Design, expression and characterization of mutants of fasciculin optimized for interaction with its target, acetylcholinesterase

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Predicting mutations that enhance protein–protein affinity remains a challenging task, especially for high-affinity complexes. To test our capability to improve the affinity of such complexes, we studied interaction of acetylcholinesterase with the snake toxin, fasciculin. Using the program ORBIT, we redesigned fasciculin’s sequence to enhance its interactions with Torpedo californica acetylcholinesterase. Mutations were predicted in 5 out of 13 interfacial residues on fasciculin, preserving most of the polar inter-molecular contacts seen in the wild-type toxin/enzyme complex. To experimentally characterize fasciculin mutants, we developed an efficient strategy to over-express the toxin in Escherichia coli, followed by refolding to the native conformation. Despite our predictions, a designed quintuple fasciculin mutant displayed reduced affinity for the enzyme. However, removal of a single mutation in the designed sequence produced a quadruple mutant with improved affinity. Moreover, one designed mutation produced 7-fold enhancement in affinity for acetylcholinesterase. This led us to reassess our criteria for enhancing affinity of the toxin for the enzyme. We observed that the change in the predicted inter-molecular energy, rather than in the total energy, correlates well with the change in the experimental free energy of binding, and hence may serve as a criterion for enhancement of affinity in protein–protein complexes. Keywords: acetylcholinesterase/binding affinity/computational protein design/fasciculin/protein–protein interactions

Introduction

Computational protein design has been used frequently to predict stabilizing mutations (Malakauskas and Mayo, 1998), to repack protein cores and to redesign entire protein sequences (Dahiyat and Mayo, 1997). Design of protein–protein binding interactions is one of the directions in the field that presents particular interest. Indeed, the capability of creating a binding partner for any given protein would allow us to construct inhibitors for a specific disease-associated pathway, thus revolutionizing the pharmaceutical industry. However, to date, only relatively few studies have reported successful redesign of protein–protein interfaces. Computational methods have been used to substantially increase protein binding specificity in calmodulin-target complexes (Shifman and Mayo, 2002, 2003; Yosef et al., 2009) and to supply proteins with novel binding specificities (Reina et al., 2002; Kortemme et al., 2004; Joachimiak et al., 2006). Introduction of computationally designed mutations at protein–protein interfaces helped to convert a native homodimer to a heterodimer (Bolon et al., 2005), and to construct chimeric proteins with novel DNA-binding properties (Chevalier et al., 2002). Optimization of electrostatic potential between two proteins was shown to increase association rates significantly (Selzer et al., 2000; Kiel et al., 2004).

It remains difficult, however, to use existing protein design methodology to predict affinity-enhancing mutations of residues located at protein–protein binding interfaces. Computational design of point mutations intended to increase binding affinity have met with variable success, while redesign of entire binding interfaces was often found to result in reduced affinities (Shifman and Mayo, 2003; Clark et al., 2006; Palmer et al., 2006; Song et al., 2006). A recent study by Kuhlman and colleagues suggests an approach that predicts single affinity-enhancing mutations by substituting polar residues at the binding interface by hydrophobic residues, and hydrophobic residues by larger hydrophobic residues (Sammond et al., 2007). This approach, probably the most successful so far, cannot, however, be applied to the redesign of entire binding interfaces, since it is inherently bound to increase the hydrophobicity of the individual proteins, thus enhancing their propensity to aggregate. In addition, removal of polar residues at the binding interface is likely to reduce the binding specificity of the bio-molecular interaction, which is usually not desirable. We set as our goal the development of a more general strategy for predicting affinity-enhancing mutations in protein complexes. Such a strategy would allow for the improvement of both polar and hydrophobic interactions at protein–protein interfaces.

As our model system we chose a complex of the synaptic enzyme, acetylcholinesterase (AChE) with a polypeptide toxin present in the venom of the green mamba, fasciculin-2 (Fas). There were several reasons for this choice. First, the interaction displays a very high affinity (Karlsson et al., 1985; Eastman et al., 1995; Radic et al., 1995), thus making...
the Fas/AChE complex an excellent and challenging system for designing enhanced protein–protein interactions. Secondly, structural studies reveal that no substantial conformational change occurs upon binding (Bourne et al., 1995; Harel et al., 1995; Kryger et al., 2000), thus simplifying the design and the analysis procedures. Lastly, a convenient assay is available for assessing the changes in the affinity of Fas for AChE.

