Electrostatic Interactions of Hsp-organizing Protein Tetratricopeptide Domains with Hsp70 and Hsp90

COMPUTATIONAL ANALYSIS AND PROTEIN ENGINEERING

Received for publication, February 24, 2009, and in revised form, June 16, 2009 Published, JBC Papers in Press, July 7, 2009, DOI 10.1074/jbc.M109.033894

Tommi Kajander‡§1, Jonathan N. Sachs¶2, Adrian Goldman‡§3, and Lynne Regan†¶

From the Departments of ‡§1Molecular Biophysics and Biochemistry and ¶Chemistry, Yale University, New Haven, Connecticut 06511, the §Institute of Biotechnology, Research Program in Structural Biology and Biophysics, University of Helsinki, Helsinki 00014, Finland, and the ¶Department of Biomedical Engineering, University of Minnesota, Minneapolis, Minnesota 55455

The Hsp-organizing protein (HOP) binds to the C termini of the chaperones Hsp70 and Hsp90, thus bringing them together so that substrate proteins can be passed from Hsp70 to Hsp90. Because Hsp90 is essential for the correct folding and maturation of many oncogenic proteins, it has become a significant target for anti-cancer drug design. HOP binds to Hsp70 and Hsp90 via two independent tetratricopeptide (TPR) domains, TPR1 and TPR2A, respectively. We have analyzed ligand binding using Poisson-Boltzmann continuum electrostatic calculations, free energy perturbation, molecular dynamics simulations, and site-directed mutagenesis to delineate the contribution of different interactions to the affinity and specificity of the TPR-peptide interactions. We found that continuum electrostatic calculations could be used to guide protein design by removing unfavorable interactions to increase binding affinity, with an 80-fold increase in affinity for TPR2A. Contributions at buried charged residues, however, were better predicted by free energy perturbation calculations. We suggest using a combination of the two approaches for increasing the accuracy of results, with free energy perturbation calculations used only at selected buried residues of the ligand binding pocket. Finally we present the crystal structure of TPR2A in complex with its non-cognate Hsp70 ligand, which provides insight on the origins of specificity in TPR domain-peptide recognition.

The Hsp-organizing protein (HOP) plays a key role in in vivo folding by bringing the chaperones Hsp70 (or its constitutively expressed isoform, Hsp70) and Hsp90 together into a complex (1) (see Fig. 1), where specific substrate proteins are passed over from Hsp70 to Hsp90. Partially folded Hsp90 client proteins are transferred from Hsp70 to Hsp90, where the final steps of folding are completed (2, 3). While bound to the Hsp90, the client protein then becomes functional, e.g. hormone receptors can bind their ligands, and then are released. Both chaperones must be brought together by their interaction with HOP to accomplish this (see Fig. 1). The whole Hsp90 functional cycle involves also other co-chaperones, such as p23, other TPR proteins, and ATP binding and hydrolysis, and is fairly complicated and incompletely understood (4). However, it is clear that HOP facilitates the interaction of Hsp70 and Hsp90 and thus is important in providing the link between the chaperones in the early part of Hsp90 functional cycle. Hsp90 client proteins include nuclear hormone receptors, p53, and other transcription factors and various kinases, such as Atk and Her2. Many of these are oncogenes, making Hsp90 a significant anti-cancer drug target (5). It is therefore of considerable importance to better understand the chaperone cycle of Hsp90 and in particular its interaction with essential co-chaperones, such as HOP. The interactions of Hsp70 and Hsp90 with HOP also provide an excellent model system in which to study the contribution of electrostatics to protein-peptide interactions, as will become evident.

The interactions of Hsp70 and Hsp90 with HOP are mediated by the interaction of their C-terminal amino acids with two HOP TPR motifs, TPR1 and TPR2A (6–8) (see Fig. 1). The C-terminal residues needed for affinity and specificity are GPTIEEVD for Hsp70 and MEEVD for Hsp90. Not surprisingly, the crystal structures of TPR1 and TPR2A in complex with their cognate peptides show that the peptides are bound in an extended conformation and that electrostatic interactions contribute significantly toward affinity in both complexes (Fig. 1) (8).

We have used continuum electrostatic calculations, molecular dynamics simulations, and free energy perturbation calculations combined with site-directed mutagenesis to analyze the binding interface of both the TPR1-Hsp70 peptide and TPR2A-Hsp90 peptide complexes. The results allowed us to delineate the contributions of specific interactions to the affinity of binding. Moreover, they allowed us to identify positions at which the electrostatic contributions are unfavorable, and we show that removing or replacing these interactions is a route to tighter binding. Finally, we have investigated not only the affinity of the TPR1 and TPR2A peptide interactions, but also their specificity. We present a high resolution crystal structure of TPR2A...
Electrostatics and Engineering of TPR Peptide Binding

bound to its non-cognate Hsp70 peptide, which allows us to give a more thorough explanation of how HOP TPR2A domain discriminates between the closely related Hsp70 and Hsp90 C-terminal peptides.

EXPERIMENTAL PROCEDURES

Electrostatic Binding Energy Calculations—The continuum electrostatic calculations were carried using the linearized Poisson-Boltzmann (PB) equation as implemented in the program DELPHI (9). Hydrogen atoms were built and optimized with the WHATIF server and CHARMM22 force field partial charges and atomic radii were used in the calculations (10). Calculations were performed with Debye-Hückel boundary conditions starting with the protein filling 30% of the box and focusing in on the protein until it filled 90% of the box (201 × 201 × 201 grid points), resulting in grid resolution of 3.617 grids/Å, leaving a Stern layer of 2 Å around the protein. The salt concentration used in the calculation was 0.15 M, chosen to match approximately both physiological conditions and the experimental conditions we used (see below). The protein interior was assigned a dielectric constant of 4. Overall electrostatic binding energies and residue contributions were calculated as described previously (11, 12). Briefly, the electrostatic binding energy (ΔΔE_{elec}) is obtained from Equation 1 as follows.

\[ \Delta \Delta E_{elec} = \Delta E_{(complex)} - \Delta E_{(protein)} - \Delta E_{(peptide)} \]  

(Eq. 1)

The energies are ΔEs because they are obtained relative to reference state that is equivalent to a hydrophobic isostere of that molecule or complex. The calculations assume no backbone differences between the free and complexed TPR structures, an assumption supported by several experimental studies (8, 13). For simplicity, crystallographic waters were excluded from the calculations.

