is to determine the structure of the specific complex formed upon interaction of two or more given proteins. However, since this is not always technically possible, the practical approach is often to locate and characterize the protein residues that are involved in the interaction. This can be achieved by experimental means at expense of time and cost, so a growing number of computer tools are becoming available to complement experimental efforts. Reported methods for interface prediction are based on sequence information or on structural data, and make use of a variety of evolutionary, geometrical, and physicochemical parameters. As we show here, computer predictions can achieve a high degree of success, and they are of practical use to guide mutational experiments as well as to explain functional and mechanistic aspects of the interaction. Interestingly, it has been found that typically only a few of the interacting residues contribute significantly to the binding energy. The identification of such hot-spot residues is important for understanding basic aspects of protein association. In addition, these residues have received recent attention as possible targets for drug design, so several computer methods have been developed to predict them. We will review here existing computer approaches for the prediction of protein binding sites and hot-spot residues, with a discussion on their applicability and limitations. © 2011 John Wiley & Sons, Ltd. *WIREs Comput Mol Sci* 2011 1 680–698 DOI: 10.1002/wcms.45

INTRODUCTION

Proteins function through interaction networks that are ubiquitously found in all essential cell processes, such as cellular communication, immunological response, and gene expression control, among others. Understanding the structure, function and mechanism of these interaction networks at molecular level is one of the current challenges in structural biology. In this regard, experimental techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) can achieve the most detailed structural knowledge of protein–protein interactions at atomic resolution. During the last decades, a significant number of complex structures have been deposited in the Protein Data Bank (PDB), which has boosted our understanding of protein–protein association. However, in crystallography structures it is often difficult to distinguish true biological

interactions from crystal packing contacts (which usually have no biological sense).^{1–3} Therefore, some computer tools have been developed based on residue conservation,⁴ interface size,⁵ or other interface descriptors to distinguish crystal packing from obligate and nonobligate interactions.⁶ Some available databases aim to compile true biological units, such as Protein Quaternary Structure (PQS) (www.ebi.ac.uk/pdbe/pqs)⁷ or ProtBuD,⁸ while others are specialized in storing and curating structural data on protein–protein interactions: 3DCOMPLEX (<http://www.3dcomplex.org/>), PiBase (<http://alto.compbio.ucsf.edu/pibase/>), Protein3D (<http://protein3d.ncifcrf.gov/~tsai/>), Structural Classification of Protein–Protein Interfaces (SCOPPI) (<http://www.scoppi.org/>), DOCKGROUND (<http://dockground.bioinformatics.ku.edu>), or Surface Properties of Interfaces - Protein–Protein interfaces (SPIN-PP) (<http://trantor.bioc.columbia.edu/cgi-bin/SPIN/>).

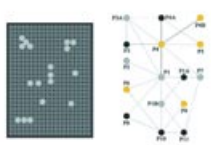
The main challenge regarding the structural comprehension of protein interactions is that the number of available three-dimensional (3D) complex structures is still very low with respect to the total number of protein–protein interactions that

*Correspondence to: juanf@bsc.es

Life Sciences Department, Barcelona Supercomputing Center, Barcelona, Spain

DOI: 10.1002/wcms.45

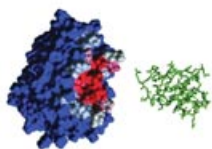
Target identification



1. Establish protein–protein interaction

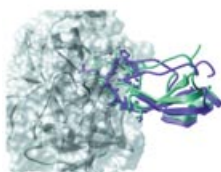
Correlated mRNA expression profiles; correlated evolution; domain fusion patterns; automated literature mining

Target characterization



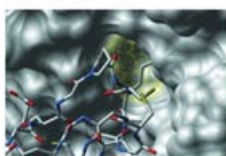
2. Locate interface

Surface analysis; hydrophobicity profiles; 3D cluster analysis; residue conservation



3. Modeling protein–protein interaction

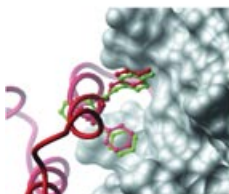
Rigid-body docking; energy minimization; side-chain refinement; flexible docking



4. Finding putative small-molecule pockets

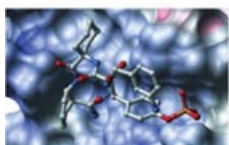
Analysis of 'hot spots'; surface concavities

Lead discovery and optimization



5. Mimicking interface

Energy minimization; graphic modeling



6. Ligand docking

Flexible ligand docking; grid or explicit receptor representations; MC minimization

FIGURE 1 | Scheme of a typical drug discovery process. Computer prediction of protein binding sites and hot-spot residues are essential for steps 2 and 4, respectively.

occur in living organisms. Thus, when the structure of a given protein–protein complex is not easily available for technical reasons, the practical approach is to characterize the interface, i.e., to identify the surface residues of the interacting proteins that are involved in the interaction. Some experimental approaches aim to characterize protein interfaces using methods that are faster than atomic-level structure determination, such as cross-linking, site-directed mutagenesis, alanine scanning, or NMR chemical shift, which in prin-

ciple could also be amenable to high-throughput application. However, given the difficulties and costs of the experimental techniques, many computer tools have been developed to complement the experimental efforts by characterizing, classifying, and predicting protein interfaces.⁹ Thus, computer prediction of interface residues is becoming an essential tool for target characterization in the first step of a drug discovery program targeting protein–protein interactions (Figure 1). The predictions can guide mutation

experiments, give biological and functional insights, reduce the complexity of computer docking simulations for binding mode modeling,⁹ (Box 1) and provide further information for drug design targeting specific protein interactions. In addition, further characterization of the protein interfaces is required to identify hot-spot residues, i.e., the largest contributors to the binding affinity. This is typically achieved through mutational studies, but recently, different computational approaches have been developed to predict putative protein–protein hot spots. The identification of such hot spots is important not only from a functional and biological point of view, but also for drug design targeting protein–protein interactions (Figure 1). In this article, we will review the main computer methods that have been reported for the prediction of protein–protein interfaces and the identification of hot-spot residues.

Dissection of Protein–Protein Binding Sites

Protein–protein interfaces are usually flat and large in comparison to small-molecule binding sites, although their residue composition and physicochemical character is quite varied. Pioneer analyses of available protein–protein complex structures attempted to find specific features that differentiated protein binding sites from other areas of the protein surface.^{25–31} However, further studies showed that shape and composition of interfaces were largely dependent on the type of interaction (Box 2). Thus, while homodimer, obligate interfaces had clearly different physicochemical composition with respect to the solvent-exposed surface, protein–protein interfaces in heterocomplexes did not seem to be easily differentiated from the rest of the surface.^{32–34} This seems to be in contradiction with the fact that chemical and physical complementarity between the interacting surfaces is essential for the formation of heterocomplexes, frequently involving nonobligate and transient interactions. Indeed, some studies based on continuum electrostatic calculations suggested that protein–protein interfaces are naturally designed to exploit electrostatic and hydrophobic forces in very different ways.³⁵

A recent work³⁶ has revisited the definition of protein–protein interface, typically divided into core and rim regions in many studies,³⁷ by including a third region called ‘support’. The results showed that core residues contribute over two-thirds of the contact surface. Interestingly, the support region composition is similar to the interior of proteins, while the rim region composition is similar to the exposed surface. The authors hypothesized that part of a protein–protein interface (support and rim) could pre-exist in a noninteracting protein surface, and thus evolving to

BOX 1: PROTEIN–PROTEIN DOCKING

Protein–protein docking aims to predict the structure of a protein–protein complex starting from the coordinates of the unbound subunits. A number of protein–protein docking methods have been reported, with reasonable predictive success (good reviews on protein–protein docking have been recently published).^{10–13} The majority of docking methods rely on the rigid-body approach, which seems valid for cases with only small side-chain movement upon binding. Many sampling methods perform exhaustive search using fast Fourier transform (FFT), spherical polar Fourier, or geometric hashing algorithm. Some of the most well-known FFT-based docking programs are FTDock,¹⁴ Zdock,¹⁵ MolFit,¹⁶ and Global Range Molecular Matching (GRAMM-X).¹⁶ Other successful shape-based methods are Hex¹⁷ and PatchDock.¹⁸ Another group of rigid-body docking methods use energy based-sampling by molecular dynamics (MD), energy minimization or Monte Carlo methodology combined with different energy-based scoring schemes such as ICM-DISCO,¹⁹ Haddock,²⁰ RosettaDock,²¹ or ATTRACT.²¹ After the initial docking step, many methods rely on the scoring and refinement of the generated docking poses. One example is pyDock,²² which uses an energy function composed of van der Waals (truncated to 1.0 kcal/mol to avoid interatomic clashes from the rigid-body approach), Coulombic electrostatics with distance-dependent dielectric constant, and atomic solvation parameter based desolvation energy. Another successful scoring method is ClusPro/SmoothDock,^{23,24} which uses an energy-based minimization function, plus an additional clustering stage. A good blind assessment of protein docking methods can be found in Critical Assessment of PRedicted Interactions (CAPRI; <http://www.ebi.ac.uk/msd-srv/capri/>).

a protein interface would only need a few mutations in order to achieve the typical core composition.