AChE is a synaptic enzyme that terminates impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter, acetylcholine (Zimmerman and Soreq, 1985); it belongs to the family of three-finger toxins that are within 4 Å of Fas in the Fas–AChE complex structure (Harel et al., 1995). In addition, electrostatic interactions between cationic residues on the interaction surface of Fas and anionic residues on the interaction surface of AChE play an important role in the binding process (Radic et al., 1997).

A few studies have been performed to determine the contribution of individual amino acid residues of Fas to the interaction between the two proteins. Karlsson and coworkers neutralized positive charges on Fas by chemical modification of single lysines or arginines, and reported a substantial decrease in Fas activity (Cervenansky et al., 1994; Cervenansky et al., 1995). Marchot et al. used structural and kinetic data to construct 14 single and double Fas mutants (Marchot et al., 1997). They assayed these mutants on mouse AChE (mAChE), measuring inhibitory activity by an indirect assay based on titration of the mutants with a polyclonal anti-Fas serum. Seven of these mutants lost some or all their affinity for the enzyme compared to that of wild-type Fas (FasWT), while four mutants showed no change in affinity. The remaining three Fas mutants surprisingly exhibited an increased inhibitory activity against mAChE. However, the authors suggested that this may reflect immuno-dominant determinants in these regions or intra-molecular rearrangements in conformation that enhance the interaction (Marchot et al., 1997). In contrast to the earlier studies, we chose to develop a quantitative computational approach in attempt to optimize the Fas–AChE interaction. Mutations in Fas that were computationally predicted to stabilize the Fas/TcAChE complex were tested experimentally by expressing the Fas mutants and measuring their binding affinity for TcAChE. To this end, we developed a reproducible and efficient methodology for overexpression and refolding of WT and mutant forms of Fas in Escherichia coli. This was a challenging task due to the small size of Fas (61 residues) and to the fact that it contains four intra-chain disulphides (Fig. 1A).

Materials and methods
Computational design
The protein redesign program ORBIT was used for Fas design (Dahiyat and Mayo, 1997). The residues on Fas that are within 4 Å of TcAChE in the Fas–TcAChE complex structure (Harel et al., 1995) were selected for optimization (positions 6, 8, 9, 11, 12, 27, 29, 32, 33, 34, 35, 37 and 61). All amino acids, except for Pro, Cys and Gly, were considered at all the design positions. The residues on TcAChE that are within 4 Å of Fas in the Fas–TcAChE complex structure were allowed to change their side-chain conformation. Rotamer libraries used for the Fas–TcAChE optimizations were based on the backbone-dependent library of Dunbrack and Karplus (Dunbrack and Karplus, 1993), with additional rotamers expanded by one standard deviation around their mean \( \chi_1 \) and \( \chi_2 \) values for all residues except Lys and Arg. A potential energy function that included terms for Van der Waals, electrostatic and hydrogen bonding interactions, and for surface area-based solvation, was used to calculate side chain/side chain and side chain/backbone pairwise interactions as described (Dahiyat and Mayo, 1997; Gordon et al., 1999; Street and Mayo, 1999). In the Fas design, we used the ORBIT energy function that was optimized to better reproduce the side-chain conformations in the data set of protein–protein interfaces (O. Sharabi and J. M. Shifman, in preparation). In this energy function, the value of the distance-dependent dielectric constant used to calculate the electrostatic interactions was set to 10r (where r is distance between two atoms) and the penalty for hydrophobic burial, \( \sigma_p \), was set to 0.005 kcal mol\(^{-1}\) Å\(^{-2}\). The calculated energies served as input to a side-chain selection

Fig. 1. (A) The amino acid sequence of Fas, showing the four intra-chain disulphide bridges and the interfacial residues chosen for redesign. (B) The Fas/TcAChE binding interface before redesign; (C) The Fas/TcAChE binding interface after redesign. Fas (pink) sits at the entrance of the narrow gorge leading to the active site of TcAChE (gray). The side-chains on Fas and TcAChE selected for computational optimization are displayed as pink and blue sticks, respectively. The numbers are shown for the Fas residues for which affinity-enhancing mutations had been predicted.
procedure that used the Dead-End Elimination theorem (Desmet et al., 1992; Gordon et al., 2003). All optimizations were performed using a cluster of Xeon computers.