The individual residue contributions to interaction energies, \( \Delta \Delta E_{int} \), were calculated as indicated above, but by charging only one residue at a time. This is possible because, using the linearized PB equation, all group contributions are additive. The sum can be described in terms of electrostatic potential (φ) distribution,

\[ \Delta E_{elec} = 1/2 \sum \Delta \phi q_i \]  

(Eq. 2)

where φ is the potential due to the charged group in question at charge \( q_i \). To get the group’s \( \Delta \Delta E_{int} \) contribution, potential distributions with only the particular group charged was calculated. The \( \Delta E_{int} \) for each component, free protein, or peptide and complex, were calculated from thus obtained potential distributions with all other sites charged. This yields \( \Delta \Delta E_{int} \) of binding (11, 12) from the difference of the interaction energies,

\[ \Delta \Delta E_{int} = \Delta E_{int}(complex) - \Delta E_{int}(protein) \]  

(Eq. 3)

or

\[ \Delta \Delta E_{int} = \Delta E_{int}(complex) - \Delta E_{int}(peptide) \]  

(Eq. 4)

The desolvation component for group contribution on complex formation was obtained with only the group in question charged, from the difference upon binding,

\[ \Delta \Delta E_{desolv} = \Delta E_{(complex)} - \Delta E_{(protein)} \]  

(Eq. 5)

or

\[ \Delta \Delta E_{desolv} = \Delta E_{(complex)} - \Delta E_{(peptide)} \]  

(Eq. 6)

depending on whether the residue is on protein or peptide. As the rest of the protein is left uncharged, \( \Delta \Delta E_{int} \) in this calculation represent only solvation changes of the group in question. Total group contribution in then given by Equation 7.

\[ \Delta \Delta E_{elec} = \Delta \Delta E_{desolv} + \Delta \Delta E_{int} \]  

(Eq. 7)

Pairwise or network interaction energies were calculated as for the whole complex, hence these energies include both coloumbic and solvation terms as for the total \( \Delta \Delta E_{elec} \) but by treating the rest of the protein outside the interaction network as uncharged (see also (11)).

Molecular Dynamics and Free Energy Perturbation Calculations—Molecular dynamics (MD) systems were constructed using CHARMM (version 27) (10). Ligand-bound proteins were solvated in water-boxes containing 7265 water molecules and 0.1 M NaCl. The total charge of the system was neutralized by adding an appropriate number of additional counter-ions to balance the charge of the protein. Periodic boundary conditions were applied using a constant number of atoms (N), temperature (T), and pressure (P), to form NPT ensembles. The temperature in each simulation was set to 303 K. All systems were run for 15 ns using NAMD v2.5 (14, 15). A cut-off of 10 Å was used for van der Waals interactions, and particle mesh Ewald summation was used for electrostatic interactions. The time step was 2 fs, and all bonds involving
Electrostatics and Engineering of TPR Peptide Binding

hydrogens were fixed using the SHAKE algorithm with a tolerance (relative deviation) of 10^{-8} Å.

Free energy perturbation calculations were performed using “alchemical” transformation and the dual-topology approach, as described (16). They followed the thermodynamic cycle shown in Fig. 2. To get the overall change in the binding free energy upon mutation (ΔΔG), we sum the effect of the mutation on the ligand-bound and unbound states. Briefly, the goal of the calculation is to gradually transform a residue from the wild type to the mutant form. We achieved this by running a series of sequential MD simulations, each of which is governed by the modified total potential energy function,

\[ U_{\text{Total}} = (1 - \lambda)U_{\text{wt}} + \lambda U_{\text{mut}} \]  
(Eq. 8)

where \( U_{\text{wt}} \) is the potential of the wild-type residue and \( U_{\text{mut}} \) is the potential of the mutant residue. Each simulation is therefore distinguished by the value of \( \lambda \), which is changed from 0 (where the potential is solely determined by the wild-type residue) to 1 (where it is solely the mutant residue) with intervening values representing an unphysical amalgam of the two potentials. For each mutation, we ran simulations of 100 ps at each of 40 \( \lambda \) values. The transformation was done in a non-linear fashion, wherein the scaling of \( \lambda \) was slower for values near 0 and 1. This is a typical approach to help in avoiding van der Waals clashes at the end-points that can lead to non-converged calculations (16, 17). Using this strategy, a set of simulations was run in the ligand-bound and ligand-unbound states, and the final free energy change was simply the sum of the two processes. We plot (Fig. 2) the calculated evolution of \( \Delta G \) versus \( \lambda \) from a typical FEP calculation (in this case, the K229A mutation), which is the cumulative sum of changes as \( \lambda \) is varied from 0 to 1.

Convergence was verified by checking change in \( \Delta G \) during simulations. A typical 100-ps time series showing the evolution and convergence of the \( \Delta G \) value at \( \lambda = 0.5 \) is given in Fig. 2. Further, we show overlap of density of states \( \rho (\Delta U(x)) \) for consecutive intermediate \( \lambda \)-states (e.g., \( \lambda = 0.5 \) and \( \lambda = 0.525 \)), which is a measure of convergence (18, 19) (supplemental Fig. S1).

Site-directed Mutagenesis, Protein Purification, and Expression—TPR2A and TPR1 were expressed in Escherichia coli BL21(DE3) as described (8, 20). All mutations were introduced by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene).

Surface Plasmon Resonance Measurements—Affinity measurements were performed using a Biacore3000 (Amersham Biosciences) instrument. All measurements were made at 25 °C. Binding buffer was 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% polysorbate 20, and 1 mM dithiothreitol. N-terminally biotinylated 24-mer peptides corresponding to the C termini of Hsp70 and Hsp90 (GGFGPGGGAPSGGASSGPTIEEVDCOOH and SAAVTEEMPLEDDDSMEVDCOOH, respectively) were prepared as previously described (20). Biotinylated peptides were coupled to CM5 or CM4 Biacore chips, which had been coated with ~2000–4000 response units of NeutrAvidin or avidin through amide coupling (with some extremely low affinity mutants, nonspecific binding to NeutrA-vidin was significant). Proteins were injected for 2 min (TPR2A) or 5 min (TPR1) over the sensor chip at a flow rate of 40 μl/min. The sensor chip surface was regenerated with injections of 1 M NaCl (2 × 40 μl). \( K_d \) values were obtained from a fit assuming 1:1 binding by plotting equilibrium response (\( R_{eq} \)) values at different protein concentrations using the BioEvaluation 3.0 software.

