Virtual Screening for Binding of Phenylalanine Analogues to Phenylalanyl-tRNA Synthetase

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Abstract: Although incorporation of nonnatural amino acids provides a powerful means of controlling protein structure and function, experimental investigations of amino acid analogues for utilization by the protein biosynthetic machinery can be costly and time-consuming. In this paper, we describe a computational protocol (HierDock) for predicting the relative energies of binding of phenylalanine analogues to phenylalanyl-tRNA synthetase (PheRS). Starting with the crystal structure of Thermus thermophilus PheRS without bound ligand, HierDock predicts the binding site of phenylalanine (Phe) within 1.1 Å of that revealed by the crystal structure of PheRS cocryostallized with Phe. The calculated binding energies of Phe analogues in PheRS, using HierDock, correlate well with the translational activities of the same analogues in Escherichia coli. HierDock identifies p-fluorophenylalanine and 3-thienylalanine as especially good substrates for PheRS, in agreement with experiment. These results suggest that the HierDock protocol may be useful for virtual screening of amino acid analogues prior to experiment.

1. Introduction

In vivo expression systems provide the most powerful means of controlling the composition, folding, and assembly of proteins. The power of such systems would be enhanced significantly by the development of an expanded set of amino acids that exhibit good translational activity in conventional expression hosts.

Various laboratories have demonstrated the ability of the wild-type translational apparatus to incorporate nonnatural amino acids containing fluorinated,1−4 unsaturated,5−8 electroactive,9 and other side chain functionalities.10−13 Nevertheless, the number of amino acids shown conclusively to exhibit transla-

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Here we assume that selective binding of the amino acid or
analogue to the aaRS is the step that determines the selectivity of amino acid incorporation into the growing polypeptide chain. This need not be the case: formation of the aminocacyl adenylate, attachment to tRNA, or editing by the aaRS could in principle constitute the product-determining step. However, selective binding is a necessary step, and we find herein that the calculated binding energies of a set of Phe analogues to PheRS correlate well with the translational activities of the analogues in vivo. When possible, we compare the calculated difference in binding energies to the differential free energies of binding determined from experimentally measured kinetic parameters that describe the activation of the amino acid substrate.

Predicting the binding site and binding energy for an amino acid or a novel amino acid analogue can easily become an intractable process. There might be many possible binding sites for the analogue (not just that occupied by the natural amino acid), and it is necessary to examine all such possibilities to determine if an analogue will bind to the appropriate site. Even with the binding site specified, there are many complications in predicting a binding free energy. There are often many possible configurations of the ligand and of the protein side chains, as well as uncertainty concerning solvation of the ligand and protein, and in identifying molecules and ions that might compete for the active site. To obtain a practical protocol for examining all possible sites, while predicting sufficiently accurate binding energies for the most favorable sites, we use the HierDock hierarchical strategy.21–24 HierDock starts with a coarse grain search of conformations for the binding site and ends with fine grain molecular dynamics (MD) optimization of the full ligand/protein complex, including solvation.

In this paper, we apply the HierDock computational protocol to screening of amino acid analogues for binding to the corresponding aaRS. To validate this protocol, we first predict the binding site of phenylalanylamine (Phe) in phenylalanyl-tRNA synthetase (PheRS) of Thermus thermophilus (T. thermophilus). The calculated binding site of Phe in PheRS is within 1.1 Å CRMS (coordinate root-mean-square error for all atoms) of the crystal structure. The HierDock procedure was then used to predict the binding site and to calculate the binding energies of various analogues of Phe to PheRS. We find that the calculated binding energies of Phe and its analogues correlate well with the results of experimental measurements of in vivo incorporation in Escherichia coli and of in vitro measurements of the differential free energy of binding. It should be noted that the binding energies were calculated for PheRS from T. thermophilus and compared to the experimental measurements of in vivo translational activities made on wild-type E. coli PheRS. This is reasonable because the sequence identity between E. coli and T. thermophilus for PheRS is 43% with 94% identity in the binding site. The HierDock procedure also yields a predicted binding site for each of the Phe analogues, which allows assessment of the feasibility of activation of each bound substrate. We suggest that the methods described here could be used for virtual screening of amino acid analogues prior to experiment. Such virtual screening procedures could considerably speed the development of libraries of analogues likely to be incorporated into protein in vivo.

