Mechanisms of Protein-Protein Binding: Double-Mutant Cycle Analysis Using the ProteOn XPR36 System

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Introduction

Complex life processes require proteins to be able to transfer specific signals, build multiprotein complexes, control the function of enzymes, and regulate all other cellular activities. Many of these tasks are performed through specific protein-protein interactions. This is feasible due to the almost unlimited potential for the generation of protein binding sites, unique sites characterized by their shape and surface chemistry. A major research area today is the investigation of the structure, mechanisms, and dynamics of protein-protein interactions. One model system being utilized for basic research into the mechanisms of protein complex formation uses the interactions between TEM1 β-lactamase enzyme (TEM1) and its inhibitor, β-lactamase inhibitor protein (BLIP), shown in Figure 1 (Albeck and Schreiber 1999; Albeck et al. 2000).

Surface plasmon resonance (SPR) technology is a central, widely used tool for kinetic studies of interactions between unlabeled biomolecules in real time. However, there has been limited analytical, high-sensitivity SPR technology for monitoring multiple kinetic interactions in parallel. The ProteOn XPR36 protein interaction array system is a new system possessing all the qualities of high-level SPR biosensing technology combined with high-throughput and multiplexing capabilities. The principles and concepts of this array-format system are provided in Bronner et al. (2006). Briefly, XPR™ technology is based on the built-in orientation-controlled multichannel module of the ProteOn XPR36 system, which allows parallel measurement of multiple binding interactions between as many as six protein pairs. This kind of multiplexing is done efficiently on the ProteOn XPR36 system using the innovative technique of one-shot kinetics, which allows simultaneous monitoring of multiple protein pair interactions (Bronner et al. 2006).

The aim of this study was to measure the cooperativity between residues on TEM1 and BLIP, with the working assumption that while a single mutation provides information about the energy consequences of changing single residues, only analysis of multiple mutations can uncover the more complex, cooperative nature of noncovalent protein-protein interactions. Schreiber and colleagues are using the multiple-mutant cycle method to evaluate cooperativity of residue binding (Reichmann et al. 2005). With this method, the binding of two mutant proteins is measured individually and together; the interaction energy between the two proteins can then be determined by subtracting the free energy difference in the binding of a double-mutant protein from that of the two single-mutant proteins. The data presented in this tech note is in the context of this larger study.

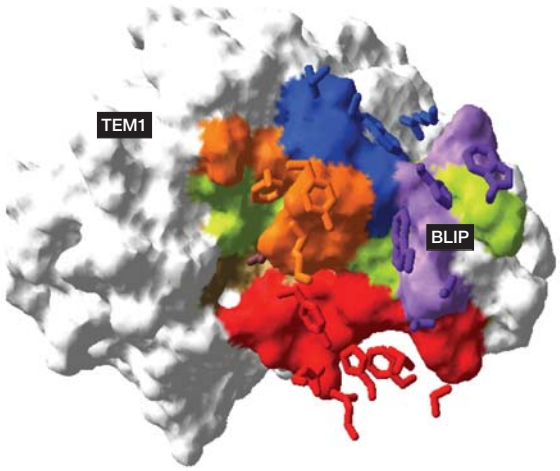

Fig. 1. Working model depicting the binding domain interactions between TEM1 and BLIP proteins.

Here, five different TEM1 mutant protein ligands and a reference buffer sample were immobilized in the six ligand channels of a sensor chip. Six wild-type BLIP concentrations were injected in the analyte channels. This multiplex analysis provided kinetic and thermodynamic data on the binding of each of the six BLIP protein concentrations with each mutant TEM1 protein in a single experimental shot.

Methods

Instrumentation and Reagents

The experiment was performed using the ProteOn XPR36 protein interaction array system and a ProteOn GLC sensor chip. ProteOn phosphate buffered saline with 0.005% Tween 20, pH 7.4 (PBS/Tween) was used as running buffer throughout the experiment, which was performed at 25°C.

The TEM1 proteins used as ligands in the larger study included wild-type TEM1 (TEM1, 29 kD) and the following mutant proteins: E104A, Y105A, E104A/Y105A, R243A, K234A, S130A, SSR (TEM1 mutated to S130A, S235A, and R243A), KSSR (TEM1 mutated to S130A, S235A, R243A, and K234A), R243A/S130A, S235A, R243A/S235A, and K234A. The BLIP proteins used as analytes included wild-type BLIP (BLIP, 17.5 kD) and the following mutants: D49A, K74A, F142A, Y143A, K74/F142A, K74A/Y143A, F142A/Y143A, and K74A/F142A/Y143A.

The protein expression and purification procedures used are described in Reichmann et al. (2005). The following reagents were used for amine coupling: 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDAC), *N*-hydroxysulfo-succinimide (sulfo-NHS), and ethanolamine HCl (ProteOn amine coupling kit).