**CD Measurements**—Wavelength scans from 260 to 190 nm and thermal scans at 222 nm for TPR2A and TPR1, and their mutants where performed from 4 °C to 95 °C in both forward and reverse directions on an Aviv model 215 CD spectrophotometer at protein concentrations of ~5–10 μM in 50 mM sodium phosphate, pH 7.0. The reversibility was not complete so an “apparent \( T_m \)” was taken as the midpoint of the transition in the forward direction from 4 °C to 95 °C in 2 °C steps with a 3-min equilibration time at each temperature.

**Crystallization and Data Collection**—TPR2A was crystallized with the 12-mer GASSGPTIEEVD peptide in a 1:1.3 molar ratio at 20–23 mg/ml from 100 mM Tris-HCl, pH 8.5, 20–25% (w/v) polyethylene glycol MME 2000, 5 mM NiCl\(_2\), 5% (w/v) xylitol. The protein-peptide complex crystallized as thin plates (~0.2 × 0.3 × 0.02 mm) in space group C2 with the same unit cell dimensions as the TPR2A-MEEVD complex (8). Data were collected to 2.05-Å resolution on a Rigaku MicroMax-007 rotating anode with a Mar345 Image plate detector (Table 1).

**Structure Solution and Refinement**—The structure was solved by molecular replacement using AMORE (21) with the TPR2A protein from the TPR2A-Hsp90 MEEVD peptide complex structure as the search model, and refined with REFMAC (22). Density was visible for the peptide in the initial \( 2F_o - F_c \) and \( F_o - F_c \) maps. After placing the peptide in the density and including it in the refinement, omit maps were calculated to verify the peptide conformation. Crystallographic water molecules were added with ARP/WARP (23) and model-edited between refinement cycles (24). The final model has \( R \)-factors of \( R_{work}/R_{free} = 17.7/24.4 \)% and good stereochemistry (Table 1).

**RESULTS**

**The Electrostatic Energy Calculations**

Overall Electrostatic Contributions to Binding—We calculated total binding energies and pairwise interaction energies for TPR1 and TPR2A peptide complexes. The energetic contributions of individual amino acids in the TPR-peptide interface were examined to gain insight into their contribution to the affinity and specificity of ligand recognition. The calculated total net electrostatic contribution to the energetics of the TPR2A-Hsp90 peptide interactions was ~4.23 kcal/mol, whereas that of the TPR1-Hsp70 interaction was unfavorable, +1.37 kcal/mol.

Although we calculate an unfavorable electrostatic contribution to binding for TPR1, this is merely because electrostatic effects represent only one component of the total free energy change \( \Delta G \) of binding. Thus, the numbers we present are meaningful on a relative scale and provide a measure of the favorability of the polar interactions in the two complexes examined. The calculated electrostatic energies are consistent with
observed affinities, because the measured affinity of TPR2A for the Hsp90 peptide is higher (Table 2) than that of TPR1 for the Hsp70 peptide.

Residue-by-Residue Contributions—We examined the contributions of individual residues to the electrostatic energy of peptide binding by TPR1 and TPR2A (Fig. 3 and Table 2). The results of these calculations clearly show that the “dicarboxylate clamp” binding residues (8), which interact with both the side chain and the free carboxylate of the C-terminal aspartate, make as expected the largest favorable contributions to the stability of both peptide complexes, in addition to one conserved arginine, Arg-77 in TPR1 and Arg-305 in TPR2A. The binding residues that contribute most significantly are Lys-8, Lys-73, and Asn-43 in TPR1, and Lys-229, Lys-301, and Arg-305 in TPR2A (Fig. 3).

In addition to identifying residues that contribute favorably to the binding energy, the calculations also allow us to identify residues that make unfavorable electrostatic contributions. Among the five most conserved peptide-binding residues, Arg-77 of TPR1 is one such residue. Electrostatic calculations predict it contributes unfavorably because of a large desolvation penalty associated with burying this residue at the interface. Analysis of other polar residues on the concave face of the TPR domain where the binding site is located reveals that Asp-334 of TPR2A contributes very unfavorably (+1.80 kcal/mol) as do Glu-83 and Glu-110 of TPR1 (+1.42 and +0.67 kcal/mol, respectively).

In addition, several other residues were predicted to contribute unfavorably to the electrostatic component of the binding energy: Glu-9 of TPR1 and its equivalent Glu-230 of TPR2A, and Glu-271 and Lys-239 of TPR2A (Fig. 3). The calculated contributions of non-conserved residues involved in TPR2A hydrogen bonding to
Electrostatics and Engineering of TPR Peptide Binding

the Hsp90-peptide were slightly favorable (Asn-298 and Asn-308) or quite unfavorable (Glu-271) (Fig. 3); therefore they do not seem important for binding compared with the conserved binding residues.

Finally, all the peptide ligand side chains were predicted to stabilize the complexes, except for Glu(–3) in TPR1-Hsp70 peptide complex (Fig. 3). This peptide side chain packs between the aliphatic portion of the side chains of Lys-73 and Gln-107.