2. Methods

2.1. Computational Methodology. We use the HierDock procedure, which has been applied successfully to study the binding of ligands to globular25–27 and membrane-bound proteins.22,24,25 The HierDock ligand screening protocol follows a hierarchical strategy for examining conformations, binding sites, and binding energies. The steps involve using coarse grain docking method to generate several conformations of protein/ligand complexes followed by molecular dynamics (MD) simulations including continuum solvation method performed on a subset of good conformations generated from the coarse grain docking. Methods combining docking and MD simulations have been tested,26 but the main drawback of these tests was that only one protein/ligand complex structure was kept from the coarse grain docking method for MD simulations. This is risky considering that the coarse grain methods do not have accurate scoring functions that include solvation. Free energy perturbation methods lead to accurate free energies of binding but are computationally intensive and not readily applicable to a wide variety of ligands.27 In this paper, we demonstrate that a hierarchical computational protocol that uses hierarchical conformation search methods along with different levels of scoring functions would allow screening of amino acid analogues for aminoacyl-tRNA synthetases. The steps in HierDock are as follows:

1. First we carry out a coarse grain docking procedure to generate a set of conformations for ligand binding. In this paper, we used DOCK 4.028 to generate and score 30,000 configurations of which 10% (3000) were ranked using the DOCK scoring function.

2. We then select the 20 best conformations for each ligand from DOCK and subject them to annealing molecular dynamics (MD) to further optimize the conformation in the local binding pocket, allowing the atoms in both the ligand and the binding cavity (residues with an atom within 5 Å of the binding ligand) to move. In this step, the ligand and the binding cavity in the protein were heated and cooled from 50 to 600 K in steps of 10 K (0.05 ps at each temperature) for five cycles. At the end of each annealing MD cycle, the best energy structure is retained. Annealing MD allows the protein cavity to readjust for interaction with the ligand. This fine grain optimization was performed using MPSim29 and full atom force field (DREIDING)30 and continuum solvation methods. We use the surface generalized Born (SGB) continuum solvent method to obtain forces and energies resulting from the polarization of the solvent by the charges of the ligand and protein. This allows us to calculate the change in the ligand structure due to the solvent field and hence obtain accurate binding energies that take into account the solvation effects on the ligand/protein structure. For the annealing MD procedure, the charges for the ligand were derived using the charge equilibration (QE) method, while charges for the protein were taken from CHARMM22.13 This procedure generated 5 × 20 = 100 good protein/ligand complexes for each ligand.

3. For the 100 structures generated by annealing MD simulations for each ligand, we minimized the potential energy (conjugate gradients) of the full ligand/protein complex in aqueous solution using SGB. This


step of protein/ligand complex optimization is critical to obtaining energetically good conformations for the complex (cavity + ligand). We then calculated binding energies as the difference between the total energy of the ligand/protein complex in solvent and the sum of the total energies of the protein and the ligand separately in solvent. The energies of the protein and the ligand in solvent were calculated after independent energy minimization of the protein and the ligand separately in water. The energy calculations used the more accurate Poisson−Boltzmann solvation method to calculate solvation energies. The nonbond interaction energies were calculated exactly using all pair interactions.

Section 3 shows that this HierDock strategy provides a practical scheme for predicting ligand binding energies and relative binding energies of Phe and its analogues to PheRS. The calculated relative binding energies correlate well with in vivo incorporation results with wild-type PheRS, reported previously.2,9,13

2.2. Procedures for Screening Analogues and Application to PheRS. PheRS offers a good test case for HierDock because a range of Phe analogues has been evaluated with respect to in vivo translational activity with wild-type PheRS. PheRS is an α2β2 heterotetrameric enzyme. The crystal structure of the T. thermophilus variant without bound Phe has been reported by Mosyak and co-workers.15 We will denote this structure (PDB code: 1PYS) as No/PheRS, where “No” indicates the crystal structure of PheRS protein with no ligand. The entity before the slash in this notation indicates the ligand, and after the slash the entity is the protein. Reshetnikova et al.36 determined the crystal structure of PheRS complexed with Phe from T. thermophilus at 2.7 Å resolution (PDB code: 1B70). We will denote this structure as Phe/PheRS, where the underline indicates that the protein structure is that of the liganded protein. The CRMS (all atoms) between the protein part of Phe/PheRS and No/PheRS is 0.4 Å, indicating that binding the ligand does not cause significant reorganization of the protein.

To determine how well the HierDock procedure works for a system in which the binding site has not been determined, we predicted the structure of PheRS with bound Phe, starting with No/PheRS. This predicted structure is denoted as Phe/PheRS. Although the binding site of Phe in PheRS is known, we did not use the explicit three-dimensional coordinates from the crystal structure in our simulations. This is because we wanted to validate how well our procedure would identify an unknown binding site and structure. This is a critical test for the procedure, because in many important applications one may not have access to a known crystal structure with the ligand bound.