**Gene construction**

The gene construct encoding Fas\(^{WT}\) was cloned into the bacterial expression vector pET-25b (Novagen). The pelB leader peptide was removed from the expression vector in the cloning process. The genes for the designed Fas mutants were constructed based on the skeleton of the WT Fas expression vector using the standard site-directed mutagenesis procedure. The full open reading frames of both Fas\(^{WT}\) and Fas mutants were encoded without additional residues.

**Expression of Fas\(^{WT}\) and mutants**

Fas\(^{WT}\) and Fas mutants were expressed using the E. coli BL21(DE3) cell line at 37°C. Induction was initiated by the addition of 0.1 mM isopropyl-D-thiogalactopyranoside (IPTG) when the optical density of the culture had reached 0.8 OD at 600 nm. 3.5 hours after induction, the cells were harvested by centrifugation, and resuspended in a lysis buffer containing 50 mM phosphate, 1% Triton X100, lysozyme (1 mg/L), 15 mM DNase I and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.8. The cells were sonicated and centrifuged at 16 000 rpm for 20 min at 4°C. The pellets containing the inclusion bodies (IBs) were collected and stored at −20°C.

**Refolding of Fas variants**

The IBs were dissolved in the denaturing buffer (6 M GdHCl, 10 mM EDTA, 50 mM phosphate, pH 8.0) by sonicating the samples at 0°C. Reduction of the disulphide bonds of the protein samples was carried out by the addition of 0.1 M 1,4-dithiothreitol (DTT), followed by shaking at room temperature for 2 h. The non-soluble material was removed by ultracentrifugation of the sample at 35 000 rpm at 4°C. The denatured and reduced Fas samples were purified by reversed-phase HPLC using an acetonitile/water gradient. The Fas samples eluted at ~30% acetonitrile. The concentration of the purified protein was determined by measuring the absorption at 280 nm. The purified Fas samples were diluted in the denaturing buffer to a final concentration of ~10 µM, and slowly dialyzed into the refolding buffer (0.5 M GdHCl, 0.3 M NaCl, 2 mM reduced glutathione, 1 mM oxidized glutathione, 5 mM EDTA, 100 mM Tris, pH 9.0). To remove the reducing agents, three more rounds of dialysis were performed against a buffer containing 0.3 M NaCl, 5 mM EDTA, 100 mM Tris, pH 9.0, at 4°C. The correct molecular mass of the refolded protein was confirmed by mass spectrometry, and the absence of free SH groups by use of the Ellman assay (Ellman, 1959).

**AChE activity assays**

Native, snake-derived fasciculin-II was purchased from Alomone Laboratories (Jerusalem, Israel). TcAChE was purified from electric organ tissue of T. californica (Sussman et al., 1988), and AChE activity assays were performed as previously described (Ellman et al., 1961). TcAChE at 0.04 nM concentration was pre-incubated with 0.6 mM DTNB for a variable period of time (usually, 10–70 s). TcAChE activity was measured immediately after the addition of 1 mM ATC. The percent of TcAChE activity for the sample pre-incubated for a certain period of time was calculated by dividing the measured TcAChE activity of the sample by that of the sample containing TcAChE in the absence of Fas. To fit the data, we assumed that Fas binding to TcAChE could be

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described by the opposing association and dissociation reactions:

\[ \text{Fas} + \text{AChE} \rightleftharpoons \text{Fas} \cdot \text{AChE} \]

When the Fas concentration is much greater than that of \( Tc\text{AChE} \), the percentage of enzymatic activity can be described by the following expression that combines the \( k_{\text{on}} \) and \( k_{\text{off}} \) rate equations (Laidler, 1965):

\[
\% \text{ activity} = 100 \left( \frac{k_{\text{off}}}{[\text{Fas}]k_{\text{on}} + k_{\text{off}}} + \frac{[\text{Fas}]k_{\text{on}}}{[\text{Fas}]k_{\text{on}} + k_{\text{off}}} \exp\left(-\left([\text{Fas}]k_{\text{on}} + k_{\text{off}}\right)t\right) \right).
\]

where \([\text{Fas}]\) is the total concentration of Fas at \( t = 0 \), and \( t \) is the time elapsed from the addition of Fas. The kinetic data were fitted using Eq. (3) in order to determine \( k_{\text{on}} \) and \( k_{\text{off}} \).