Protein-Protein Interaction Experiment

For immobilization of the different TEM1 mutant proteins, the ligand channel surfaces were activated by amine coupling using 100 mM EDAC and 25 mM sulfo-NHS. The TEM1 mutant protein samples (180 µl, 1 µM prepared in sodium acetate buffer, pH 4.0) and a buffer sample (for use as a reference in the sixth channel) were injected in parallel in a single injection step at a flow rate of 30 µl/min. Ethanolamine HCl (1 M, pH 8.5) was then injected to deactivate any remaining surface groups in the five ligand channels.

A 2-fold dilution series of six wild-type BLIP concentrations bracketing the K_D value (ranging from 0.1 to 10 times the K_D value) were prepared in PBS/Tween, pH 7.4. These six BLIP samples (250 µl) were injected into the six analyte channels orthogonal to the ligand channels at a flow rate of 50 µl/min, generating the 36-element interaction array (Bronner at al. 2006). The clustering studies and methods used are described in Reichmann et al. (2005).

Kinetic Analysis

Kinetic analysis was performed by fitting curves describing a simple 1:1 bimolecular reaction model to the resulting sensorgrams (Bronner et al. 2006). In addition to reference subtraction using a dedicated reference channel, in some experiments, reference subtraction was performed using interspot references. Interspot references are computed by averaging the background signal obtained from the ligand-free spots on either side of each protein interaction spot.

Results and Discussion

Whereas the interface of protein-protein interactions is traditionally described by single-mutation analysis, in our larger study we analyzed a network of interactions in a multiplex method of multiple-mutant cycles. Such cycles reveal whether the contributions from a pair of residues are additive or whether the effects of mutations are coupled. Standard clustering techniques were used to separate the network of TEM1-BLIP interactions into five connecting binding units, or clusters. Of the five clusters (Reichmann et al. 2005), clusters C1 and C2 were investigated to determine (in terms of free energy of binding) the inter- and intracluster relationships of the TEM1 and BLIP residues. Various combinations of residues (single, double, and multiple) located in clusters C1 and C2 were analyzed using the ProteOn XPR36 system.

Figure 2 shows sensorgrams of the analysis of the TEM1-BLIP interactions. Each of the five panels shows six sensorgrams representing the interactions between six different wild-type BLIP concentrations and the TEM1 mutant protein indicated. Kinetic analysis of the interaction between the different ligand-analyte pairs is shown, and the kinetic properties of the different interactions are readily compared. The association rate constant, k_a, dissociation rate constant, k_d, and equilibrium dissociation constant, K_D, for these TEM1-BLIP interactions are presented in Table 1.

Table 1. Kinetic constants for the interactions between mutants of TEM1 and wild-type BLIP. The equilibrium dissociation constant, K_D, was calculated from k_d/k_a.

TEM1 Mutant	k_a (M^{-1} sec^{-1})	k_d (sec^{-1})	K_D (M)
R243A/S235A	1.61×10^4	4.48×10^{-4}	2.78×10^{-8}
K234A	2.11×10^4	8.86×10^{-4}	4.20×10^{-8}
R243A/S130A	1.29×10^4	1.22×10^{-3}	9.46×10^{-8}
S235/S130A	3.02×10^4	9.11×10^{-4}	3.02×10^{-8}
E104A	1.66×10^5	7.46×10^{-3}	4.49×10^{-8}

Similar experiments were performed for all combinations of TEM1 and BLIP wild-type and mutant proteins, and detailed results and conclusions of this larger study can be found in Reichmann et al. (2005). Briefly, the results indicated that interactions within a cluster are nonadditive, since the sum of ΔΔG values of the individual mutations is much larger than the value measured for the multiple-mutant protein.

This effect is clearly demonstrated when an entire cluster is mutated to alanine. Summing up the loss of free energy of binding of five single mutants of C2 (BLIP K74A, F142A, and Y143A; and TEM1 E104A and Y105A) yields a value of 31.1 kJ/mol. This number is composed of an additive loss of 25.3 kJ/mol for the three BLIP mutant proteins, and 5.8 kJ/mol for the two TEM1 mutant proteins. The ΔΔG value of the triple mutant BLIP was 16.3 kJ/mol, and that of the double mutant TEM1 was 4.3 kJ/mol. However, removing all five residues simultaneously (by mutation to alanine) resulted in a loss of only 10.1 kJ/mol of binding free energy. The same phenomenon was detected for the C1 cluster.