We validated the force field used here by performing energy minimization on two crystal structures. We started with the No/PheRS structure (shown in red in Figure 1) and added explicit hydrogens to all heavy atoms. To represent the solvation shell expected near33 the charged residues, we neutralized the acidic residues by adding Na+ counterions and basic residues by adding Cl− counterions. The energy of this structure was then minimized with the conjugate gradient method while including solvation via the SGB continuum solvation model. We found a 0.8 Å CRMS difference between the coordinates of all atoms in the minimized structure and the crystal structure (as shown in Figure 1), which is well within the resolution of the crystal structure (2.9 Å).

To further validate our force field, we also performed a similar minimization on the 1B70 (Phe/PheRS) structure. The CRMS difference in the coordinates of all atoms between the crystal structure and the force field-minimized structure is 0.7 Å. The above two tests, summarized in Table 1, indicate that the force field, charges, and solvation description are satisfactory. The notations used for the minimized and other structures of PheRS used or generated in this study are given in Table 2.

For all of the docking studies, we started with the unliganded No/PheRS(min) structure. This was to remove any bias that might be introduced by using the crystal structure with Phe bound. Because the α chain contains the binding site, we used just this chain for the docking studies. Crystallographic water molecules were removed to allow the volume of the receptor site to be explored completely.

Table 1. Comparison of Various Predicted and Experimental Structures for PheRS

<table>
<thead>
<tr>
<th>system</th>
<th>CRMS (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1PYS and 1PYS minimized</td>
<td>0.8</td>
</tr>
<tr>
<td>1B70 and 1B70 minimized</td>
<td>0.7</td>
</tr>
<tr>
<td>predicted Phe/PheRS with 1B70 crystal structure</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 2. Notation Used To Denote the Different Ligand/Protein Complex in This Paper

<table>
<thead>
<tr>
<th>system</th>
<th>notation</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. Thermophilus PheRS crystal structure with no bound Phe (pdb: 1PYS)</td>
<td>No/PheRS</td>
</tr>
<tr>
<td>energy minimized(min) structure starting with the No/PheRS structure</td>
<td>No/PheRS(min)</td>
</tr>
<tr>
<td>No/PheRS(min) structure after MD simulations at 300 K</td>
<td>No/PheRS(dyn)</td>
</tr>
<tr>
<td>crystal structure of PheRS (T. Thermophilus) with bound Phe (pdb: 1B70)</td>
<td>Phe/PheRS</td>
</tr>
<tr>
<td>force field energy minimized structure of Phe/PheRS</td>
<td>Phe/PheRS(min)</td>
</tr>
<tr>
<td>predicted structure of Phe bound to PheRS starting from No/PheRS(dyn)</td>
<td>Phe/PheRS</td>
</tr>
</tbody>
</table>

Figure 2. α-Subunit of T. thermophilus PheRS from the crystal structure (No/PheRS, 1PYS). The dots show the potential binding sites, and the box shows the binding region used for HierDock.

We then carried out a coarse grain search using “DOCK 4.0”. The docking site was limited to a 10 Å cube (the box shown in Figure 2) in the region of the binding site for Phe. However, we made no use of the coordinates from the crystal structure with Phe bound. The DOCK search was done with the following controls:

1. Mapping Possible Binding Regions. The negative image of the receptor molecular surface (using the Connolly method) was filled with a set of overlapping spheres. A probe of 1.4 Å radius was used to generate a molecular surface. These spheres represent potential ligand binding sites. Sphere clusters were generated for the whole binding site using the program “Sphgen” from the DOCK suite of programs.

2. Defining Regions for Docking. The sphere-filled volume representing the void space or the potential binding region in the protein around the binding site was chosen from the description of the binding site as shown in Figure 2.

3. Generating Docked Conformations of the Receptor/Ligand Complexes. Starting with an arbitrary conformation of Phe, several orientations of Phe within the receptor were generated using DOCK 4.0. Here we used flexible docking with torsion minimization of ligands, a nonbond distance dependent dielectric constant of 1, and a cutoff of 10 Å for energy evaluation.