TABLE 1
Data collection and structure refinement

| TPR2A-Hsp70 GASSGPTIEEVD |
|---------------------------|
| Space group              | C2 |
| Unit cell                | 73.46, 48.02, 37.90, β = 91.56 |
| Resolution               | 40-2.05 Å |
| Unique reflections       | 8178 |
| Redundancy               | 2.5 |
| R_refm/ R/ I/          | 7.4 (23.9)%/ 13.8 (3.9)% |
| Model                    | |
| Number of atoms          | 1191 |
| Protein                  | 1100 |
| Waters                   | 90 |
| Ni²⁺                    | 1 |
| R/ R_free (average)      | 17.7/24.4% |
| B-factors (average)      | |
| Protein                 | 20.7 |
| Peptide                 | 17.6 |
| Water                   | 34.9 |
| Rmsd bonds              | 0.024 |
| Rmsd angles             | 1.827 |
| Ramachandran plot (most favored) | 94.2% |

TABLE 2
Kᵥ values (µM) for TPR1 and TPR2A ligand binding (Hsp70 or Hsp90 peptide)

The corresponding ΔΔG (kcal/mol) calculated from the Kᵥ value are also given relative to WT value. The values from FEP calculations (ΔΔGexp) to binding to Hsp90 peptide are listed for comparison for TPR2A. Also, ΔΔF_elec (the residue contributions) values are listed for all sites in the table for which these were calculated (note that these are not values for mutants, but for the contributions of the original residues mutated). In the last column the T_m values for the thermal denaturation of the variants are given.

| TPR1          | Kᵥ Hsp70 | ΔΔG_exp  | ΔΔF_elec   | T_m  |
|---------------|----------|----------|------------|------|
| WT            | 50 ± 10  | –0.65    | ND         | 50   |
| S16K          | 16.7 ± 7 | –0.15    | –0.49      | 51   |
| R77M⁺         | –        | 1.65(1)  | –          | 54   |
| R77A⁻         | –        | –        | 1.42(1)    | 52   |
| E83Q          | 35       | –0.30    | 0.67(1)    | ND   |
| Q107A         | 39 ± 3   | –        | –          | 51   |
| E110K         | 30 ± 10  | –        | –          | 51   |
| E110K/E83Q    | 11 ± 1   | –0.90    | –          | 51   |
| E110K/E83Q/S16K | 7 ± 2   | –1.16    | –          | 52   |

| TPR2A        | Kᵥ Hsp90 | ΔΔG_exp  | ΔΔG_TEP  | ΔΔF_elec   | T_m  |
|---------------|----------|----------|----------|------------|------|
| WT            | 5 ± 2    | 3.8      | –3.48(1) | –          | 59   |
| K229A         | 820 ± 20 | 3.8      | 2.07(1)  | 25.5 ± 5   | –0.10 | 48   |
| E203A         | 16.8     | –0.25    | –0.59(1) | ND         | 57.5  |
| E203Q         | 0.43     | 2.70(1)  | 37.7     | 0.14       | 54   |
| N233A⁺        | 300 ± 100| 2.42     | –0.05(1) | –          | 51.5  |
| K239F         | 3.3      | 1.02(1)  | ND       | 12         | –0.54 | ND   |
| N264A⁻        | 160      | 2.05     | 0.80(1)  | ND         | 57    |
| E271A⁻        | 0.14     | 2.70(1)  | 37.7     | 0.14       | 54   |
| E271Q⁺        | 0.42     | 2.70(1)  | 37.7     | 0.14       | 54   |
| N298A         | 12.5 ± 7 | 0.54     | –0.75(1) | 35 ± 5     | 0.09  | ND   |
| K301A         | 200 ± 20 | 2.18     | 6.99      | –2.92(1)   | ND    | 56   |
| R305A⁻        | 700      | 1.44(1)  | –        | –          | 54   |
| N308A         | 22 ± 10  | 0.88     | –0.77(1) | 46         | 0.25  | 53   |
| T332R         | 1.9 ± 0.1| –0.57    | –0.95     | 24         | ND    | 56   |
| D334A         | 1 ± 0.5  | –0.57    | –1.65     | 1.81(1)    | –     | 54   |
| D334N         | 0.25 ± 0.05| –1.77  | –        | –          | ND    | 56   |
| D334K         | 0.325 ± 0.75| –1.62 | –        | –0.25     | 46    | 52   |
| D334K/T332R   | 3.2 ± 0.01| –2.62    | –        | –0.25     | 46    | 52   |

*“(1)” marks predicted favorable contribution to affinity, and “(1)” marks predicted unfavorable contribution by the residue. The actual charge removing/reversing mutation will have its sign will opposite, if in keeping with the prediction.

ND, not determined.

Indicates residues that have charged groups buried in the interface (>75% buried accessible surface area).
kcal/mol, very similar to the TPR2A value. The interaction of Lys-73 with the D(0) side chain carboxylate gave a stabilization of just 0.136 kcal/mol even though TPR1 Lys-73 is equivalent to Lys-301 in TPR2A and both bind to the peptide Asp(0) side-chain carboxylate. This difference is probably due to the differences in the orientation and environment in the two binding sites for this particular residue in the otherwise conserved framework (Fig. 4).

Nonetheless, the total calculated free energy of the two C-terminal dicarboxylate clamp networks in TPR1 around the peptide D(0) is −1.66 kcal/mol, which again is more than the sum of the individual interactions but much less than that of analogous TPR2A network. This is in agreement with their affinities for the ligands. Arg-77 (equivalent to Arg-305 in TPR2A) binding to the peptide backbone carbonyls at Glu(−2) and Glu(−3) (Fig. 4) gave +0.83 kcal/mol. These arginines are in both TPR domains buried in the interface.

**Alanine-scanning Mutagenesis of Predicted Stabilizing Interactions**

Based on the crystal structures of the TPR-peptide complexes, sequence conservation of binding residues, and electrostatic calculations, we individually mutated several of the residues that were predicted to contribute favorably to the TPR-peptide interaction to alanine. The mutations were mainly made to TPR2A. We measured the affinity of the mutated TPR domains to the peptide by surface plasmon resonance and checked the stability and folding of the mutant proteins by thermal denaturation (Table 2 and supplemental Figs. S2 and S3).