Given that most of the analyses of protein binding sites are based on available X-ray data, flexibility aspects have been typically overlooked in spite of its enormous importance. Molecular dynamics (MD) simulations have shown the existence of anchor residues for molecular recognition,³⁸ which are more rigid than the rest of the interface and correlate well with conserved hot-spot residues (see the following sections).³⁹ Moreover, a recent systematic analysis of the dynamic properties of interface residues has shown correlation with the interface type, size, and nature of the complex.⁴⁰ Allosteric effects are also important in order to understand (and predict) protein binding sites, although many questions still remain before we are able to fully describe their mechanisms.⁴¹ Other aspects that are usually

BOX 2: TYPES OF PROTEIN–PROTEIN INTERFACES

In the analysis of protein interaction sites and hot spots, it is important to distinguish between different types of protein–protein interfaces. An early work classified protein–protein interactions into permanent complexes (components are not structurally stable as monomers) and nonobligatory or transient complexes (components are stable as monomers).⁴⁷ A more recent study has classified protein–protein interfaces in four different types: homo-obligomers, homocomplexes, hetero-obligomers, and heterocomplexes.⁴⁸ The meaning of obligomers and complexes was similar to permanent and nonobligatory, respectively.⁴⁷ They also defined two extra types of interfaces: intradomain (interactions within the same domain) and domain–domain (interactions within the same chain), but although they might be evolutionary close to protein–protein interactions, they are not directly relevant for protein association. Of course, one could argue that domain–domain interactions are not much different from obligomers in the sense that the separated subunits are not stable, but the difference is that obligomers are formed by two separated entities (even though they do not need to be structurally stable when separated) while domain interactions have additional physical constraints, such as the peptide/s linker, which brings other considerations into the equation. Perhaps, the most reasonable choice is to classify protein interactions according to several criteria, regarding (1) the similarity of the subunits (homo-oligomeric and hetero-oligomeric complexes), (2) the thermodynamics of the association (nonobligate and obligate complexes), or (3) the kinetics of the interaction (transient and permanent complexes).⁴⁹ More recent interface classifications have been based on geometrical and evolutionary criteria aiming to the automated high-throughput annotation of new interfaces.⁵⁰

difficult to address from analyses purely based on crystallographic data are those related to transient interactions,⁴² some of which are mediated by linear motifs,⁴³ or to hub proteins in protein interaction networks,⁴⁴ which show shared binding sites for different interaction partners.^{45,46}

Computer Prediction of Protein Binding Sites

Based on the above considerations, different strategies have been developed for the specific prediction of protein–protein binding sites (which is technically quite different from the prediction of ligand-binding sites).⁵¹ Table 1 shows a compilation of available web servers for protein–protein interface prediction, but

there are more exhaustive reviews on the subject.^{51–54} From a practical point of view, we have divided the methods into those that make their predictions based on the sequence of the protein, and those that use the protein structure. Within each broad category, there is a variety of methodological approaches that we will review in detail.

Binding Site Prediction Based on the Protein Sequence

Very few methods for interface prediction are based solely on sequence information. Among them, correlated mutations, identified from multiple sequence alignments, have been used to detect putative protein–protein interfaces from sequences,⁷⁷ based on the hypothesis that residues involved in intermolecular contacts tend to mutate simultaneously during evolution. In a different approach, receptor-binding domains were predicted by analyzing hydrophobicity distribution on protein sequences.⁷⁸ The predictions had between 59 and 80% coverage (sensitivity), depending on the set of protein interactions used. This shows how dependent the predictive results are on the data set used and warns against the use of reported success rates for comparing methods. A method using support vector machines (SVMs) for interface prediction entirely based on the protein sequence showed similar sensitivity with rather low positive predictive value.⁷⁹

More recently, a machine learning-based method called Interaction Sites Identified from Sequence (ISIS) was developed to identify interacting residues from protein sequences only (Table 1).⁵⁵ They combined predicted structural features with evolutionary information with no reference to the 3D structure of the protein, and the strongest interface residue predictions reached a very high positive predictive value at the expense of a very low sensitivity. Interestingly, the fact that the method predicted only a very few residues, but with high accuracy, suggests that these residues might be truly important for the binding. We will see, in next section, how these predictions are related to the so-called hot-spot residues.

Binding Site Prediction Based on the Protein Structure

The advantage of sequence-based methods for interface prediction is that they can potentially be applied to a broader set of cases. However, their predictive results are usually lower than those obtained from methods based on structural information. Not surprisingly, there are many more reported prediction methods based on protein structure. We will classify all these methods according to the methodology used.

TABLE 1 | Available Web Servers for Prediction of Protein Binding Sites

Name of Method	Input Data	Methodology	Details	Web
ISIS ⁵⁵	Sequence	Neural network	Predicted structural features, evolutionary information	http://cubic.bioc.columbia.edu/services/isis/
TreeDet ⁵⁶	Sequence, structure	Scoring function	Sequence and structural alignments	http://treedetv2.bioinfo.cnio.es/treedet/index.html
Promate ⁵⁷	Structure	Scoring function	Secondary structure, sequence conservation, residue type	http://bioportal.weizmann.ac.il/promate
PINUP ⁵⁸	Structure	Scoring function	Side-chain energy score, propensity, sequence conservation	http://sparks.informatics.iupui.edu/PINUP/
InterProSurf ⁵⁹	Structure	Scoring function	Solvent accessibility, propensities	http://curie.utmb.edu/
PRISM ⁶⁰	Structure	Scoring function	Geometric complementarity, conservation	http://prism.ccb.ku.edu.tr/prism/
ConSurf ⁶¹	Structure	Scoring function	Conservation	http://consurf.tau.ac.il/
ET ⁶²	Structure	Scoring function	Multiple sequence alignments	http://mammoth.bcm.tmc.edu/traceview/
JET ⁶³	Structure	Scoring function	Structural and functional conservation	http://www.ihes.fr/~carbone/data.htm
WHISCY ⁶⁴	Structure	Scoring function	conservation, surface properties	http://www.nmr.chem.uu.nl/Software/whiscy/startpage.htm
PIER ⁶⁵	Structure	Scoring function	Atomic statistical propensities	http://abagyan.ucsd.edu/PIER/
SiteEngines ⁶⁶	Structure	Hierarchical scoring function	Structural matching, physicochemical properties	http://bioinfo3d.cs.tau.ac.il/SiteEngine/
PPI-Pred ⁶⁷	Structure	SVM	surface shape, electrostatic potential	http://bioinformatics.leeds.ac.uk/ppi-pred
cons-PPISP ^{68,69}	Structure	Neural network	PSI-Blast sequence profile and solvent accessibility	http://pipe.scs.fsu.edu/ppisp.html
SPPIDER ⁷⁰	Structure	Neural Network	Solvent accessibility and other features	http://sppider.cchmc.org/
Patch Finder Plus ⁷¹	Structure	Neural Network	Conservation, concavity, area, H-bond, residue frequency	http://pfp.technion.ac.il/
meta-PPISP ⁷²	Structure	Meta web server	Cons-PPISP, Promate and PINUP	http://pipe.scs.fsu.edu/meta-ppisp.html
PI ² PE ⁷³	Structure	Meta web server	Cons-PPISP, WESA, DISPLAY	http://pipe.scs.fsu.edu/
SHARP ⁷⁴	Structure	Energy based, scoring function	Desolvation, hydrophobicity, ASA, propensity, surface shape	http://www.bioinformatics.sussex.ac.uk/SHARP2/sharp2.html
ODA ⁷⁵	Structure	Energy based	Desolvation energy	http://www.molsoft.com/oda.html
NIP ⁷⁶	Structure	Energy based	Docking simulations	http://mmb.pcb.ub.es/PyDock

Empirical Scoring Function

Can protein–protein interfaces be predicted from the structures of their components based on any specific characteristic of protein–protein interfaces? We saw in previous sections that it has been difficult to extract common physicochemical or structural properties from protein–protein complexes in order to find simple patterns on protein surfaces that can identify protein binding sites. However, some specific features can be observed in certain types of interactions. Many groups have developed knowledge-based functions that can be used for binding site predictions, such as interface propensities derived from complex structures, or based on conservation scores, etc. In this line, an early method based on residue interface propensities and surface patch physicochemical properties (solvation potential, hydrophobicity, planarity, protrusion, and accessible surface area) was benchmarked on a set of 59 complexes, which included homodimers, heterocomplexes, and antibody–antigen complexes. They showed some correlation with the real interfaces.²⁹

Another method using interface residue propensity values derived from datasets of structures is InterProSurf (Table 1), based on a propensity scale and solvent accessibility of residues plus further clustering.⁵⁹ SiteEngines⁶⁶ (Table 1) used hierarchical scoring schemes to combine different descriptors, such as low-resolution surface representation of physicochemical properties and surface shape. The Protein intErface Recognition (PIER) method (Table 1) has been recently proposed for the identification of interface residues,⁶⁵ based on the statistical properties of each surface atom type and subsequent clustering in patches generated as in the optimal docking area (ODA) method.⁷⁵ Predictions were actually very similar to those of the ODA method (more details in next section). Another recent work suggested the possibility of using intramolecular pairwise contacts to improve the prediction of protein–protein interfaces with respect to using the individual interface propensities.⁸⁰ The research found some pairwise preferences, but when the values were corrected to remove the effect of the individual propensities, the significance was very marginal to be of practical use.

Sequence Conservation

Many methods include sequence conservation or evolutionary information in addition to other descriptors. For instance, a 3D cluster analysis of residue conservation scores based on the alignment of homologous sequences was shown to identify protein–protein interfaces and functional residues, as benchmarked on a set of 35 protein families.⁸¹ The Evolutionary Trace

(ET) method (Table 1) identified functional residues, potentially involved in protein–protein interactions, based on analysis of sequence alignments, mapping of conserved/unconserved residues in the 3D structure, and clustering.^{62,82} ConSurf (Table 1) is another interface prediction method based on sequence conservation.^{61,83} The procedure analyzed multiple sequence alignments in search of conserved functional regions that were then mapped on the 3D structure of the interacting proteins. A recent method, based also on sequence alignment data, further exploited the information of spatially conserved residues of similar structures.⁸⁴ In this line, the recently reported Joint Evolutionary Trees (JET) method (Table 1) based on the ET method focused on improving the sequence alignments and the functional and structural detection of conserved residues.⁶³ Similarly, the TreeDet server (Table 1) predicts functional sites based on conservation and evolutionary information,⁵⁶ and the related ‘specificity determining positions’ (SPDs) can be applied to identify protein–protein binding sites.⁸⁵ The input can be either a sequence or a structure, but it needs sufficient number of homologues to generate reliable multiple alignments.