**Results**

We used the computational design program ORBIT (Dahiyat and Mayo, 1997) to optimize the Fas sequence for interaction with \( Tc\text{AChE} \). Starting from the X-ray structure of the Fas–\( Tc\text{AChE} \) complex (Harel et al., 1995), we defined the Fas–\( Tc\text{AChE} \) binding interface by selecting the residues on each protein that are within 4 Å of the second protein in the complex (Fig. 1B). The 13 Fas residues belonging to the binding interface were simultaneously redesigned, using all amino acids as possible candidates except for Pro, Cys and Gly. At the same time, the side chains of 28 residues on \( Tc\text{AChE} \) were allowed to change their conformations (Fig. 1B). The optimal Fas sequence was selected by minimizing the total energy of the Fas–\( Tc\text{AChE} \) complex. The calculation was performed using the energy function optimized for design of partially polar protein–protein interfaces (see Materials and methods). The optimization yielded a Fas mutant, Fas\text{des}, with five interfacial mutations with respect to Fas\text{WT}: T8V, T9N, R11K, H29R and K32R (Fig. 1C).

To access experimental parameters for the binding of Fas\text{des} to \( Tc\text{AChE} \), we first had to develop a strategy for expression of Fas mutants. Fas\text{WT} from the green mamba venom could be obtained from a commercial source, while recombinant Fas\text{WT} and Fas mutants had been previously expressed only in mammalian cells (Marchot et al., 1997). We chose, however, to express Fas variants in \( E.coli \). The main challenge in making this choice is the presence of four intra-chain disulfide bonds in a protein as small as Fas, making it prone to mis-folding when expressed in a prokaryotic host. Initially, we attempted to express Fas\text{WT} in strains of \( E.coli \) in which disulfide bond formation is enhanced. Both periplasmic expression and expression in cells in which mutations had been introduced into the thioredoxin reductase (trxB) and glutathione reductase (gor) genes (e.g. Rosetta-Gami(DE3)) were unsuccessful. Although we were able to obtain some soluble Fas\text{WT}, the protein displayed a large content of free thiol groups, and exhibited very weak inhibition of AChE, indicating that the Fas\text{WT} produced was largely misfolded (data not shown). Hence, we decided to pursue an alternative strategy that involved expressing of the Fas\text{WT} as IBs, followed by a refolding procedure. The gene corresponding to Fas\text{WT} was cloned into the pET-25b vector and expressed in BL21(DE3) cells. A large amount of the Fas polypeptide indeed expressed as IBs. The IBs were denatured and reduced, the Fas was purified by reverse phase HPLC and was then slowly refolded making use of a GSH-GSSG redox buffer. The refolded Fas\text{WT} migrated identically to commercially available Fas\text{WT} on SDS–PAGE (insert to Fig. 2). The correct molecular mass of the refolded protein was confirmed by mass spectrometry, and the absence of free thiol groups by the Ellman assay (Ellman, 1959). Since the formation of all four disulphide bonds is highly unlikely to occur in a misfolded species, the absence of free SH groups in the purified protein provides strong evidence that the Fas\text{WT} had indeed folded correctly. To further verify the correctness of the Fas fold, we compared the inhibitory activity of our refolded Fas\text{WT} against \( Tc\text{AChE} \) to that of the commercially available Fas\text{WT}. Figure 2 shows that the inhibitory activities of the two Fas samples are equal within experimental error. The above experiments clearly demonstrate that our expression/refolding procedure yields the correctly folded and fully active Fas\text{WT} in reasonable quantities (~1 mg of protein from 1.5 l of \( E.coli \) culture).

Next, we used the same procedure to obtain Fas\text{des}. Less than 3% of free SH groups were detected in the protein sample after refolding, indicating that the correct Fas structure had been attained for the vast majority of the Fas\text{des} molecules.