First we mutated the key ligand binding residues in TPR2A. The mutant proteins K229A, K301A, and R305A all had dramatically decreased peptide binding affinities. Alanine mutations at two other conserved binding residues, Asn-233 and Asn-264, also had large effects on affinity (Table 2). These results are consistent with our electrostatic calculations: the network of interactions formed by these five residues with the peptide is important for binding the EEVD-peptides. This result is also in agreement with previous protein design experiments (20). Our calculations suggested that the non-conserved residues Gln-298 and Asn-308, which interact with the ligand via hydrogen bonds, also contribute to binding (Fig. 3). We therefore mutated these two to alanines. Neither of these mutations alone, however, contributed very significantly to the binding affinity, but the $K_d$ values were slightly higher (~2-fold and 4-fold) than for wild type.
Electrostatics and Engineering of TPR Peptide Binding

![Image](https://example.com/image.png)

**FIGURE 4.** Interaction networks at the binding sites of TPR2A and TPR1. A, the carboxylate clamp binding residues and hydrogen bonds on TPR-Hsp90 MEEVD complex. B, the environment around the conserved Arg-305 in TPR2A. C, carboxylate clamp binding residues in TPR1. D, hydrogen bonding interactions around Arg-77 in TPR1 (analogous of Arg-305 of TPR2A).

**TABLE 3** Electrostatic energies calculated for peptide interaction networks of TPR1-Hsp70 and TPR2A-Hps90 complexes

| Ionic network                  | \( \Delta G_{\text{elec}} \) kcal/mol |
|--------------------------------|--------------------------------------|
| TPR2A                         |                                      |
| D(-1)-side chain-K301-N298     | -1.50                                |
| D(-1)-side chain-K229-N233-N264| -0.92                                |
| D(-1)-K301-N298-K229-N233-N264 | -2.83                                |
| R305-E(-2)-E(-3)               | +0.74                                |
| TPR1                          |                                      |
| D(-1)-side chain-K73           | -0.90                                |
| D(-1)-side chain-K8-N12-N43    | -0.14                                |
| D(-1)-K73-K8-N12-N43           | -1.68                                |
| R77-E(-2)-E(-3)                | +0.83                                |

**Protein Design for Increased Affinity Based on Electrostatic Calculations**

The predictions of the electrostatic contributions were experimentally tested by purifying the appropriate mutant proteins and assessing their stability and binding properties. All the mutants were properly folded and not significantly destabilized relative to their parent TPR domain, as indicated by their \( T_m \) values (Table 2 and supplemental Figs. S2 and S3).

Residues that were predicted by calculations to contribute unfavorably to the electrostatic interaction energy were mutated as follows: TPR1 Arg-77 to Met/Ala, Glu-83 to Gln, and Glu-110 to Lys; and TPR2A Asp-334 to Ala/Asn/Lys, Glu-271 to Gln, and Glu-230 to Ala. Lys-239 was mutated to Tyr in TPR2A, as this could provide a bigger hydrophobic pocket for the peptide M(-4) side chain. We combined the single mutants that were experimentally shown to have increased affinity into double and triple mutants (Table 2).

To optimize binding interactions further, we designed additional variants using a slightly different strategy. Guided by the co-crystal structures and the results of the electrostatic calculations, we replaced polar uncharged residues with a positively charged residue at two positions that were not in contact with the ligand. These side chains of the new residues could be modeled in one of the common rotamer conformations (25) with no steric clashes, and they could be placed in salt bridge/hydrogen bonding distance (<3.7 Å) from the ligand carboxylates. The changes were in TPR2A T332R and in TPR1 S16K. The total \( \Delta G_{\text{elec}} \) was calculated for the mutants as for the wild-type complexes. In both cases the mutations are predicted to increase the affinity of the TPR-peptide interaction: for T332R, \( \Delta G_{\text{mutation}} = -1.02 \text{ kcal/mol} \) and for S16K, \( \Delta G_{\text{mutation}} = -1.06 \text{ kcal/mol} \). These mutations were then tested together with the mutations prepared based on the unfavorable residue contribution calculations, as described earlier.

The greatest increase in affinity for the double and triple mutants tested was achieved with the D334K/T332R double mutant of TPR2A. This resulted in an 80-fold increase in affinity for the Hsp90 peptide (Fig. 5 and Table 2). Interestingly, the mutation improved not only the affinity but also the specificity of peptide binding: it has 50-fold higher specificity for Hsp90 peptide versus Hsp70 than wild-type TPR2A does. Also, replacing surface residues around the binding site of TPR1 (Glu-83 to Gln, Glu-110 to Lys, and Ser-16 to Lys) resulted in some increase in affinity (7-fold) (Table 2).

The strategies for optimization of stability of the complex for the two proteins differed. In TPR2A, the mutations changed close range interactions; whereas in TPR1 most mutations were close to the binding site, but not within hydrogen bonding distance of the ligand.

Once we had successfully designed the higher affinity double mutant of TPR2A (T332R/D334K), it was of interest to ask whether it would be possible to combine these mutations with mutations at the dicarboxylate clamp binding site to change specificity while retaining affinity toward different novel ligands (e.g. peptide substrates that would not be bound at their C terminus, or other novel substrates). This is of interest in terms of trying to understand the determinants of substrate specificity in TPR domain peptide recognition. To examine the effect of the clamp-binding residues in the engineered TPR2A double mutant, we created the T332R/D334K/K301A and T332R/D334K/K229A triple mutants and measured their affinity for Hsp90 and Hsp70 peptides. The affinity of the triple mutants for the Hsp90 ligand was similar to wild-type: K229A-triple mutant had a \( K_d \) of 8.0 \( \mu \text{M} \) and Lys-301-triple mutant a \( K_d = 2.5 \text{ \mu M} \) as measured by the surface plasmon resonance assay. Thermal unfolding experiments by CD confirmed that the proteins were stable and correctly folded (\( T_m \) values were 52.5 °C and 48 °C, respectively).

**Free Energy Perturbation Calculations and MD Simulations**

In addition to the continuum calculations we used MD an explicit water model to calculate the free energy of binding. We sought to compare the results from implicit and explicit models against experimental results, because it appeared the continuum methods failed for some of the predictions, mainly where charges are partially buried (see above). We were thus interested in testing whether the free energy perturbation (FEP) method would better predict the residue contributions to binding free energies in such cases.

We used FEP to calculate the effects of alanine substitutions on different types of residues in the TPR2A ligand binding pocket, based on the continuum electrostatic calculations and
experimental mutagenesis studies. We tested the following mutations: K229A and K301A, as results indicate they are clearly important (Table 2); R305A and E271A, both mispredicted by continuum electrostatic calculations; and D334A, where the continuum calculations worked well. The results of these calculations give ΔΔGs that are in good agreement with experiment (Table 2).