The Promate server (Table 1) uses a combination of conservation, physical, and empirical parameters to predict protein–protein interfaces with a Naïve Bayesian approach.⁵⁷ The Crescendo method,⁸⁶ initially devised to detect functional sites based on multiple sequence alignments and environment-specific substitution tables (ESSTs), was adapted for protein interface prediction by spatial clustering of the predicted residues and further filter by Z-score and Accessible Surface Area (ASA).⁸⁷ The predictions showed high positive predictive value, and the predicted residues were used as distance restraints to rescore docking results within the pyDockRST method, with excellent results on the tested cases. Protein Interface residUe Prediction (PINUP) (Table 1) used a combination of parameters based on residue energy, interface propensity, and conservation scores optimized on a dataset of 57 proteins, in which the method yielded 44.5% positive predictive value and 42.2% sensitivity.⁵⁸ However, these numbers dropped to 29.4% positive predicted value (PPV) and 30.5% sensitivity on an independent test set of 68 proteins, which again shows very clearly how the accuracy numbers reported by any given method should be taken with caution, since they largely depend on the test set used, the conditions of the experiment, and the definition of a hit. PRotein-protein Interaction prediction by Structural Matching (PRISM) server (Table 1) is based on geometric complementarity and residue conservation. The server can also be applied to

the challenging task of identifying whether two given proteins interact or not.^{60,88} The WHat Information does Surface Conservation Yield? (WHISCY) server (Table 1) uses surface conservation and structural information to predict interface residues achieving more than three times higher accuracy than random predictions.⁶⁴ In Ref 64, the authors combined their predictions with those from ProMate server, with the goal of defining distance restraints to guide docking simulations.

Machine Learning Techniques

The method cons-Protein-Protein Interaction Site Prediction (PPISP) (Table 1) is based on a neural network that uses sequence profiles and solvent exposure of neighboring residues.^{68,69} The method was trained on 615 pairs of nonhomologous protein-protein complexes (homodimers and heterodimers), and was tested on different sets of bound and unbound proteins. In the case of unbound proteins, 70% of the predicted residues were correctly located at the protein-protein interfaces. Patch Finder Plus (Table 1) is a neural network method that combines residue conservation (arginine, positive, and aromatic residues), frequency and composition (number of lysine and polar residues), surface concavity, accessible area and H-bond potential, with the goal of finding large electrostatic patches. The method was developed to find DNA-binding regions, but in some cases these regions can also overlap to protein binding sites.⁷¹ Another neural network method based on evolutionary information and chemicophysical surface properties has been reported with a high predictive coverage.⁸⁹

The web server Protein-Protein Interface Prediction (PPI-PRED) (Table 1) uses SVM method to evaluate different parameters such as surface shape, solvent accessible surface area, conservation, electrostatic potential, hydrophobicity, and interface residue propensity. Their reported success rates using leave-one-out cross-validation are difficult to compare to other methods because of the nonstandard definition of a correct prediction (i.e., a patch over 50% positive predictive value and 20% sensitivity was ranked in the top three) and the used test set, composed of transient and obligate interfaces.⁶⁷ The method solvent accessibility based Protein-Protein Interface iDentification and Recognition (SPPIDER) (Table 1) uses machine learning approaches, such as SVM and Neural Networks to evaluate relative solvent accessibility predictions as a fingerprint for interaction sites together with a number of parameters (up to 19 features in the final predictor).⁷⁰ They showed that this method improved the predictions obtained with other parameters such

as evolutionary conservation, physicochemical character, and structural features. Another SVM method, based on evolutionary conservation signals and local surface properties and trained on a nonredundant dataset of 1494 protein-protein interfaces, showed 39% positive predictive value and 57% sensitivity at residue-level predictions in cross-validation tests on the bound conformations of a total of 632 dimers (from which 518 were homodimers).⁹⁰

Meta-Servers

A meta-server called meta-PPISP⁷² (Table 1) combined the neural network method cons-PPISP⁶⁸ with other different web servers such as ProMate⁵⁷ and PINUP.⁵⁸ The different scores were combined with weighting factors obtained by a linear regression method trained on 35 nonredundant proteins. The cross-validation predictive results improved over those of the individual servers (PPV increased by 4.8 to 18.2 percentage points). Another meta-server is PI²PE⁷³ (Table 1), which provides a pipeline to use cons-PPISP⁶⁸ and other servers from the same authors, DNA-Interaction Site Prediction from a List of Adjacent Residues (DISPLAR) and Weighted Ensemble Solvent Accessibility (WESA).⁹¹ The latter is, in turn, a meta-predictor that uses an ensemble of five methods for solvent accessibility predictions from protein sequences. However, no data is available on whether the predictive results improved the individual servers or not. In general, meta-servers can be a convenient way of accessing several different servers, but caution is advised when interpreting the results to evaluate the contribution of each individual method.

Energy-Based Methods

Other methods for predicting protein binding sites have been based on energy considerations. For instance, the Solvation potential, Hydrophobicity, Accessible surface area, Residue interface propensity, Planarity and Protrusion (SHARP²) server⁷⁴ (Table 1) combines solvation potential and hydrophobicity calculations with other geometric descriptors and propensity scores.

The ODA (Table 1) is based on the hypothesis that desolvation must play a central role during protein-protein binding. It is based on a computer algorithm that identifies continuous surface patches of optimal docking desolvation energy (Figure 2). The size of the patches is not fixed and it is calculated through an iterative procedure until finding the circular surface patch with the most favorable desolvation energy from each starting point (these starting points can be defined either from the expanded protein solvent-accessible surface, or from the center

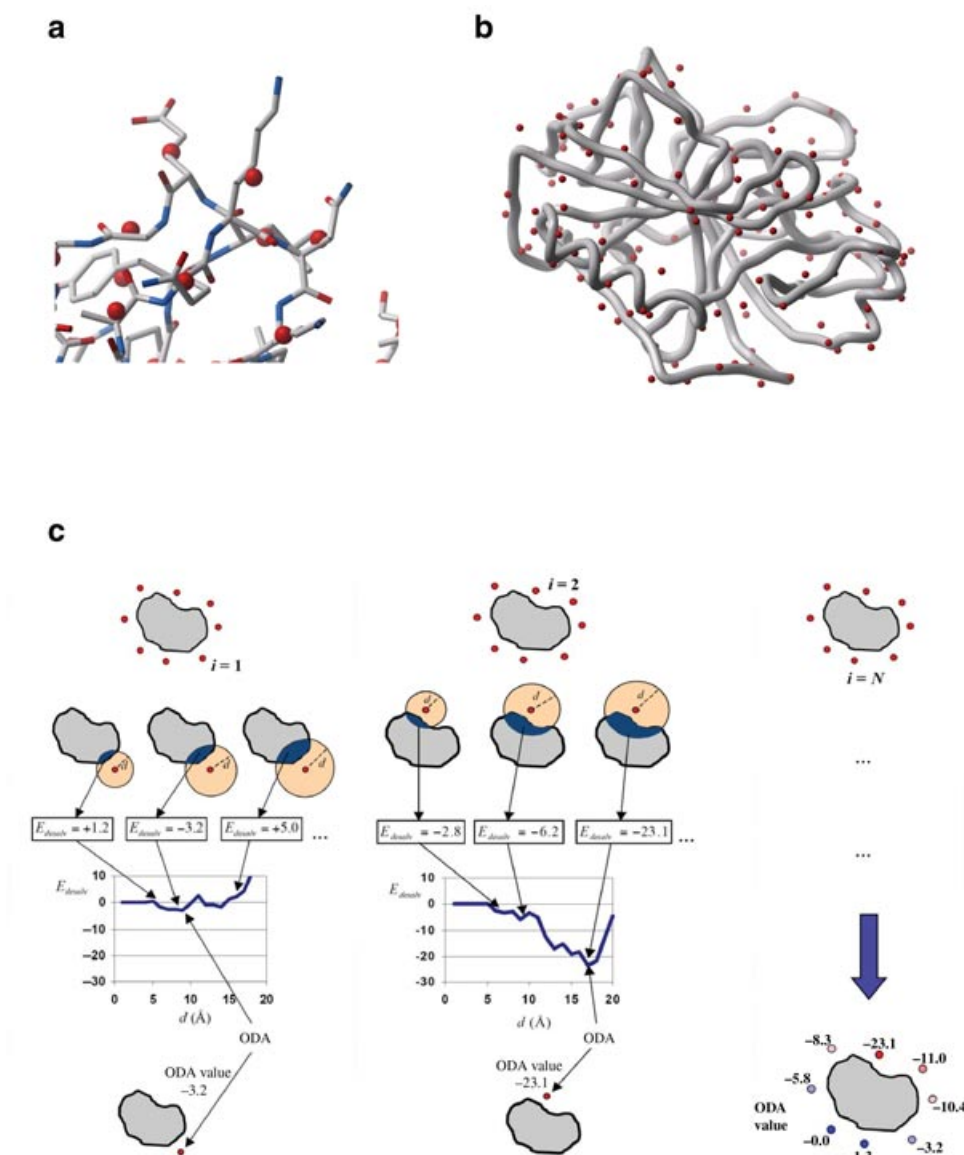


FIGURE 2 | Scheme of the optimal docking area (ODA) method for interface prediction. (a) Starting points are defined in the center of each residue side-chain; (b) representation of starting points around a protein; (c) for each point, surface patches of different size are defined and their desolvation energy calculated until finding the best value. Each starting point gets the optimal desolvation (ODA) value of the optimal patch generated from it.

of coordinates of each residue side-chain; Figure 2). This method was reportedly benchmarked on 66 unbound nonredundant protein structures involved in nonobligate protein–protein heterocomplexes, where the ODA-based predicted regions corresponded with real interfaces in 80% of the cases (Figure 3).⁷⁵ The limitation is that this method can only apply to cases where desolvation effect is important, and thus, in approximately half of the cases, in which electrostatics role is more evident, there is no predictive signal. The method has been applied to numerous cases of biological and therapeutic interest,

with excellent predictive results (Figure 4).^{92–95} Recently, this method has been implemented in the SEQMOL package (<http://biochemlabsolutions.com/FASTAandPDB.html>).