Using DOCK 4.0 to dock Phe to the No/PheRS(min) structure did not give properly bound structures because most of the structures generated were outside of the binding site. This was because the binding cavity in No/PheRS(min) was too narrow for Phe to bind. To relax the binding cavity in No/PheRS(min), we docked Phe with reduced van der Waals radii for the Phe atoms (by 50% of the original DREIDING radii) while keeping the van der Waals radii of the protein atoms unchanged. Phe was then found in the binding site. Using the scoring function from DOCK 4.0, we then selected the best structure and changed the vdW parameters back to the original values of the Phe atoms. Subsequently, the best docked conformation of the protein/ligand complex was optimized using constant temperature and constant volume MD simulations on the protein cavity at 300 K for 100 ps. This procedure relaxed the binding site of Phe in the No/PheRS(min) structure. We denote this structure as No/PheRS(dyn), implying that PheRS structure is the one after 100 ps of MD simulations. The CRMS difference of the binding cavity before and after MD is 2.1 Å, indicating rearrangement to accommodate the ligand. Figure 3 shows the relaxed binding cavity No/PheRS(dyn) in yellow as compared to the No/PheRS(min) in red. Figure 3 also compares No/PheRS(dyn) to Phe/PheRS(min) in green. The overall CRMS difference between these structures is only 1.1 Å which is within the resolution of the crystal structure, 2.5 Å. Such a relaxation procedure is applicable to proteins that do not undergo major conformational changes on ligand binding. Using the relaxed cavity predicted for No/PheRS(dyn), we then redocked Phe and each of the seven analogues shown in Scheme 1, using the same set of DOCK parameters. The analogue structures were built using PolyGraf (MSI, San Diego) and optimized in solution (SGB) using the DREIDING FF30 and charges from charge equilibration (QEq). The best 10—30 configurations for each analogue in the binding region were selected using the energy scores from DOCK 4.0 and used as input for the subsequent annealing MD step of HierDock.

Next, we carried out the fine grain HierDock procedure to select the optimum configuration for binding each ligand. Thus, the binding site was determined by considering the 100 best scoring structures of Phe in PheRS after MD annealing and optimizing the structures with minimization. This procedure was repeated to determine the binding site and binding energies of the seven Phe analogues shown in Scheme 1. As described below, the calculated binding energies correlate well with the experimental in vivo results on incorporation of these analogues into recombinant proteins. Further more, we compared the calculated binding energies with the relative free energies of binding estimated from the kinetics of pyrophosphate exchange.

2.3. Measurement of Relative Free Energies. In vitro measurements of relative free energies were performed after the theoretical predictions were made to enable direct comparison of the calculated
binding energies to the measured free energy of binding. The PheRS gene was cloned directly from *E. coli* genomic DNA with flanking primers encoding the restriction sites SacI and HindIII (primer 1, 5′-CAC CAC TGA CAC AAT GAG CTC AAC CAT GTC ACA TCT CG-3′; primer 2, 5′-CAT ATG GCT AGC AAG CTT CAT AGG TTC AAT CCC-3′). The resulting 3500 base-pair DNA fragment was gel-purified, digested with SacI and HindIII, and ligated into the expression plasmid pQE30 (Qiagen) to yield pQE-pheST, which encodes both the α and the β subunits of *E. coli* PheRS. The integrity of the cloned gene was confirmed by DNA sequencing. The cloned enzymes contained the N-terminal leader sequence MRGSHHHHHHTD-PHASST. pQE-pheST was transformed into XL-1 (Stratagene) to yield the expression strain. Protein expression was induced at OD600 = 0.6 with 1 mM IPTG. After 3 h, the cells were harvested. The enzyme was purified using Ni-NTA agarose resin under native conditions according to the manufacturer’s instructions (Qiagen), and protein was stored in Buffer A (50 mM Tris-HCl, 1 mM DTT)/50% glycerol. Aliquots were flash frozen and stored at −80 °C. The concentration of the purified enzyme was determined by absorbance at 280 nm under denaturing conditions.

Measurement of relative free energy of binding was performed by determining the kinetics of the ATP-PP i exchange. The assay buffer contained 30 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM DTT, 2 mM ATP, and 2 mM [32P]-PP i (0.5 TBq/mol). The enzyme concentration was 100 nM. The amino acid concentration varied depending on the activity of the enzyme toward the substrate (Phe, 10–500 μM; others, 30 μM–10 mM). Aliquots (20 μL) of reaction mixture were quenched into 500 μL of quench buffer (200 mM PP i, 7% w/v HClO4, and 3% activated charcoal). The charcoal was washed twice with wash buffer (10 mM PP i, 0.5% HClO4) and counted. The data reported here were the average value from duplicated experiments. The kinetic parameters were obtained by nonlinear regression fitting analysis according to the Michaeli–Menten kinetics model.