To measure the binding affinity of Fas\text{WT} and Fas\text{des} for \( Tc\text{AChE} \), we performed \( Tc\text{AChE} \) activity assays in the presence of each Fas variant. A large range of Fas concentrations were explored to obtain a full \( Tc\text{AChE} \) inhibition profile (Fig. 3A). Since binding of Fas to \( Tc\text{AChE} \) inactivates the enzyme almost completely, the fractional enzymatic activity is inversely proportional to the Fas-bound enzyme species and can thus be used to calculate the binding affinity (\( K_d \) of...
To determine whether the increase in $\Delta G_{\text{bind}}$ displayed by Fas$^{\text{des}}$ is due to a single mutation that is highly destabilizing for the Fas–TcAChE complex or to the sum of several slightly destabilizing mutations, we constructed all single mutants derived from the sequence of Fas$^{\text{des}}$. In addition, we made a double mutant, T8V/T9N, to explore the possibility that these two spatially proximal mutations are energetically coupled. TcAChE activity measurements in the presence of single and double Fas mutants are shown in Fig. 3A, while changes in binding affinity ($K_d$) and free energy of binding to TcAChE are summarized in Table I and Fig. 3B. Two mutations, K32R and T9N, proved to be rather deleterious for Fas binding to TcAChE, resulting in increases of 1.2 and 1.3 kcal/mol, respectively, in $\Delta G_{\text{bind}}$. Mutation R11K destabilized the Fas–TcAChE complex very slightly, while mutation T8V was moderately stabilizing. Mutation H29R, however, improved the binding affinity for TcAChE considerably, producing a 1.1 kcal/mol decrease in $\Delta G_{\text{bind}}$. The double mutant, T8V/T9N, exhibited a 0.4 kcal/mol increase in $\Delta G_{\text{bind}}$. Simultaneous introduction of the T8V and T9N mutations destabilized the Fas–TcAChE complex by a smaller amount compared to the sum of $\Delta G_{\text{bind}}$s observed for the two individual mutations (0.75 kcal/mol). Hence, these two mutations are indeed energetically coupled. The rest of the single mutations were additive, producing a 0.84 kcal/mol increase in $\Delta G_{\text{bind}}$ for the sum of all the designed mutations in the Fas$^{\text{des}}$ sequence (T8V/T9N+R11K+H29R+K32R) compared to 0.82 kcal/mol measured for Fas$^{\text{des}}$. In an attempt to obtain a multiple Fas mutant that would bind to TcAChE better than Fas$^{\text{WT}}$, we constructed the Fas$^{\text{des}}$R32K variant. In this variant, all the designed mutations were incorporated except for K32R, which had proved to be the most deleterious mutation. Fas$^{\text{des}}$R32K showed an enhanced binding affinity for TcAChE corresponding to a 0.4 kcal/mol decrease in $\Delta G_{\text{bind}}$ (Fig. 3B and Table I).

Our computational design procedure seeks to increase the overall stability of the Fas–TcAChE complex, but cannot make predictions with respect to changes in the rates of association and dissociation of the two proteins ($k_{\text{on}}$ and $k_{\text{off}}$, respectively). Nevertheless, it is interesting to measure the effect of the predicted Fas mutations on the kinetics of the Fas–TcAChE interaction. To determine $k_{\text{on}}$ and $k_{\text{off}}$ for interaction of the Fas mutants with TcAChE, we performed the TcAChE activity assays under conditions in which the interaction of the two proteins approaches equilibrium. In these experiments, a Fas mutant was pre-incubated with TcAChE activity measurements in the presence of single and double Fas mutants [see Eq. (2) in Materials and methods]. The $K_d$ of Fas$^{\text{WT}}$ for TcAChE was found to be 0.31 ± 0.07 nM, very similar to the value of 0.4 nM reported previously (Weiner et al., 2009). Fas$^{\text{des}}$ bound to TcAChE with a $K_d$ of 1.2 ± 0.2 nM, corresponding to a ~0.8 kcal/mol increase in the free energy of binding, $\Delta G_{\text{bind}}$, compared to Fas$^{\text{WT}}$ (Fig. 3B).
quintuple mutant Fas\textsuperscript{des}. $k_{\text{off}}$ was unaltered for the H29R and R11K mutants. The rest of the Fas mutants (T8V/T9N, K32R and Fas\textsuperscript{des}) displayed a several-fold increase in $k_{\text{off}}$. The $K_d$ values calculated from the kinetic constants are in good agreement with the $K_d$ values obtained in the equilibrium experiments (Table I).