The above described desolvation energy descriptor has been also included as part of a method to predict protein interaction sites based on clefts in protein surfaces using Q-SiteFinder.⁹⁶ This desolvation descriptor was compared to several others and achieved excellent predictive results in all types of complexes, being the top predictor in antibody, antigen, and the ‘other’ type of complexes. Interestingly,

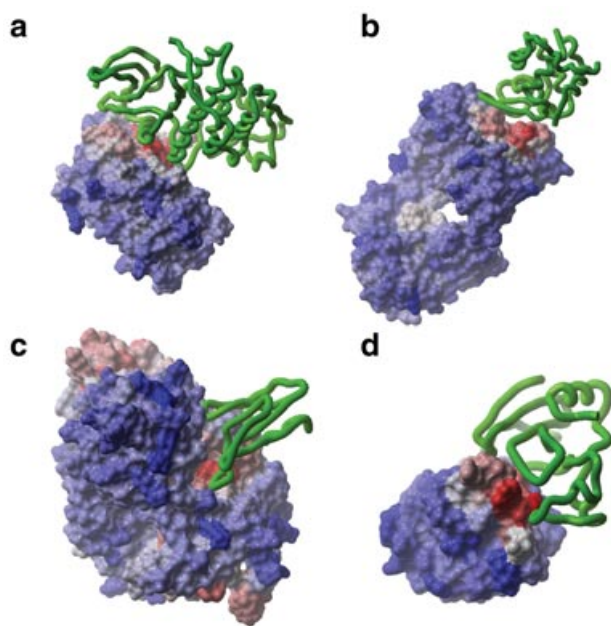


FIGURE 3 | Examples of optimal docking area binding site predictions on a variety of unbound proteins: (a) bovine cyclin A3 (PDB 1VIN); (b) antibody Fab D44.1 (PDB 1MLB); (c) acetylcholinesterase (PDB 1ACL); (d) barstar (PDB 1A19). The position of the partner molecule in the complex structure is shown in green ribbon, for comparison purposes. Figure prepared with ICM software (www.molsoft.com).

the cleft-based approach used in that work seemed even more general than the original ODA circular patches.

Energy-based docking simulations have also been used to identify protein interfaces. Although the challenging goal of docking is predicting the binding mode of two interacting proteins, it has been observed that the docking solutions sample more frequently the interface regions even when using a low-resolution protein representation.⁹⁷ This is consistent with the fact that conformational changes upon protein–protein association are often limited to local movements, which suggests that in many cases protein–protein association can be represented by rigid-body fit.^{34, 98–100} The inclusion of energy-based descriptors to sample and score rigid-body docking poses improved the docking energy landscapes and the tendency towards the real interfaces.⁷⁶ This led to the development of a residue-based normalized interface propensity (NIP) parameter (Table 1), computed from the ensemble of the 100 lowest-energy docking solutions, which was used to identify surface residues potentially involved in protein–protein interactions (Figure 5).⁷⁶ A NIP cutoff of 0.4 was reported to predict known protein–protein interfaces on unbound proteins with PPV of over 80%, although

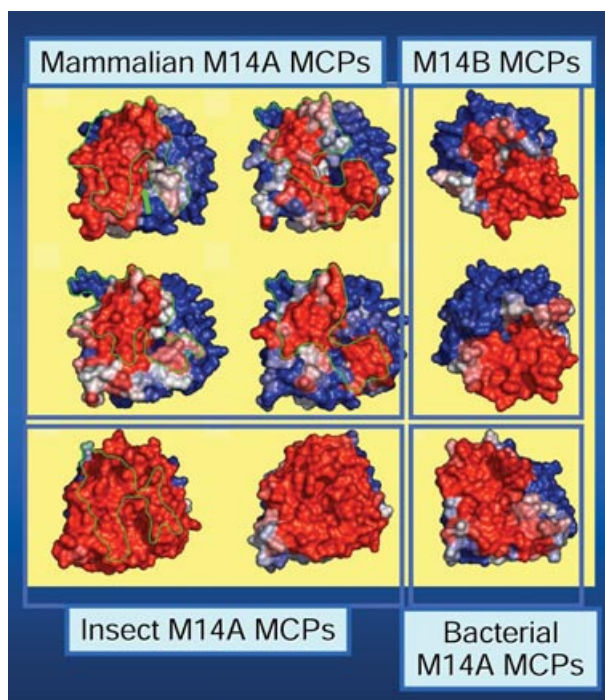


FIGURE 4 | An example of the application of the optimal docking area prediction method to a case of biological interest. Interface predictions (in red) on different carboxypeptidases show that their surfaces contribute differently (in shape and intensity) to the interaction with the corresponding partners (inhibitors and/or N-terminal pro domains), according to each sub-family. The interest here is to show that interface predictions can help in protein classification. In green is shown the contour of the real interface (if known) for comparison purposes. Positive predictive values of the predictions are 60, 80, 87, 90, and 90%, from left to right and top to bottom, respectively.⁹⁴

with quite low sensitivity.⁷⁶ The method was able to identify only a few residues of the interface, but its high accuracy suggested that these residues might be the important ones for the interaction. We will see in next sections how these residues corresponded to actual hot-spot residues.

Dissection of Hot-Spot Residues at Protein Interfaces

Protein–protein interfaces are formed by a high variety of residues, many of which are important for specificity or for dynamic considerations. But it has been reported that most of the binding free energy is usually contributed by a few number of residues, also called ‘hot-spots’. Pioneer computational studies on N9 neuraminidase complexes estimated the contribution of different interface residues to complex formation, and certain residues were experimentally confirmed to cause a marked reduction in the

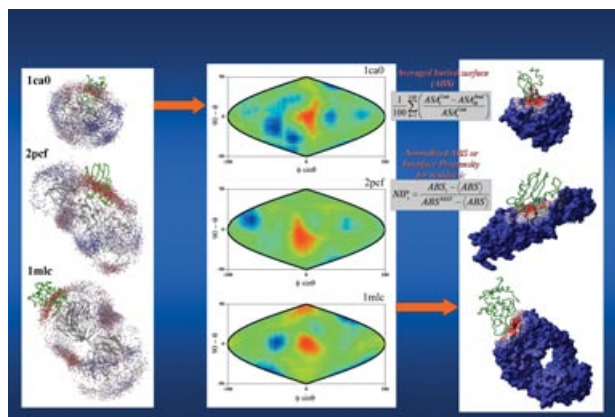


FIGURE 5 | Scheme of the normalized interface propensity method for prediction of interface and hot-spot residues. The rigid-body docking poses are sorted by binding energy and mapped onto the surface of the interacting proteins, by computing the average relative buried surface per residue. The residues more often involved in the docking interfaces are shown in red and indicate hot-spot residues.

experimental binding energy when mutated.^{101–103} The term ‘hot-spot’ was later used to describe the key residues in the complex formed by the human growth hormone and its receptor.¹⁰⁴ In Ref 104, the authors mutated all interface residues to alanine in order to measure the contribution of each specific side-chain to the binding free energy. They identified two tryptophan residues that were responsible for the majority of the total binding energy ($\Delta\Delta G$ upon mutation > 4.5 kcal.mol⁻¹). Since then, hot-spot residues have been thoroughly studied, and they are usually defined as the residues contributing in more than 1–2 kcal/mol to the free energy of binding. The hot-spot residues are highly conserved and typically surrounded by other moderately conserved residues, forming highly cooperative interactions.¹⁰⁵ Structurally, they are generally located around the center of the interface and they are protected from bulk solvent by energetically less important residues forming a hydrophobic O-ring. Tryptophan, arginine, and tyrosine are the ones most frequently found as hot-spot residues, whereas leucine, serine, threonine, and valine are the least frequent.^{106, 107} Hot spots have been recently shown to correlate with relevant nodes of residue networks in protein interfaces.¹⁰⁸ Interestingly, in hub proteins at protein–protein networks, different hot regions can be used to bind to different partners.¹⁰⁹ Regarding flexibility, MD simulations have shown that hot spots are quite rigid as compared to the surrounding interface residues.^{38, 39}

These hot-spot residues are generating a great interest because they are expected to be suitable targets for the challenging task of disrupting protein–

protein interactions with small molecules.^{110, 111} But identification of hot spots for a given interaction is not straightforward. Experimental measurement of the energetic contribution of each residue can be done by alanine scanning mutagenesis combined with a variety of biophysical methods.^{111–114} There are databases of experimentally calculated binding energies of hot-spot residues, such as ASEdb (<http://nic.ucsf.edu/asedb/>),¹⁰⁶ Binding Interface Database (BID) (<http://tsailab.tamu.edu/BID/>),¹¹⁵ or HotSprint (<http://prism.cccb.ku.edu.tr/hotsprint/>).¹¹⁶ However, the experimental characterization of protein interfaces in search of hot spots is still costly and technically cumbersome, so several computational methods have been developed for the prediction of hot spots in protein–protein interactions.

Prediction of Hot Spots

Different scoring schemes for computational hot-spot prediction have been reported, based on residue conservation, hydrogen bonding, or complete energy binding (Table 2). Other approaches have tried a combination of all these features with machine learning techniques. Although a few methods can predict hot spots based only on protein sequences, most of the available methods need some structural information as input.

Hot-Spot Prediction Based on the Protein Sequence

Very few methods have been reported to make hot-spot predictions based only on the protein sequences. A neural network method called ISIS (Table 2), initially designed to predict interface residues from protein sequences,⁵⁵ was also applied to predict hot spots on a dataset of 296 mutations from 30 different complexes.¹²⁴ The remarkable reported high predictive rates obtained only from the protein sequence can be explained because of the restricted definition of positive and negative predictions they used in their benchmark. They considered hot spots as those residues with $\Delta\Delta G$ upon mutation > 2.5 kcal/mol, whereas non-hot-spot residues were only those with $\Delta\Delta G = 0$ kcal/mol. Therefore, they were leaving out of the test all the mutants with $\Delta\Delta G < 0$ (even though these residues should be considered as non-hot spots) or $0 < \Delta\Delta G \leq 2.5$, which can be in fact of high interest in a realistic situation and perhaps the most difficult residues to be classified as hot spots. Most of the other methods establish a single cutoff to classify the predictions as hot spot or non-hot spot, and thus the success rates reported by the ISIS method cannot be compared to them.

TABLE 2 | Available Servers for Prediction of Hot-spots

Name of Method	Input Data	Methodology	Details	Sensitivity	PPV	Availability
ISIS ⁵⁵	Sequence	Neural network	Predicted structural features, evolutionary information	15%	89%	http://cubic.bioc.columbia.edu/services/isis/
FOLDEF ¹¹⁷	Complex structure	Energy based	Alanine scanning	45–72% ¹	61–73% ¹	http://foldx.crg.es/
ROBETTA ¹¹⁸	Complex structure	Energy based	Alanine scanning	28–69% ²	60–71% ²	http://robeta.org/submit.jsp
K-FADE ¹¹⁹ /K-CON/ROBETTA	Complex structure	Machine learning algorithm	Physical–biochemical features	48%	53%	http://kfc.mitchell-lab.org
MAPPIS ¹²⁰	Complex structure	Evolutionary conservation	Multiple alignments, 3D clustering	66%	63%	http://bioinfo3d.cs.tau.ac.il/MAPPIS
HotPoint ¹²¹	Complex structure	Empirical model	Accessibility, knowledge-based potentials	59%	70%	http://prism.ccbb.ku.edu.tr/hotpoint
pyDockNIP ¹²²	Unbound protein structure	Energy based	Docking simulations	42–43%	68–75%	http://mmb.pcb.ub.es/PyDock

¹ Calculated based on data from several studies.^{117, 122, 124}² Calculated based on data from several studies.^{118, 119, 120, 124}

Hot-Spot Prediction Based on the Complex Structure

Most of the available hot-spot prediction methods are based on the 3D structure of the complex. We can roughly classify such methods into those that are based on an empirical function, very often using conservation data, and those based on energy considerations.