In contrast to the intracluster mutations showing nonadditive relationships, all the tested combinations of intercluster mutations were additive. These measurements were between five different TEM1 mutant proteins in cluster C1 (three single mutations S130A, K234A, and R243A, and two multiple mutations K234A, S130A, S235A, and R243A, and R243A, S130A, and S235A) and the seven BLIP proteins with

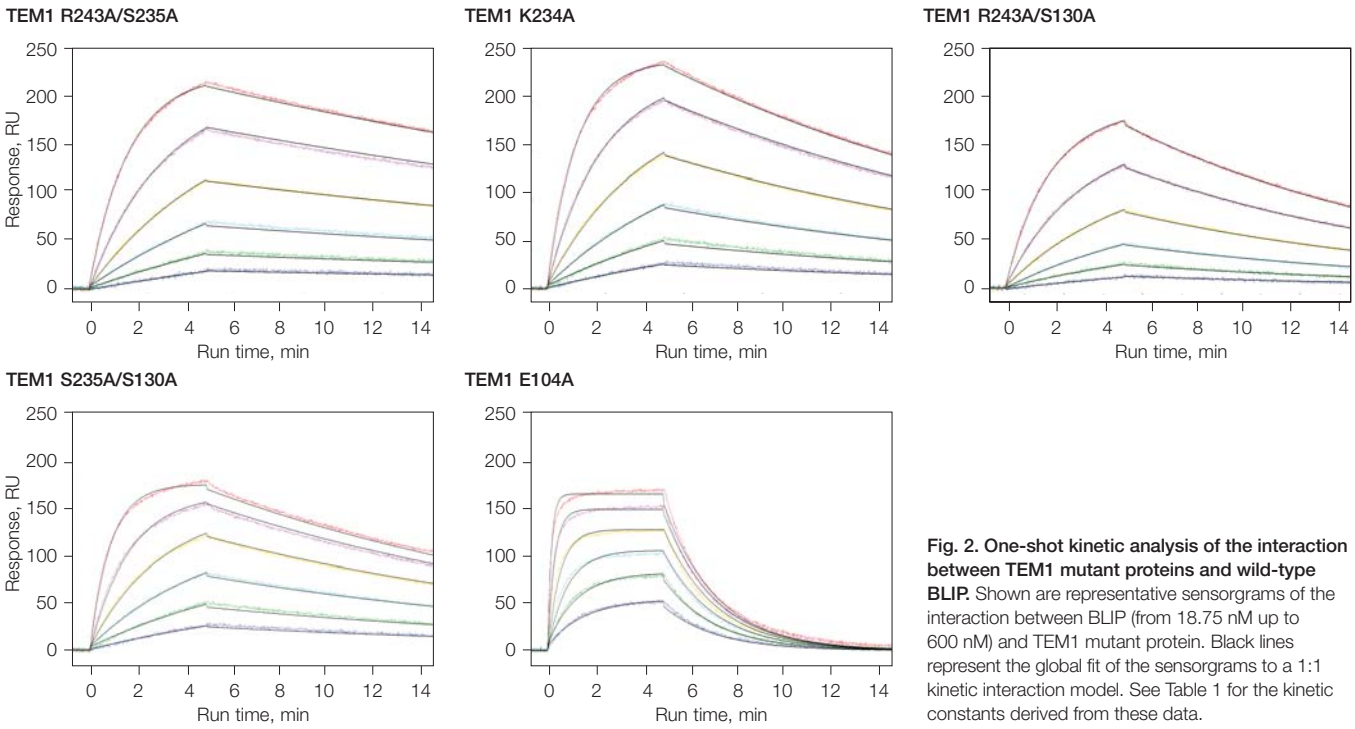

Fig. 2. One-shot kinetic analysis of the interaction between TEM1 mutant proteins and wild-type BLIP. Shown are representative sensorgrams of the interaction between BLIP (from 18.75 nM up to 600 nM) and TEM1 mutant protein. Black lines represent the global fit of the sensorgrams to a 1:1 kinetic interaction model. See Table 1 for the kinetic constants derived from these data.

mutations in C2 (three single mutations, K74A, F142A, and Y143A, and four multiple mutations, K74A and F142A, F142A and Y143A, K74A and Y143A, and K74A, F142A, and Y143A). Although the clusters are in close structural proximity, they are energetically independent. In other words, mutations in C1 do not affect residues in C2, and vice versa.

Extensive multiple mutant analysis of the two TEM1-BLIP clusters, C1 and C2, indicated that residue clusters are energetically independent of each other but have a high degree of cooperativity within each cluster. Additional detailed results and conclusions of this study can be reviewed in Reichmann et al. (2005). Results using interspot references were comparable to those using a dedicated reference channel, which confirms that the ProteOn XPR36 system can be used for analysis of a complete set of 36 protein-protein interactions.

Conclusions

In this study, the cooperativity between residues on TEM1 and BLIP was measured. The method most commonly used to analyze the contribution of residues toward the stability of a protein-protein complex involves evaluating the loss in free energy of binding upon mutation. However, this method is not without problems, because the loss in the measured free energy of binding caused by single mutations can equal, or even exceed, that of removing the entire cluster. Therefore, multiple mutants were analyzed efficiently and rapidly using the ProteOn XPR36 system. The results indicated that the sum of the loss in free energy of all of the single mutations within the C1 and C2 clusters far exceeds (up to 4-fold) the loss in free energy generated when all of the residues of the cluster are mutated simultaneously. These results demonstrate that the protein-protein interface is built in a modular fashion, where each cluster is an independent binding unit, and that two of these clusters (C1 and C2) show intracluster cooperativity as well as intercluster additivity between the protein residues.

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