3. Results and Discussion

3.1. Prediction of the Binding Site of Phe in PheRS. Figure 4 shows the predicted bound structure (Phe/PheRS) starting from the No/PheRS(min) structure. The substrate Phe forms seven H-bonds to the residues Trp149, Arg204, His178, Gln218, Ser180, Glu220, and His178. It is important to note that (1) Gln218 and Glu220 make two hydrogen bonds with the amino group of Phe; (2) the carboxyl oxygen of Phe hydrogen bonds with the backbone amide group of Trp149; and (3) the side chains of Phe258, Phe260, Val261, Gly282, Ala283, Gly284, Phe315, and Gly316 form a hydrophobic pocket that sandwiches the phenyl ring of the ligand.

Table 3 lists the distances of the hydrogen bonds to Phe in Phe/PheRS. These hydrogen bond distances agree well with the Phe/PheRS(min) structure. These distances also agree well with the hydrogen bonds in the Phe/PheRS (pdb code: 1B70) crystal structure, column 5 in Table 3. The calculated hydrogen bonds in Phe/PheRS are typically shorter than those in the Phe/PheRS structure, probably because the vibrations at finite temperature lead to an expansion in the anharmonic hydrogen bonds. Table

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**Table 3.** Hydrogen Bond Distances and vdW Interactions in the Phe Binding Sites of PheRS/Phe Complexes

<table>
<thead>
<tr>
<th>substrate</th>
<th>Phe residue atom</th>
<th>Phe/PheRS</th>
<th>Phe/PheRS(min)</th>
<th>Phe/PheRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe−O</td>
<td>Trp149 Nε1</td>
<td>2.97</td>
<td>2.97</td>
<td>3.32</td>
</tr>
<tr>
<td>Phe−O</td>
<td>Arg204 Nε1</td>
<td>3.07</td>
<td>2.87</td>
<td>3.28</td>
</tr>
<tr>
<td>Phe−O</td>
<td>Gln218 Nε2</td>
<td>2.93</td>
<td>3.02</td>
<td>3.46</td>
</tr>
<tr>
<td>Phe−O</td>
<td>His178 Nδ1</td>
<td>2.93</td>
<td>3.40</td>
<td>3.50</td>
</tr>
<tr>
<td>Phe−N</td>
<td>Glu220 Oε2</td>
<td>4.49</td>
<td>4.32</td>
<td>3.28</td>
</tr>
<tr>
<td>Phe−N</td>
<td>Gln181 Oε1</td>
<td>2.99</td>
<td>3.08</td>
<td>3.31</td>
</tr>
</tbody>
</table>

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**Note:**

support protein synthesis in an *E. coli* p

...mutant form of PheRS that exhibits relaxed substrate specific-... into protein in conventional expression strains. The analogues to the left of the line in Figure 5 are observed from in vivo experiments with conventional *E. coli* strains to...analogues that are not incorporated experimentally. Most importantly, *p*-fluorophenylalanine and 3-thienylalanine, both of which have been shown experimentally...
to incorporate into recombinant proteins in vivo, are predicted to exhibit the highest binding energies. It is noteworthy that histidine, a natural amino acid, shows an unfavorable binding energy as compared to Phe. This correlation with experimental results suggests that this virtual screening procedure may be useful for screening analogues for other aaRS.

Table 5 analyzes the contributions to the binding energy from the Coulomb, van der Waals, and solvation terms. We see that desolvation and Coulomb interactions favor p-fluorophenylalanine, p-chlorophenylalanine, and p-bromophenylalanine. On the other hand, van der Waals interactions favor p-fluorophenylalanine, Phe, and 3-thienylalanine but are not as favorable for p-chlorophenylalanine or the bulky p-bromophenylalanine. Figure 6 shows the van der Waals surface (as dotted pink spheres) of the bromine atom in p-bromophenylalanine in its binding cavity as predicted in the present study. We see that the van der Waals surface of bromine clashes with that of the methyl group of the Ala314 (green dots in Figure 6). This is consistent with the binding energy values, which show that the van der Waals interactions in the hydrophobic pocket of the binding cavity distinguish the various analogues. Differential solvation is also critical to these predictions, indicating that it is important to obtain fast and accurate predictions of solvation (for both structure optimization and scoring).