Empirical Function

Different studies have tried to find characteristic structural features in the known hot spots. The current view is that protein–protein interfaces are composed of a variety of residues involved in the specificity of the interaction, with a group of quite conserved hot-spot residues acting as binding site anchors that are required to stabilize the complex. One basic observation is that the number of hot spots increases with the size of the interface.¹⁰⁵ Structurally, hot spots are surrounded by moderately conserved and energetically less important residues forming a hydrophobic O-ring responsible for bulk solvent exclusion.^{106, 110} They appear to be clustered in tightly packed regions in the center of the interface.¹⁰⁵ However, it has not been found any single attribute as shape, charge, or hydrophobicity that can unequivocally define a hot spot by itself.^{34, 47, 111, 124}

Given the energetic importance of hot-spot residues, it is expected that they will be conserved at the interfaces along the members of a given family.^{107, 125} This can be used to computationally identify hot spots, as in the recently reported method Multiple Alignment of Protein-Protein InterfaceS (MAPPIS) (Table 2), which aims to predict hot spots by detecting spatially conserved patterns applying multiple alignment of physicochemical interactions and binding properties in the 3D space.¹²⁰ MAPPIS success rates on predicting hot spots have been analyzed on a dataset of 440 mutants from 10 different complexes,¹²⁰ yielding quite good predictive rates (for further information, PPV was recalculated in a recent study).¹²⁶ However, this method needs a sufficient number of high-resolution complex structures of functionally similar proteins in order to build reliable structural alignments. A recently reported web server, HotPoint,¹²¹ predicts hot spots using an empirical model, based on relative accessibility in the complex state and knowledge-based pair potentials.

Energy Based

Several methods for hot-spot prediction are based on the computational alanine scanning of a protein–protein complex. This approach consists in

computing the variation of binding affinity ($\Delta\Delta G$) upon *in silico* modification of a given residue to alanine. One of such methods uses the energy-based scoring function in ROBETTA (Table 2) to predict hot spots, with reported high success (PPV was recalculated in a recent study)¹²⁶ on a data set of 380 mutants from 19 different complexes.^{118,127} However, this method gave slightly worse success rates on different data sets,^{119,123} which again shows the difficulties of comparing methods when using different test sets. Another method, FOLD-X Energy Function (FOLDEF) (Table 2) with the FOLD-X energy function, has also been used to provide a fast and quantitative evaluation of the interactions involved in a protein–protein complex.¹¹⁷ On a set of 40 single mutations to alanine from three complexes, this approach yielded 61% PPV and 72% sensitivity. However, on larger benchmark sets^{122,123} the success rates were different (slightly higher PPV but significantly lower sensitivity).

Using a different energy-based approach, two different machine learning algorithms called K-FADE and K-CON (Table 2) have been recently reported to predict hot-spot residues based on the use of physical/biochemical features.¹¹⁹ A combination of both models gave better results than the individual ones, and even better when they were integrated with ROBETTA. More sophisticated energy analyses have been reported, but they have not been benchmarked on large datasets of cases. For instance, a fully atomistic method based on MD with generalized Born model in a continuum medium (MM-PBSA) obtained high PPV for the prediction of hot-spot residues (defined by $\Delta\Delta G > 2$ kcal/mol) but only on a reduced set composed of three complexes.¹²⁸ Another interesting study estimated the effect of point mutations in the binding free energies of protease–inhibitor interactions using MD simulations combined with the linear interaction energy (LIE) approach.¹²⁹ This study underlined the importance of considering the preorganization of the binding surface when modeling point mutations.

Hot-Spot Prediction Based on the Unbound Protein Structure

All of the above described methods can give reliable predictions of hot spots on a given protein–protein complex. However, a major limitation is that they need the 3D structure of the complex or that of a closely homologue. Unfortunately, in most of the protein–protein interactions the 3D structure of the complex is not yet available, and thus the above

described methods are of limited applicability. Very few hot-spot prediction methods have been reported based only on the structure of the unbound proteins.

An interface prediction method based on computational protein–protein docking simulations, and therefore suitable for cases in which the structure of the complex is not available, has been recently applied to hot-spot prediction.¹²² The pyDockNIP method (Table 2) was a variation of the original algorithm for interface prediction.⁷⁶ In the original NIP calculations, a longer docking approach was used, based on ICM pseudo-Brownian rigid-body docking search with a complete energy function, including van der Waals, hydrogen bonding, electrostatics and desolvation. This rather sophisticated docking and scoring scheme was replaced by a simpler one, which achieved similar docking results in previous tests, based on faster FFT-based docking search (FTDock¹⁴ and ZDOCK¹⁵) and a simple energy function implemented in pyDock.²² These new NIP from the FFT-based rigid-body docking and pyDock scoring predicted known hot-spot residues on a benchmark of 586 mutations from 21 complexes with 68% PPV and 43% sensitivity (Figure 6). The method was also applied to homology-based models of the interacting proteins, with similar predictive rates.¹²² This method was the first reported systematic application of protein–protein docking calculations to the identification of hot-spot residues. This kind of approach can be especially helpful in drug design projects targeting protein–protein interactions in cases with no structural information about the complex. The NIP method is a description of the distribution of docking poses around certain residues, based on the percentage of low-energy docking poses in which a given surface residue is involved in the docking interface. Thus, another interesting aspect of the NIP values is that they can be also seen as the residue binding free energies estimated from the Boltzmann population of the two states in which a given residue can be found after the docking simulations: either exposed or involved in the docking interface. A similar approach, but based on the distribution of MD conformations of organic solvents around the protein, has also shown hot-spot detection on selected test cases.¹³⁰

Mechanistic Considerations and Practical Applications

Mechanistic Considerations

It is interesting to find that two independent and quite different approaches for hot-spot predictions, ISIS¹²⁴

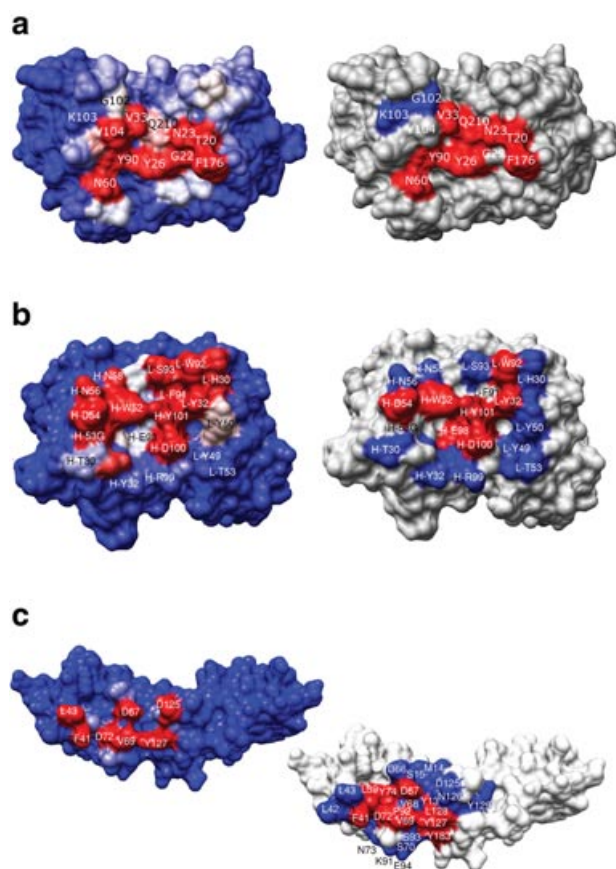


FIGURE 6 | Examples of hot-spot predictions by pyDockNIP method (in red predicted residues, left panel). (a) SEC3 super antigen (hot spots for interaction with T-Cell β -chain; complex PDB 1JCK); (b) D1.3 IgG1 (hot spots for interaction with lysozyme; complex PDB 1VFB); (c) IL-4 receptor α -chain (hot-spots for interaction with IL-4; complex PDB 1IAR). In the right panel, the experimentally known hot spots (in red) and the non-hot spots (in blue) are shown. Residues in white have not been tested experimentally.

and pyDockNIP,¹²² were based on methods previously devised for interface prediction. Both showed high positive predictive value and low sensitivity in their interface predictions, which is compatible with the hypothesis that they were identifying only the few residues that were truly important for the interaction (i.e., the hot spots). The rationale is that any descriptor that may be useful for predicting the tendency of a residue to be at interface might be also useful to detect hot-spot residues, since these should have increased signal for that descriptor. On the other side, these methods based on interface prediction descriptors will necessarily miss those hot-spot residues that form specific interactions, since these are not included in the predictor. This logical chain of thoughts may lead to the conclusion that hot-spot prediction meth-

ods that are based on the structure of the complex will be more accurate than those based on sequence alone or on the unbound structures. However, the comparison of their predictive success rates (Table 2) does not support this conclusion. In a seemingly contradiction, methods based on the unbound structures show similar success rates to those based on the complex structure. This suggests that calculations on a single complex configuration are not sufficiently accurate for a correct description of specific interactions, mostly because dynamic and plasticity of protein interfaces should play a relevant role. Indeed, a detailed study about the effect of point mutations on protein–protein interaction energies achieved good correlation between computational and experimental energies only after inclusion of MD for the consideration of the preorganization energy.¹²⁹

Comparison of Predictive Methods

One of the difficulties when comparing published methods is that every laboratory may use different evaluation measures and statistical parameters to assess the success rates of their predictions. Perhaps the most useful measure for the experimentalists that want to test the predictions is the statistical parameter called positive predictive value (Eq. 1), which indicates the reliability of the predictions and the expected outcome of mutation experiments. However, one could also be interested in finding as many interface residues or hot spots as possible, in which case the best measure to assess the outcome of the predictions would be the statistical parameter called sensitivity (Eq. 2). For the sake of clarity, here are the definitions used in the statistical field for these commonly used measures to assess the predictive success rates:

$$\text{Positive predictive value} = \frac{TP}{TP + FP} 100, \quad (1)$$

$$\text{Sensitivity} = \frac{TP}{TP + FN} 100, \quad (2)$$

where TP is the number of true positives (i.e., predicted residues that are correctly located at interfaces or hot spots), FP is the number of false positives (i.e., predicted residues that are not interface or hot-spot residues), and FN the number of false negatives (i.e., interface or hot-spot residues that have not been predicted). However, the absence of a common accepted standard for these definitions makes the comparison problem even more difficult. As a note of caution, positive predictive values can also be called precision in some studies (or even accuracy or specificity, whose statistical definition is different from that in other

fields). Similarly, sensitivity is also called coverage or recall in different studies.