Thus, for buried charges, the free energy perturbation calculations agree better with experiment than the continuum electrostatics calculations (Table 2 and below). Both perform well for the exposed binding residues.

MD simulations (15 ns) on the complex stabilizing double mutant T332R/D334K were also conducted to obtain information about the effect of the mutations on structure. The simu-
Electrostatics and Engineering of TPR Peptide Binding

For the HOP Hsp70 and Hsp90 recognition, both the affinity and the specificity of the TPR-peptide interaction are important, because the substrates of the two TPR domains are highly similar. Therefore, for each mutant we measured binding not only to its cognate peptide, but also to its non-cognate peptide (Table 2). Wild-type TPR2A discriminates between the cognate, Hsp90 peptide (MEEVD) and the non-cognate Hsp70 peptide (PTGIEEVD) with a difference in binding affinity of ~6-fold (Table 2).

To better understand the non-cognate interaction, we crystallized TPR2A with the C-terminal 12-mer peptide of Hsp70 (GASSPTGEVEVD). The overall structure of the TPR domain was very similar to that seen in the TPR2A-Hsp90 MEEVD peptide complex (r.m.s.d. 0.138 Å for 122 Cα atoms of the TPR domain). Also, the Hsp70 peptide adopts an extended conformation that is very similar to that of the Hsp90 peptide. The dicarboxylate clamp interacting side chains of TPR2A align closely in the two structures (Fig. 6), but the Glu(−2) side chain of the peptide substrate assumes a different conformation. MD simulation results suggest that Glu(−2) is hydrogen-bonded to Arg-332 and Lys-334 in the double mutant in complex with the Hsp90 peptide. The different conformation preferred for this side chain in the Hsp70 EEVD ligand could in part explain the increase in the variant’s specificity for Hsp90, as it was designed to bind to Glu(−2) based on the peptide conformation in the TPR2A-Hsp90 complex.

The most significant difference between the structures of TPR2A with cognate versus non-cognate peptide is near the N terminus of the peptide. In the TPR2A-Hsp90 peptide complex, the methionine side chain fits snugly into a pocket on the protein. By contrast, the β-branched isoleucine at this position in the Hsp70 peptide is not able to fit into the same hydrophobic pocket, and consequently the peptide backbone “lifts up” from the protein, beginning at the isoleucine residue (Fig. 6).

DISCUSSION

Computational Analysis of Electrostatic Interactions—We have investigated the electrostatic contributions of residues at the TPR-peptide interfaces for the two independent TPR domains of HOP, TPR1 and TPR2A. These two systems are of interest, because, although their ligands are very similar the TPRs discriminate between them both in vitro and in vivo (1, 26).

As a matter a fact, Hsp70 has similar affinity for TPR1 and TPR2A (Table 2), however, in all cases the interactions are shortly lived based on the observed kinetics (supplemental Fig. S1). Most importantly the biologically relevant configuration, where Hsp70 binds to TPR1 together with substrate, and TPR2A domain is bound to Hsp90, is further stabilized by the interactions between Hsp70 and Hsp90 and the substrate. Hence, the overall affinity of the HOP for Hsp70-substrate-Hsp90 complex is higher than for individual HOP-Hsp70/Hsp90 interactions, which would favor formation of correct intermediate complex.

It is also probably necessary that the interactions are fairly transient (27), in order for the substrate to be passed on through the Hsp90 supported substrate maturation and trafficking pathway, and not get trapped in a particular intermediate state. In the Hsp90 functional cycle HOP is often replaced by TPR domain containing immunophilins in the chaperone complex (28, 29).

The TPR binding surface and the peptide ligands are highly charged, indicating that electrostatic interactions must play a key role in defining the binding affinity and specificity. Therefore, we were interested in performing a detailed analysis of the electrostatic component of binding.

Based on the continuum calculations, electrostatics in the TPR1 complex are not as optimized as in the TPR2A complex, possibly because fewer polar residues in the binding site and the larger amount of surface area buried in the interface. This could
contribute to the increased desolvation penalties at some positions, e.g. at TPR2A Lys-301 versus the equivalent TPR1 Lys-73 and TPR2A Arg-305 versus Arg-77 in TPR1. The combined effects at various positions then lead to higher $K_d$ for TPR1 for its cognate ligand compared with TPR2A.

Our calculations agreed well with previous suggestions that the four conserved carboxylic clamp binding residues are important for binding (8, 30). They were also verified by alanine-scanning mutagenesis and binding studies on TPR2A. Indeed, our earlier protein design results suggested that these, together with the fifth conserved arginine, were sufficient for low affinity recognition of Hsp90 by a 3-repeat consensus sequence TPR template (20).

To further explain differences in affinity, we studied residues other than the five conserved polar residues in the binding site. In TPR2A there are additional polar ligand binding residues compared with TPR1: Glu-271, Gln-298, and Asn-308 (Fig. 4).

Glu-271 of TPR2A, according to PB results, contributes unfavorably to interaction energy (Fig. 3), whereas FEP calculations suggest its contribution is essentially zero. Experimental studies verify that FEP calculations give a more correct picture for Glu-271 (2-fold higher $K_d$ value of 10 μM, with FEP ΔΔG = +0.14 kcal/mol and experimental ΔΔG = +0.41 kcal/mol).

Gln-298 and Asn-308 bind to the peptide at the D(0) and E(−3) side chains, respectively. Both peptide residues are predicted to contribute favorably to binding (Fig. 3). Asn-308 clearly has some importance as the N308A $K_d$ is reduced by 4-fold. The experimentally determined effect of Asn-298 was small (Table 2). However, based on measured changes in affinity, mutations E271A, N298A, and N308A together would give a 16-fold loss in affinity (i.e. from 5 μM to ~80 μM), assuming simple additivity. Cooperative effects may further reduce affinity. Therefore small contributions by these residues that are different in the two systems may together produce the 10-fold difference in the affinity observed between TPR2A and TPR1 for their ligands (Table 2).