Table 5 indicates that Phe has a binding energy (computational) that is 3.79 kcal/mol more favorable than p-fluorophenylalanine and 9.40 kcal/mol better than that of 3-thienylalanine. To test these predictions, the experimental kinetics of pyrophosphate exchange were analyzed to yield the differential free energy of binding via eq 2

$$K_m^{\text{a}/K_m^{\text{b}}} = \exp(\Delta \Delta G/(RT)) \tag{2}$$

in which the superscript a refers to the cognate amino acid, and the superscript b refers to the amino acid analogue. The quantity $\Delta \Delta G$ is the difference in the free energies of binding of the analogue ($\Delta G^a$) and the cognate amino acid ($\Delta G^b$), that is, $\Delta \Delta G = \Delta G^a - \Delta G^b$.39 The kinetic analysis yields differential binding energies considerably smaller than those predicted computationally; p-fluorophenylalanine and 3-thienylalanine bind just 0.98 and 1.09 kcal/mol, respectively, more weakly than phenylalanine. Part of this difference arises because the energies from the theory are for minimized structures (0 K), while the experimental results are at ~300 K. Molecular dynamics studies at 300 K would lead to wide excursions of the molecules over the binding site, leading to less differentiation between the energies of the various bound states. However, the energies obtained from the HierDock method refer only to the binding event and do not take into account changes in the activation energy of the catalytic step.39 Thus, while the qualitative conclusions drawn from the computational work are of substantial value, quantitative prediction of differential binding energies will require further refinement of these computational methods.

3.3. Predicted Binding Sites for p-Fluorophenylalanine and 3-Thienylalanine. Comparison of the best predicted structures of Phe/PheRS with those of p-fluorophenylalanine and 3-thienylalanine in their respective binding sites shows that the overall CRMS difference between the binding site of p-fluorophenylalanine and Phe/PheRS is 0.4 Å, identical to that for 3-thienylalanine. However, the CRMS difference between the binding sites of 3-thienylalanine and p-fluorophenylalanine is 0.1 Å, much less than the CRMS between Phe/PheRS and the corresponding binding sites of p-fluorophenylalanine and 3-thienylalanine. Tables 6 and 7, respectively, list all of the hydrogen bonds and van der Waals contacts that p-fluorophenylalanine and 3-thienylalanine make in their respective binding cavities. Figure 7 compares the binding pockets of p-fluorophenylalanine and 3-thienylalanine to Phe/PheRS. It is seen from list 2 in Table 7 that the side chain of 3-thienylalanine makes a more favorable van der Waals contact to Phe260 (3.8 Å) as compared to p-fluorophenylalanine (4.3 Å) with Phe260. However, p-fluorophenylalanine makes close van der Waals contacts with Ala314 (3.4 Å) and Val261 (3.4 Å) as compared to Phe/PheRS. It is also seen that the amino terminus of 3-thienylalanine is farther from Arg204 and His178 as compared to p-fluorophenylalanine. This could be due to the larger size of the fluorine

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39 Fersht, A. Structure and Mechanism in Protein Science; W. H. Freeman and Co.: New York, 1999. Fersht has pointed out that use of the ratio of Michaelis constants to calculate relative binding energies neglects the contribution of binding to catalysis. He recommends instead use of the ratio of specificity constants, $(k_{cat}/K_m^a)/(k_{cat}/K_m^b)$. However, the energies obtained from the HierDock method refer only to the binding event and do not take into account changes in the activation energy of the catalytic step. If we apply the Fersht treatment to the data in Table 5, the values of $\Delta \Delta G$ are 1.35 and 2.13 kcal/mol for 3-thienylalanine and p-fluorophenylalanine, respectively. The latter value is in excellent agreement with that reported by Gabius et al. (Gabius, H. J.; von der Haar, F.; Cramer, F. Biochemistry 1983, 22, 2331–2339).
atom, which moves the \( p \)-fluorophenylalanine more toward Arg204 and His178.

4. Summary

The HierDock protocol predicts the binding site for Phe in PheRS to within 0.7 Å CRMS of the crystal structure, suggesting that it might correctly predict the binding site for Phe analogues in PheRS. Using this procedure, we predicted the binding sites and binding energies of seven Phe analogues. The two predicted to have the most favorable binding energies, \( p \)-fluorophenylalanine and 3-thienylalanine, are the only analogues that have been incorporated into recombinant proteins in an \textit{E. coli} host harboring an unmodified PheRS. These results suggest that the HierDock protocol may be useful as a virtual screening tool for designing nonnatural amino acid analogues for protein engineering. Further refinement will be required for accurate prediction of differential binding energies of amino acid substrates.

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