An additional problem for comparing methods is the different test sets used for benchmarking. An ideal comparative study of different methods would require performing calculations in the same conditions and on the same data set. Unfortunately, there are not many benchmark studies of this type. A recent critical assessment of different methods for prediction of protein binding sites has been published.^{53,131} Regarding hot spots, there is a recent comparative analysis of predictive methods, which included the recalculation of their success rates considering the same data sets whenever was possible.¹²⁶ In any case, the field would largely benefit from a world-wide community assessment in the spirit of Critical Assessment of protein Structure Prediction (CASP)¹³² or Critical Assessment of PRedicted Interactions (CAPRI).^{133,134} Actually, the previously reported use of docking results to predict binding interfaces,⁷⁶ has also suggested the possibility of further assessing the docking submissions in CAPRI for interface prediction.¹³⁵ The necessity of objective benchmarking and comparative assessment is even more evident in the hot-spot prediction field, given the limited availability of experimental data, which hampers correct training of the developed methods. In addition, these methods are currently tested only on the very few residues that have been experimentally described. Thus, the majority of predictions for a given benchmark case remain untested and it is impossible to know the true PPV and sensitivity values for all predictions. In comparison, in the case of interface prediction there are usually available data for all the residues in a given benchmark complex, which makes it possible to test all the predictions. It is true that there could be interface residues yet to know (e.g., multiple interfaces),⁵³ but this would only make actual PPV values higher than the obtained ones.

Use of Predicted Hot Spots for Drug Discovery

The prediction of hot-spot residues for protein–protein binding opens interesting applications in drug discovery. The design of small molecules capable of inhibiting or modulating protein–protein interfaces is a long-awaited goal of the pharmaceutical field. However, the main difficulty for using protein–protein interfaces with therapeutic purposes is that they are quite large as compared to small-molecule binding sites, and in addition, they lack clear cavities that might serve as targets for drug design.^{30,47} Of course, one can always try to apply the same techniques that

are commonly used to determine the ‘druggability’ of a standard surface, by analyzing the whole surface in search of possible binding pockets or cavities on the interacting surfaces so that classical rational drug design can be applied.¹³⁶ Indeed, the protein–protein interfaces that have already been targeted with drugs typically contain a sufficiently deep surface pocket suitable for small molecule binding.¹³⁷ Thus, experimental and computational prediction of binding pockets on the surface of proteins has been successfully used in rational drug design.^{138–143} However, the flatness, large size, and lack of clear pockets of most protein–protein interfaces makes it much more efficient to focus the druggability analysis on the existence of possible hot spots, where the computer methods can be helpful. Indeed, a specific interaction may be disrupted with small molecules by targeting one or several of the hot-spot residues found in protein–protein interfaces,^{106,111} and actually some of these small molecules targeting hot spots to disrupt protein–protein interactions are currently in clinical trials.¹¹²

CONCLUSION

Protein–protein interfaces are usually large and formed by a variety of residues, which is needed in order to achieve high affinity and specificity in protein–protein recognition. The physicochemical features of protein interfaces strongly depend on the type of association, ranging from obligate complexes in which the separated components are not stable, to transient interactions in which the lifetime of the complex is extraordinarily small. Based on observed structural and physicochemical patterns, conservation, and energy considerations, a number of computer methods have been reported for the prediction of protein binding sites, which we have reviewed here. Most of the methods show quite good predictive success rates, so interface prediction is becoming a common tool to help characterizing a given protein–protein interaction. However, important challenges remain, such as the impossibility of identifying the relevant interface for each partner in cases of shared binding sites or multiple interfaces, or the lack of truly negative data in benchmark tests (some authors discuss about whether there is really any residue that is not involved in any interaction). Future efforts should focus on including flexibility and allosteric considerations in the predictions, as well as to improve affinity and specificity predictions when dealing with multiple interfaces.

In spite of the variety of protein interfaces, it has been observed that most of the binding affinity usually arises from only a few residues, so called hot spots, which are important from a functional and practical point of view and can be used as starting points for drug discovery targeting protein–protein interactions. We report here a survey of methods that have been developed to predict such hot spots. The majority of them need the structure of the protein–protein complex, although a few of the methods are able to identify hot spots on the structures of the

unbound proteins or homology-based models, which opens the door to large-scale identification of hot-spot residues in protein interaction networks. However, current limited availability of experimental data is hampering further advancement in method development. One of the goals of the binding site and hot-spot prediction methods is to help in the therapeutic drug discovery programs. The field is highly promising, and several small molecules have already been reported to disrupt protein interactions of therapeutic interest.

REFERENCES

1. Janin J, Rodier F. Protein–protein interaction at crystal contacts. *Proteins* 1995, 23:580–587.
2. Janin J. Specific versus non-specific contacts in protein crystals. *Nat Struct Biol* 1997, 4:973–974.
3. Bahadur RP, Chakrabarti P, Rodier F, Janin J. A dissection of specific and non-specific protein–protein interfaces. *J Mol Biol* 2004, 336:943–955.
4. Elcock AH, McCammon JA. Identification of protein oligomerization states by analysis of interface conservation. *Proc Natl Acad Sci USA* 2001, 98:2990–2994.
5. Valdar WS, Thornton JM. Conservation helps to identify biologically relevant crystal contacts. *J Mol Biol* 2001, 313:399–416.
6. Zhu H, Domingues FS, Sommer I, Lengauer T. NOX-class: prediction of protein–protein interaction types. *BMC Bioinformatics* 2006, 7:27.
7. Henrick K, Thornton JM. PQS: a protein quaternary structure file server. *Trends Biochem Sci* 1998, 23:358–361.
8. Xu Q, Canutescu A, Obradovic Z, Dunbrack RL Jr. ProtBuD: a database of biological unit structures of protein families and superfamilies. *Bioinf* 2006, 22:2876–2882.
9. Huang B, Schroeder M. Using protein binding site prediction to improve protein docking. *Gene* 2008, 422:14–21.
10. Smith GR, Sternberg MJ. Prediction of protein–protein interactions by docking methods. *Curr Opin Struct Biol* 2002, 12:28–35.
11. Bonvin AM. Flexible protein–protein docking. *Curr Opin Struct Biol* 2006, 16:194–200.
12. Gray JJ. High-resolution protein–protein docking. *Curr Opin Struct Biol* 2006, 16:183–193.
13. Ritchie DW. Recent progress and future directions in protein–protein docking. *Curr Protein Pept Sci* 2008, 9:1–15.
14. Gabb HA, Jackson RM, Sternberg MJ. Modelling protein docking using shape complementarity, electrostatics and biochemical information. *J Mol Biol* 1997, 272:106–120.
15. Chen R, Weng Z. A novel shape complementarity scoring function for protein–protein docking. *Proteins* 2003, 51:397–408.
16. Katchalski-Katzir E, Shariv I, Eisenstein M, Friesem AA, Aflalo C, Vakser IA. Molecular surface recognition: determination of geometric fit between proteins and their ligands by correlation techniques. *Proc Natl Acad Sci USA* 1992, 89:2195–2199.
17. Ritchie DW, Kemp GJ. Protein docking using spherical polar Fourier correlations. *Proteins* 2000, 39:178–194.
18. Schneidman-Duhovny D, Inbar Y, Nussinov R, Wolfson HJ. PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic Acids Res* 2005, 33:W363–W367.
19. Fernandez-Recio J, Totrov M, Abagyan R. Soft protein–protein docking in internal coordinates. *Protein Sci* 2002, 11:280–291.
20. Dominguez C, Boelens R, Bonvin AM. HADDOCK: a protein–protein docking approach based on biochemical or biophysical information. *J Am Chem Soc* 2003, 125:1731–1737.
21. Gray JJ, Moughon S, Wang C, Schueler-Furman O, Kuhlman B, Rohl CA, Baker D. Protein–protein docking with simultaneous optimization of rigid-body displacement and side-chain conformations. *J Mol Biol* 2003, 331:281–299.
22. Cheng TM, Blundell TL, Fernandez-Recio J. pyDock: Electrostatics and desolvation for effective scoring of rigid-body protein–protein docking. *Proteins* 2007, 68:503–515.
23. Camacho CJ, Gatchell DW. Successful discrimination of protein interactions. *Proteins* 2003, 52:92–97.