Increasing the atomicity of the system (in FEP) improved the results for buried charged residues, apparently due to the fact that it takes into account the heterogeneity of the local environment. Although FEP calculations do not only include electrostatic contributions of exposed and buried polar residues, it has earlier been shown that more microscopic definition of the system predicts better the contribution by buried polar residues (31, 32), as also we observe.

Only a few such direct comparisons between implicit and explicit solvent models have been made on the residue-by-residue binding energy contributions, but our results are in accord with previous reports (33). We therefore suggest FEP-type calculations are a more accurate means for predicting contributions by buried polar residues.

**Engineering and Analysis of Mutations with Increased Affinity**—A key motivation for estimation of the group contributions was to find clues how to improve affinity by removing unfavorable interactions. PB calculations have been used successfully to engineer proteins with higher stability (34, 35), but to best of our knowledge the same method as here has not been used much for engineering higher affinity for ligand binding. A related Tanford-Kirkwood electrostatic model (36) has been used in design of specificity of coiled-coil recognition, whereas a previous study (37) used continuum calculations to design cytokine variants with pH-dependent loss of affinity by a histidine-switch. The most significant results on affinity improvement via electrostatic optimization have been obtained by Schreiber and co-workers, who showed that the coulombic interaction component of the electrostatic energy for encounter complex can be used for optimization of the on-off-rate of binding (38).

We could optimize the binding sites for both peptide ligands based on the PB model, including its desolvation component. However, in several cases PB calculations overestimated the penalty from desolvation of charges at the interface. Further changes, specifically the addition of positive charges (Arg-332 and Lys-334 in TPR2A and Lys-16 in TPR1), allowed further enhancement of binding affinity. In the case of the TPR2A T332R/D334K double mutant, it appears that the increased affinity is due to the formation of extra close range interactions, not just an overall increase in positive charge in the binding site for a negatively charged ligand. This was shown by the surface plasmon data; the off-rate is clearly slower, indicating that new short-range interactions have formed, while the effect on the on-rates is unclear (Fig. 5). MD simulations predict the same effect.

Unlike in some previous experiments, where mutations around the binding site were designed to increase the on-rate (38), increasing the overall charge around the binding site seemed partly to fail: mutating TPR2A Glu-230 or TPR1 Glu-83 or Glu-110 did not significantly improve affinity of binding. However, the E83Q-E110K double mutant did have 5-fold increased affinity (Table 2).

It would seem that, when the binding site is already highly charged, increasing the affinity by increasing the charge (39) may not be very fruitful, even if the affinity is only moderate, as optimization of charge distribution and interface complementarity becomes rather challenging. Our tightest binder was obtained here by engineering close range interactions (e.g. TPR1 S16K). This may depend on the system; perhaps in this case as the kinetics are fast, affecting the off-rate by creating close range interactions works best.

**Structural Basis of Specificity**—The three high resolution peptide complex structures now available help in understanding why affinities of TPR1 and TPR2A for their ligands differ, and why TPR2A has higher affinity for both ligands.

In the TPR2A-Hsp90 complex, there is a total of eleven hydrogen bonds between the protein and the peptide ligand, whereas there are only seven in the TPR1 complex. Also, the peptide conformation in TPR1 case may not be as favorable. In particular Glu(−3) carboxylate group on the peptide does not interact with anything on the protein, but it is in close contact with the hydrocarbon side chains.

Probably due to the conformational differences in the complexes (Fig. 6), TPR1 can discriminate effectively against the Hsp90 ligand and has no observable affinity for it. Also, for TPR1, residues beyond the carboxylate clamp binding ones, such as Lys-50, are crucial for discriminating against the non-cognate ligand (40). The structure of the TPR2A-Hsp70 peptide complex explains why changes in the charged residues that predominantly interact with the peptide dicarboxylic clamp have
Electrostatics and Engineering of TPR Peptide Binding

similar effects whether TPR2A interacts with cognate or with non-cognate peptide ligands (Table 2).

If the TPR2A and TPR1 domains are aligned, and the peptide ligand along with them, it is clear that the Hsp70-peptide cannot fit in the same conformation into the TPR2A binding pocket, as it does into the TPR1 binding pocket (Fig. 6). Both the Hsp70 and Hsp90 ligand bind in the same conformation to TPR2A, suggesting furthermore that the protein behaves rather rigidly on binding. Our structure of the non-cognate pair of TPR2A further confirms that specificity in TPR2A case is mainly due to the methionine of MEEVD sequence fitting its pocket better than the corresponding isoleucine in the Hsp70 C-terminal GPTIEEVD peptide.

In summary, we have used PB calculations to design variants with increased affinity and specificity in two related highly charged peptide-binding complexes of significant biological interest, the TPR1 and TPR2A domains of HOP co-chaperone of the Hsp70-Hp90 chaperone system. Overall, the most important contributions are fairly well described by the PB calculations, which can thus be powerful in guiding protein engineering to enhance ligand binding affinity, as shown in the TPR2A case with 80-fold increase achieved in affinity. The cases tested here also show that FEP calculations are a powerful tool in aiding in electrostatic design for buried residues.

The results on charge residue contributions toward binding reveal interesting charge-charge repulsion at TPR2A Asp334-Hsp90 Glu (−2), which may have relevance on regulating the affinity of the interaction. It may be important for functional reasons to keep the interaction short-lived for other TPR cofactors to be able to bind and the functional cycle to proceed (27). Otherwise the data confirm the conserved interactions on EEVD peptide interaction as the most important ones and indicate that difference in affinity toward their cognate ligand between TPR1 and TPR2A are due to differences in electrostatic optimization.

Recently, Yi and Regan (41) and Cortajarena et al. (42) have shown that TPR domains or small molecule inhibitors can be used to inhibit TPR2A-Hsp90 interaction and the Hsp90 function. Herein, we have in detail examined the binding site for hot spots, as possible sites for inhibitor intervention, and engineered a tight binding variant of TPR2A, indicating it is possible to design higher affinity inhibitory TPR domains. The present data thus help in targeting Hsp90 function for biomedical applications.