**Discussion**

Our optimization produced a Fas mutant, Fas\textsuperscript{des}, with a binding interface composition 62% identical to that of Fas\textsuperscript{WT}. In this design procedure, we achieved a wild-type recovery rate higher than that observed on the average in protein design studies (51 and 27% in protein cores and surfaces, respectively) (Kuhlman and Baker, 2000). In addition, the optimization almost completely preserved the polar/hydrophobic content of the Fas\textsuperscript{WT}/Tc\textsubscript{AChE} interface, although no hydrophobic patterning had been imposed during the design. These two results indicate that our energy function was able to reproduce successfully most of the favorable interactions in a high-affinity protein–protein complex. Nevertheless, Fas\textsuperscript{des}, with the five designed mutations, exhibited a $\sim$4-fold reduction in affinity for Tc\textsubscript{AChE} compared to Fas\textsuperscript{WT} when measured experimentally. This reduction is attributed to an increase in $k_{\text{off}}$.

Examination of single and double Fas mutants derived from the Fas\textsuperscript{des} sequence revealed that the reduced binding affinity is due primarily to one mutation, K32R, and removal of this mutation indeed resulted in a Fas mutant displaying increased affinity for Tc\textsubscript{AChE}. At least one designed mutation, H29R, enhanced the affinity of the Fas–Tc\textsubscript{AChE} complex significantly, due to enhancement of $k_{\text{on}}$. It should be noted that this mutation involves substitution of a polar residue by another polar residue, and does not increase the buried hydrophobic surface area of the Fas–Tc\textsubscript{AChE} interface, in contrast to the strategy previously suggested for enhancing protein binding affinity (Sammond \textit{et al.}, 2007). A closer look at the design results revealed that our best Fas mutant, H29R, is predicted to considerably improve intermolecular interactions between Fas and Tc\textsubscript{AChE}. Our worst Fas mutant, K32R, in contrast, is predicted to improve interactions within Fas, while slightly destabilizing the Fas–Tc\textsubscript{AChE} complex. To better understand the shortcomings of our computational procedure, we calculated the relative energetic contributions of inter- and intra-molecular interactions for each of the designed Fas mutations. In doing so, we summed all the molecular interactions considered in the ORBIT energy function, including van der Waals interactions, electrostatic interactions, hydrogen bonding and solvation. In addition, using the structure of the Fas–m\textsubscript{AChE} complex, we could determine the energetic contributions of the various Fas mutants. Error bars represent the standard deviation obtained by repeating the experiments shown in (A). The experiment for K32R was performed only once.
Table I. Binding parameters for interaction of Fas mutants with Tc-AChE

| Mutant  | $K_d^{\text{equilibrium}}$, nM | $\Delta G_{\text{bind}}^d$, kcal/mol | $K_d$, nM | $k_{\text{off}}$, $\times 10^7$, M$^{-1}$ s$^{-1}$ | $k_{\text{on}}^{\text{tot}}$, $\times 10^2$, s$^{-1}$ |
|---------|---------------------------------|-------------------------------------|----------|-----------------------------------------------|-----------------------------------------------|
| Fas$^{\text{WT}}$ | 0.31 ± 0.07                     | $-$                                 | 0.37 ± 0.07 | 6.8 ± 1.5                                     | 2.5 ± 0.9                                    |
| Fas$^{\text{des}}$ | 1.2 ± 0.2                       | 0.82                               | 1.02 ± 0.02 | 11.1 ± 0.6                                    | 11.3 ± 0.5                                   |
| T8V     | 0.125 ± 0.005                   | $-$                                 | $-$        | $-$                                           | $-$                                          |
| T9N     | 2.8 ± 0.2                       | 1.3                                | $-$        | $-$                                           | $-$                                          |
| T8V/T9N | 0.64 ± 0.09                     | 0.42                               | 0.60 ± 0.05 | 8.5 ± 0.7                                     | 5.05 ± 0.05                                  |
| R11K    | 0.53 ± 0.05                     | 0.31                               | 0.63 ± 0.05 | 4.7 ± 0.4                                     | 2.93 ± 0.02                                  |
| H29R    | 0.052 ± 0.009                   | $-$                                 | 0.03 ± 0.02 | 39.0 ± 3.5                                    | 1.27 ± 0.9                                   |
| K32R    | 2.3 ± 0.2                       | 1.2                                | 2.6        | 3.5                                           | 9.0                                          |
| Fas$^{\text{des}}$ R32K | 0.16 ± 0.02                   | $-$                                 | $-$        | $-$                                           | $-$                                          |

$^{a}K_d^{\text{equilibrium}}$ was calculated by fitting the data on Fig. 3A to Eq. (2).