24. Camacho CJ. Modeling side-chains using molecular dynamics improve recognition of binding region in CAPRI targets. *Proteins* 2005, 60:245–251.
25. Young L, Jernigan RL, Covell DG. A role for surface hydrophobicity in protein–protein recognition. *Protein Sci* 1994, 3:717–729.
26. Argos P. An investigation of protein subunit and domain interfaces. *Protein Eng* 1988, 2:101–113.
27. Janin J, Miller S, Chothia C. Surface, subunit interfaces and interior of oligomeric proteins. *J Mol Biol* 1988, 204:155–164.
28. Jones S, Thornton JM. Protein–protein interactions: a review of protein dimer structures. *Prog Biophys Mol Biol* 1995, 63:31–65.
29. Jones S, Thornton JM. Prediction of protein–protein interaction sites using patch analysis. *J Mol Biol* 1997, 272:133–143.
30. Jones S, Thornton JM. Analysis of protein–protein interaction sites using surface patches. *J Mol Biol* 1997, 272:121–132.
31. Glaser F, Steinberg DM, Vakser IA, Ben-Tal N. Residue frequencies and pairing preferences at protein–protein interfaces. *Proteins* 2001, 43:89–102.
32. Chothia C, Janin J. Principles of protein–protein recognition. *Nature* 1975, 256:705–708.
33. Janin J, Chothia C. The structure of protein–protein recognition sites. *J Biol Chem* 1990, 265:16027–16030.
34. Conte LL, Chothia C, Janin J. The atomic structure of protein–protein recognition sites. *J Mol Biol* 1999, 285:2177–2198.
35. Sheinerman FB, Honig B. On the role of electrostatic interactions in the design of protein–protein interfaces. *J Mol Biol* 2002, 318:161–177.
36. Levy ED. A simple definition of structural regions in proteins and its use in analyzing interface evolution. *J Mol Biol* 2010, 403:660–670.
37. Chakrabarti P, Janin J. Dissecting protein–protein recognition sites. *Proteins* 2002, 47:334–343.
38. Rajamani D, Thiel S, Vajda S, Camacho CJ. Anchor residues in protein–protein interactions. *Proc Natl Acad Sci USA* 2004, 101:11287–11292.
39. Yogurtcu ON, Erdemli SB, Nussinov R, Turkay M, Keskin O. Restricted mobility of conserved residues in protein–protein interfaces in molecular simulations. *Biophys J* 2008, 94:3475–3485.
40. Zen A, Micheletti C, Keskin O, Nussinov R. Comparing interfacial dynamics in protein–protein complexes: an elastic network approach. *BMC Struct Biol* 2010, 10:26.
41. del Sol A, Tsai CJ, Ma B, Nussinov R. The origin of allosteric functional modulation: multiple pre-existing pathways. *Structure* 2009, 17:1042–1050.
42. Perkins JR, Diboun I, Dessailly BH, Lees JG, Orengo C. Transient protein–protein interactions: structural, functional, and network properties. *Structure* 2010, 18:1233–1243.
43. Stein A, Pache RA, Bernado P, Pons M, Aloy P. Dynamic interactions of proteins in complex networks: a more structured view. *Febs J* 2009, 276:5390–5405.
44. Turner B, Razick S, Turinsky AL, Vlasblom J, Crowdy EK, Cho E, Morrison K, Donaldson IM, Wodak SJ. iRefWeb: interactive analysis of consolidated protein interaction data and their supporting evidence. *Database (Oxford)* 2010, 2010:baq023.
45. Ozbabacan SE, Gursoy A, Keskin O, Nussinov R. Conformational ensembles, signal transduction and residue hot spots: application to drug discovery. *Curr Opin Drug Discov Devel* 2010, 13:527–537.
46. Tuncbag N, Kar G, Gursoy A, Keskin O, Nussinov R. Towards inferring time dimensionality in protein–protein interaction networks by integrating structures: the p53 example. *Mol Biosyst* 2009, 5:1770–1778.
47. Jones S, Thornton JM. Principles of protein–protein interactions. *Proc Natl Acad Sci USA* 1996, 93:13–20.
48. Ofra Y, Rost B. Analysing six types of protein–protein interfaces. *J Mol Biol* 2003, 325:377–387.
49. Nooren IM, Thornton JM. Diversity of protein–protein interactions. *Embo J* 2003, 22:3486–3492.
50. Kim WK, Henschel A, Winter C, Schroeder M. The many faces of protein–protein interactions: a compendium of interface geometry. *PLoS Comput Biol* 2006, 2:e124.
51. Leis S, Schneider S, Zacharias M. In silico prediction of binding sites on proteins. *Curr Med Chem* 17:1550–1562.
52. de Vries SJ, Bonvin AM. How proteins get in touch: interface prediction in the study of biomolecular complexes. *Curr Protein Pept Sci* 2008, 9:394–406.
53. Ezkurdia I, Bartoli L, Fariselli P, Casadio R, Valencia A, Tress ML. Progress and challenges in predicting protein–protein interaction sites. *Brief Bioinform* 2009, 10:233–246.
54. Tuncbag N, Kar G, Keskin O, Gursoy A, Nussinov R. A survey of available tools and web servers for analysis of protein–protein interactions and interfaces. *Brief Bioinform* 2009, 10:217–232.
55. Ofra Y, Rost B. ISIS: interaction sites identified from sequence. *Bioinf* 2007, 23:e13–e16.
56. Carro A, Tress M, de Juan D, Pazos F, Lopez-Romero P, del Sol A, Valencia A, Rojas AM. TreeDet: a web server to explore sequence space. *Nucleic Acids Res* 2006, 34:W110–W115.
57. Neuvirth H, Raz R, Schreiber G. ProMate: a structure based prediction program to identify the location of protein–protein binding sites. *J Mol Biol* 2004, 338:181–199.

58. Liang S, Zhang C, Liu S, Zhou Y. Protein binding site prediction using an empirical scoring function. *Nucleic Acids Res* 2006, 34:3698–3707.
59. Negi SS, Schein CH, Oezguen N, Power TD, Braun W. InterProSurf: a web server for predicting interacting sites on protein surfaces. *Bioinf* 2007, 23:3397–3399.
60. Ogmen U, Keskin O, Aytuna AS, Nussinov R, Gursoy A. PRISM: protein interactions by structural matching. *Nucleic Acids Res* 2005, 33:W331–W336.
61. Glaser F, Pupko T, Paz I, Bell RE, Bechor-Shental D, Martz E, Ben-Tal N. ConSurf: identification of functional regions in proteins by surface-mapping of phylogenetic information. *Bioinf* 2003, 19:163–164.
62. Morgan DH, Kristensen DM, Mittelman D, Lichtarge O. ET viewer: an application for predicting and visualizing functional sites in protein structures. *Bioinf* 2006, 22:2049–2050.
63. Engelen S, Trojan LA, Sacquin-Mora S, Lavery R, Carbone A. Joint evolutionary trees: a large-scale method to predict protein interfaces based on sequence sampling. *PLoS Comput Biol* 2009, 5:e1000267.
64. de Vries SJ, van Dijk AD, Bonvin AM. WHISCY: what information does surface conservation yield? Application to data-driven docking. *Proteins* 2006, 63:479–489.
65. Kufareva I, Budagyan L, Raush E, Totrov M, Abagyan R. PIER: protein interface recognition for structural proteomics. *Proteins* 2007, 67:400–417.
66. Shulman-Peleg A, Nussinov R, Wolfson HJ. Recognition of functional sites in protein structures. *J Mol Biol* 2004, 339:607–633.
67. Bradford JR, Westhead DR. Improved prediction of protein–protein binding sites using a support vector machines approach. *Bioinf* 2005, 21:1487–1494.
68. Zhou HX, Shan Y. Prediction of protein interaction sites from sequence profile and residue neighbor list. *Proteins* 2001, 44:336–343.
69. Chen H, Zhou HX. Prediction of interface residues in protein–protein complexes by a consensus neural network method: test against NMR data. *Proteins* 2005, 61:21–35.
70. Porollo A, Meller J. Prediction-based fingerprints of protein–protein interactions. *Proteins* 2007, 66:630–645.
71. Stawiski EW, Gregoret LM, Mandel-Gutfreund Y. Annotating nucleic acid-binding function based on protein structure. *J Mol Biol* 2003, 326:1065–1079.
72. Qin S, Zhou HX. meta-PPISP: a meta web server for protein–protein interaction site prediction. *Bioinf* 2007, 23:3386–3387.
73. Tjong H, Qin S, Zhou HX. PI2PE: protein interface/interior prediction engine. *Nucleic Acids Res* 2007, 35:W357–W362.
74. Murakami Y, Jones S. SHARP2: protein–protein interaction predictions using patch analysis. *Bioinf* 2006, 22:1794–1795.
75. Fernandez-Recio J, Totrov M, Skorodumov C, Abagyan R. Optimal docking area: a new method for predicting protein–protein interaction sites. *Proteins* 2005, 58:134–143.
76. Fernandez-Recio J, Totrov M, Abagyan R. Identification of protein–protein interaction sites from docking energy landscapes. *J Mol Biol* 2004, 335:843–865.
77. Pazos F, Helmer-Citterich M, Ausiello G, Valencia A. Correlated mutations contain information about protein–protein interaction. *J Mol Biol* 1997, 271:511–523.
78. Gallet X, Charlotiaux B, Thomas A, Brasseur R. A fast method to predict protein interaction sites from sequences. *J Mol Biol* 2000, 302:917–926.
79. Res I, Mihalek I, Lichtarge O. An evolution based classifier for prediction of protein interfaces without using protein structures. *Bioinf* 2005, 21:2496–2501.
80. de Vries SJ, Bonvin AM. Intramolecular surface contacts contain information about protein–protein interface regions. *Bioinf* 2006, 22:2094–2098.
81. Landgraf R, Xenarios I, Eisenberg D. Three-dimensional cluster analysis identifies interfaces and functional residue clusters in proteins. *J Mol Biol* 2001, 307:1487–1502.
82. Lichtarge O, Sowa ME. Evolutionary predictions of binding surfaces and interactions. *Curr Opin Struct Biol* 2002, 12:21–27.
83. Armon A, Graur D, Ben-Tal N. ConSurf: an algorithmic tool for the identification of functional regions in proteins by surface mapping of phylogenetic information. *J Mol Biol* 2001, 307:447–463.
84. Chung JL, Wang W, Bourne PE. Exploiting sequence and structure homologs to identify protein–protein binding sites. *Proteins* 2006, 62:630–640.
85. Rausell A, Juan D, Pazos F, Valencia A. Protein interactions and ligand binding: from protein subfamilies to functional specificity. *Proc Natl Acad Sci USA* 2010, 107:1995–2000.
86. Chelliah V, Chen L, Blundell TL, Lovell SC. Distinguishing structural and functional restraints in evolution in order to identify interaction sites. *J Mol Biol* 2004, 342:1487–1504.
87. Chelliah V, Blundell TL, Fernandez-Recio J. Efficient restraints for protein–protein docking by comparison of observed amino acid substitution patterns with those predicted from local environment. *J Mol Biol* 2006, 357:1669–1682.
88. Keskin O, Nussinov R, Gursoy A. PRISM: protein–protein interaction prediction by structural matching. *Methods Mol Biol* 2008, 484:505–521.