Acknowledgments—We thank Dr. Chris Wilson for making the original expression vectors of TPR2A and TPR1 and the Regan laboratory members for critical discussion. Computational resources for molecular dynamics runs were provided by Yale University, with the support of Nicholas Carriero and Robert Bjornson. J. N. S. thanks Jérôme Hénin for valuable discussion and advice on the FEP calculations.

REFERENCES
1. Chen, S., and Smith, D. F. (1998) J. Biol. Chem. 273, 35194–35200
2. Grad, I., and Picard, D. (2007) Mol. Cell Endocrinol. 275, 2–12
3. Pearl, L. H., and Prodromou, C. (2006) Annu. Rev. Biochem. 75, 271–294
4. Richter, K., and Buchner, J. (2001) J. Cell Physiol. 188, 281–290
5. Neckers, L. (2002) Trends Mol. Med. 8, 555–561
6. Demand, J., Lüders, J., and Hörfeld, J. (1998) Mol. Cell Biol. 18, 2023–2028
7. Young, J. C., Obermann, W. M., and Hartl, F. U. (1998) J. Biol. Chem. 273, 18007–18010
8. Scheufler, C., Brinker, A., Bourenkov, G., Pgoraro, S., Moroder, L., Bar- tunik, H., Hartl, F. U., and Moarefi, I. (2000) Cell 101, 199–210
9. Gilson, M. K., and Honig, B. H. (1987) Nature 330, 84–86
10. MacKerell, A. D., Jr., Bashford, D., Bellott, M., Dunbrack, R. L., Jr., Evan- seck, J. D., Field, M. J., Fischer, S., Gao, I., Guo, H., Ha, S., Joseph-Mc- Tyherty, D., Kuchin, L., Kuczera, K., Lau, F. T., Mattos, C., and Karplus, M. (1998) J. Phys. Chem. B 102, 3586–3616
11. Sheinerman, B. F., and Honig, B. (2002) J. Mol. Biol. 318, 161–177
12. Hendsch, Z. S., and Tidor, B. (1999) Protein Sci., 8, 1381–1392
13. Cortajarena, A. L., and Regan, L. (2006) Protein Sci. 15, 1193–1198
14. Kále, L., Scek, R., Bhandarkar, M., Brunner, R., Gusaroy, A., Krawetz, N., Phillips, J., Shinozaki, A., Varadarajan, K., and Schulten, K. (1999) J. Comput. Phys. 151, 283–312
15. Bhandarkar, M., Brunner, R., Chipot, C., Dalke, A., Dixin, S., Grayson, P., Gulingsrud, J., Gusaroy, A., Hardy, D., Humphrey, W., Hurwitz, D., Krawetz, N., Nelson, M., Phillips, J., Shinozaki, A., Zheng, G., and Zhu, F. (2003) NAMD User’s Guide, Version 2.5, University of Illinois, Urbanna, IL
16. Henin, J., Pohorilie, A., and Chipot, C. (2005) J. Am. Chem. Soc. 127, 8478–8484
17. Beutler, T. C., Mark, A. E., van Schaik, R. C., Gerber, P. R., and van Gun- steren, W. F. (1994) Chem. Phys. Lett. 222, 529–539
18. Lu, N., Kofke, D. A., and Woolf, T. B. (2004) J. Comput. Chem. 25, 28–39
19. Chipot, C. (2008) J. Chem. Theory Comp. 4, 2150–2159
20. Cortajarena, A. L., Kajander, T., Pan, W., Cocco, M. J., and Regan, L. (2004) Protein Eng. Des. Sel. 17, 399–409
21. Navaza, J., Paneppucci, E. H., and Martin, C. (1998) Acta Crystallogr. D Biol. Crystallogr. 54, 817–821
22. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. D Biol. Crystallogr. 53, 240–255
23. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) Nat. Struct. Biol. 6, 1041–1043
24. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132
25. Lovell, S. C., Word, J. M., Richardson, J. S., and Richardson, D. C. (2000) Proteins 40, 389–408
26. Lässle, M., Blatch, G. L., Kundra, V., Takatori, T., and Zetter, B. B. (1997) J. Biol. Chem. 272, 1876–1884
27. Pratt, W. B., and Toft, D. O. (2003) Exp. Biol. Med. (Maywood) 228, 111–133
28. Wandinger, S. K., Richter, K., and Buchner, J. (2008) J. Biol. Chem. 283, 18473–18477
29. Pratt, W. B., Galigniana, M. D., Morishima, Y., and Murphy, P. J. (2004) Essays Biochem. 40, 41–58
30. Brinker, A., Scheufler, C., Von Der Mulbe, F., Fleckenstein, B., Herrmann, C., Jung, G., Moarefi, I., and Hartl, F. U. (2002) J. Biol. Chem. 277, 19265–19275
31. Sham, Y. Y., Muegge, I., and Warshel, A. (1998) Biophys. J. 74, 1744–1753
32. Simonson, T. (2001) Curr. Opin. Struct. Biol. 11, 243–252
33. Spector, S., Wang, M., Capp, S. A., Robblee, J., Hendsch, Z. S., Fairman, R., Tidor, B., and Raleigh, D. P. (2000) Biochemistry 39, 872–879
34. Strickler, S. S., Gribenko, A. V., Gribenko, A. V., Keiffer, T. R., Tomlinson, J., Reible, T., Lolasde, V. V., and Makhadzade, G. I. (2006) Biochemistry 45, 2761–2766
35. Havernek, J. I., and Harbury, P. B. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 11145–11150
36. Sarkar, C. A., Lowenauer, K., Horan, T., Boone, T. C., Tidor, B., and Lauffenburger, D. A. (2002) Nat. Biotechnol. 20, 908–913
37. Selzer, T., Albeck, S., and Schreiber, G. (2000) Nat. Struct. Biol. 7, 537–541
38. Selzer, T., and Schreiber, G. (1999) J. Mol. Biol. 287, 409–419
39. Odunuga, O. O., Hornby, J. A., Bies, C., Zimmermann, R., Pugh, D. J., and Blatch, G. L. (2003) J. Biol. Chem. 278, 6896–6904
40. Yi, F., and Regan, L. (2008) ACS Chem. Biol. 3, 645–654
41. Cortajarena, A. L., Yi, F., and Regan, L. (2008) ACS Chem. Biol. 3, 161–166