$^{b}$The $\Delta G_{\text{bind}}$ is the difference in the experimental binding free energy between the Fas mutant and Fas$^{\text{WT}}$. It was calculated from $K_d^{\text{equilibrium}}$.

$^{c}$These values were calculated by fitting the kinetic data presented in Fig. 4A to Eq. (3).

In conclusion, using our computational design procedure, we were able to identify a single Fas mutant with a significantly enhanced binding affinity for Tc-AChE, and a quadruple Fas mutant with a slightly enhanced binding affinity for TcAChE. Inspection of our data revealed a better correlation.

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Fig. 5. Correlation between the experimentally observed change in free energy of binding, $\Delta G_{\text{bind}}^d$, and the change in the calculated energies for the Fas–Tc-AChE complex. (A) Correlation with the total energy change, $\Delta E_{\text{tot}}$. (B) Correlation with the inter-molecular energy change, $\Delta E_{\text{inter}}$. (Closed circle) Fas mutants tested for binding to TcAChE in this study. (Closed inverted triangle) Fas mutants previously tested for binding to mAChE. We excluded Fas mutants with amino acid deletions or with mutations outside the Fas–mAChE interface, as well as mutants for which an exact value of $K_d$ had not been reported. The lines represent the linear fitting of all the data points except for that for Fas$^{\text{des}}$. 

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Complex, we calculated the same energetic contributions for Fas mutants whose inhibitory activity towards mAChE had been reported previously (Marchot et al., 1997). For both Fas–AChE complexes, we observed only a weak correlation between the experimentally observed $\Delta G_{\text{bind}}$ and the change in the total calculated energy of the complex ($\Delta E_{\text{tot}}$) that was used as a design criterion in this study (Fig. 5A). However, the correlation improved greatly when the inter-molecular energy term ($\Delta E_{\text{inter}}$) alone was considered (Fig. 5B). A correlation coefficient of 0.80 was obtained for the single, double and quadruple Fas mutants reported in this study. This correlation coefficient was slightly reduced to 0.74 if the data for the previously reported Fas mutants were also incorporated. A slightly worse correlation between the experimental $\Delta G_{\text{bind}}$ and the calculated $\Delta E_{\text{inter}}$ was observed for the quintuple mutant Fas$^{\text{des}}$. One possible reason for this discrepancy is accumulation of errors in our predictions due to slight overestimation of $\Delta E_{\text{inter}}$ for each individual mutation (Fig. 5B). Another source of error might arise from slight changes in the backbone conformation of the Fas–TcAChE complex that is not modeled in our study. Such changes are much more likely to occur when multiple, rather than single, mutations are introduced into the Fas sequence.

In our recent study in which calmodulin (CaM) was optimized for interaction with a target peptide, a very strong correlation was observed between the experimental $\Delta G_{\text{bind}}$ and the calculated $\Delta E_{\text{inter}}$, which is in contrast to our present findings (Yosef et al., 2009). This discrepancy can be explained simply if one takes into account that CaM undergoes a large conformational change upon binding. Hence, improvement in binding of CaM to its targets can be achieved not only by introduction of mutations that improve inter-molecular interactions, but also by introduction of mutations that stabilize CaM in its target-bound conformation [see also (Shimaoka et al., 2000)]. In the Fas/TcAChE complex, for which no substantial conformational change is believed to occur, mutations that stabilize Fas alone are thus not expected to improve its binding affinity for TcAChE.
of the experimental $\Delta\Delta G_{\text{bind}}$ with the calculated inter-
molecular energy $\Delta E_{\text{inter}}$ than with $\Delta E_{\text{total}}$. Thus, $\Delta E_{\text{inter}}$ may
serve as a better predictive measure for redesign of protein–
protein complexes in which no substantial conformational
changes occur upon binding. A more extensive mutational
study of the Fas–AChE binding interface is under way in
order to test this prediction.

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