89. Fariselli P, Pazos F, Valencia A, Casadio R. Prediction of protein–protein interaction sites in heterocomplexes with neural networks. *Eur J Biochem* 2002, 269:1356–1361.
90. Bordner AJ, Abagyan R. Statistical analysis and prediction of protein–protein interfaces. *Proteins* 2005, 60:353–366.
91. Chen H, Zhou HX. Prediction of solvent accessibility and sites of deleterious mutations from protein sequence. *Nucleic Acids Res* 2005, 33:3193–3199.
92. Casal E, Federici L, Zhang W, Fernandez-Recio J, Priego EM, Miguel RN, DuHadaway JB, Prendergast GC, Luisi BF, Laue ED. The crystal structure of the BAR domain from human Bin1/amphiphysin II and its implications for molecular recognition. *Biochemistry* 2006, 45:12917–12928.
93. Bolanos-Garcia VM, Fernandez-Recio J, Allende JE, Blundell TL. Identifying interaction motifs in CK2beta—a ubiquitous kinase regulatory subunit. *Trends Biochem Sci* 2006, 31:654–661.
94. Fernandez D, Vendrell J, Aviles FX, Fernandez-Recio J. Structural and functional characterization of binding sites in metalloproteases based on Optimal Docking Area analysis. *Proteins* 2007, 68:131–144.
95. Chandran V, Poljak L, Vanzo NF, Leroy A, Miguel RN, Fernandez-Recio J, Parkinson J, Burns C, Carpousis AJ, Luisi BF. Recognition and cooperation between the ATP-dependent RNA helicase RhlB and ribonuclease RNase E. *J Mol Biol* 2007, 367:113–132.
96. Burgoyne NJ, Jackson RM. Predicting protein interaction sites: binding hot-spots in protein–protein and protein–ligand interfaces. *Bioinf* 2006, 22:1335–1342.
97. Vakser IA, Matar OG, Lam CF. A systematic study of low-resolution recognition in protein–protein complexes. *Proc Natl Acad Sci USA* 1999, 96:8477–8482.
98. Gabdoulline RR, Wade RC. Protein–protein association: investigation of factors influencing association rates by Brownian dynamics simulations. *J Mol Biol* 2001, 306:1139–1155.
99. Janin J. The kinetics of protein–protein recognition. *Proteins* 1997, 28:153–161.
100. Norel R, Petrey D, Wolfson HJ, Nussinov R. Examination of shape complementarity in docking of unbound proteins. *Proteins* 1999, 36:307–317.
101. Novotny J, Brucoleri RE, Saul FA. On the attribution of binding energy in antigen–antibody complexes McPC 603, D1.3, and HyHEL-5. *Biochemistry* 1989, 28:4735–4749.
102. Nuss JM, Whitaker PB, Air GM. Identification of critical contact residues in the NC41 epitope of a subtype N9 influenza virus neuraminidase. *Proteins* 1993, 15:121–132.
103. Tulip WR, Harley VR, Webster RG, Novotny J. N9 neuraminidase complexes with antibodies NC41 and NC10: empirical free energy calculations capture specificity trends observed with mutant binding data. *Biochemistry* 1994, 33:7986–7997.
104. Clackson T, Wells JA. A hot spot of binding energy in a hormone–receptor interface. *Science* 1995, 267:383–386.
105. Keskin O, Ma B, Nussinov R. Hot regions in protein–protein interactions: the organization and contribution of structurally conserved hot spot residues. *J Mol Biol* 2005, 345:1281–1294.
106. Bogan AA, Thorn KS. Anatomy of hot spots in protein interfaces. *J Mol Biol* 1998, 280:1–9.
107. Ma B, Elkayam T, Wolfson H, Nussinov R. Protein–protein interactions: structurally conserved residues distinguish between binding sites and exposed protein surfaces. *Proc Natl Acad Sci USA* 2003, 100:5772–5777.
108. Tuncbag N, Salman FS, Keskin O, Gursoy A. Analysis and network representation of hotspots in protein interfaces using minimum cut trees. *Proteins* 2010, 78:2283–2294.
109. Cukuroglu E, Gursoy A, Keskin O. Analysis of hot region organization in hub proteins. *Ann Biomed Eng* 2010, 38:2068–2078.
110. Moreira IS, Fernandes PA, Ramos MJ. Hot spots—a review of the protein–protein interface determinant amino-acid residues. *Proteins* 2007, 68:803–812.
111. DeLano WL. Unraveling hot spots in binding interfaces: progress and challenges. *Curr Opin Struct Biol* 2002, 12:14–20.
112. Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein–protein interfaces. *Nature* 2007, 450:1001–1009.
113. Weiss GA, Watanabe CK, Zhong A, Goddard A, Sidhu SS. Rapid mapping of protein functional epitopes by combinatorial alanine scanning. *Proc Natl Acad Sci USA* 2000, 97:8950–8954.
114. Erlanson DA, Braisted AC, Raphael DR, Randal M, Stroud RM, Gordon EM, Wells JA. Site-directed ligand discovery. *Proc Natl Acad Sci USA* 2000, 97:9367–9372.
115. Fischer TB, Arunachalam KV, Bailey D, Mangual V, Bakhru S, Russo R, Huang D, Paczkowski M, Lalchandani V, Ramachandra C, et al. The binding interface database (BID): a compilation of amino acid hot spots in protein interfaces. *Bioinf* 2003, 19:1453–1454.
116. Guney E, Tuncbag N, Keskin O, Gursoy A. Hot-Sprint: database of computational hot spots in protein interfaces. *Nucleic Acids Res* 2008, 36:D662–D666.
117. Guerois R, Nielsen JE, Serrano L. Predicting changes in the stability of proteins and protein complexes: a study of more than 1000 mutations. *J Mol Biol* 2002, 320:369–387.

118. Kortemme T, Baker D. A simple physical model for binding energy hot spots in protein–protein complexes. *Proc Natl Acad Sci USA* 2002, 99:14116–14121.
119. Darnell SJ, Page D, Mitchell JC. An automated decision-tree approach to predicting protein interaction hot spots. *Proteins* 2007, 68:813–823.
120. Shulman-Peleg A, Shatsky M, Nussinov R, Wolfson HJ. Spatial chemical conservation of hot spot interactions in protein–protein complexes. *BMC Biol* 2007, 5:43.
121. Tuncbag N, Keskin O, Gursoy A. HotPoint: hot spot prediction server for protein interfaces. *Nucleic Acids Res* 2010, 38 Suppl:W402–W406.
122. Grosdidier S, Fernandez-Recio J. Identification of hot-spot residues in protein–protein interactions by computational docking. *BMC Bioinformatics* 2008, 9:447.
123. Li L, Zhao B, Cui Z, Gan J, Sakharkar MK, Kangueane P. Identification of hot spot residues at protein–protein interface. *Bioinformation* 2006, 1:121–126.
124. Ofra Y, Rost B. Protein–protein interaction hotspots carved into sequences. *PLoS Comput Biol* 2007, 3:e119.
125. Hu Z, Ma B, Wolfson H, Nussinov R. Conservation of polar residues as hot spots at protein interfaces. *Proteins* 2000, 39:331–342.
126. Grosdidier S, Fernandez-Recio J. Docking and scoring: applications to drug discovery in the interactions era. *Expert Opin Drug Discov* 2009, 4:673–686.
127. Kortemme T, Kim DE, Baker D. Computational alanine scanning of protein–protein interfaces. *Sci STKE* 2004, 2004:pl2.
128. Moreira IS, Fernandes PA, Ramos MJ. Computational alanine scanning mutagenesis—an improved methodological approach. *J Comput Chem* 2007, 28:644–654.
129. Almlof M, Aqvist J, Smalas AO, Brandsdal BO. Probing the effect of point mutations at protein–protein interfaces with free energy calculations. *Biophys J* 2006, 90:433–442.
130. Seco J, Luque FJ, Barril X. Binding site detection and druggability index from first principles. *J Med Chem* 2009, 52:2363–2371.
131. Zhou HX, Qin S. Interaction-site prediction for protein complexes: a critical assessment. *Bioinf* 2007, 23:2203–2209.
132. Moult J, Fidelis K, Kryshtafovych A, Rost B, Tramontano A. Critical assessment of methods of protein structure prediction—round VIII. *Proteins* 2009, 77 Suppl 9:1–4.
133. Janin J. The targets of CAPRI rounds 13–19. *Proteins* 2010, 78:3067–3072.
134. Lensink MF, Wodak SJ. Docking and scoring protein interactions: CAPRI 2009. *Proteins* 2010, 78:3073–3084.
135. Lensink MF, Wodak SJ. Blind predictions of protein interfaces by docking calculations in CAPRI. *Proteins* 2010, 78:3085–3095.
136. Laurie AT, Jackson RM. Methods for the prediction of protein–ligand binding sites for structure-based drug design and virtual ligand screening. *Curr Protein Pept Sci* 2006, 7:395–406.
137. Li S, Gao J, Satoh T, Friedman TM, Edling AE, Koch U, Choksi S, Han X, Korngold R, Huang Z. A computer screening approach to immunoglobulin superfamily structures and interactions: discovery of small non-peptidic CD4 inhibitors as novel immunotherapeutics. *Proc Natl Acad Sci USA* 1997, 94:73–78.
138. Miranker A, Karplus M. Functionality maps of binding sites: a multiple copy simultaneous search method. *Proteins* 1991, 11:29–34.
139. Caflisch A, Miranker A, Karplus M. Multiple copy simultaneous search and construction of ligands in binding sites: application to inhibitors of HIV-1 aspartic proteinase. *J Med Chem* 1993, 36:2142–2167.
140. Bohm HJ, Klebe G. What can we learn from molecular recognition in protein–ligand complexes for the design of new drugs? *Angewandte Chemie-International Edition in English* 1996, 35:2589–2614.
141. English AC, Done SH, Caves LS, Groom CR, Hubbard RE. Locating interaction sites on proteins: the crystal structure of thermolysin soaked in 2% to 100% isopropanol. *Proteins* 1999, 37:628–640.
142. English AC, Groom CR, Hubbard RE. Experimental and computational mapping of the binding surface of a crystalline protein. *Protein Eng* 2001, 14:47–59.
143. Schubert CR, Stultz CM. The multi-copy simultaneous search methodology: a fundamental tool for structure-based drug design. *J Comput Aided Mol Des* 2009.