

# A diverse family of thermostable cytochrome P450s created by recombination of stabilizing fragments

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**Thermostable enzymes combine catalytic specificity with the toughness required to withstand industrial reaction conditions<sup>1,2</sup>. Stabilized enzymes also provide robust starting points for evolutionary improvement of other protein properties<sup>3</sup>. We recently created a library<sup>4</sup> of at least 2,300 new active chimeras of the biotechnologically important<sup>5–9</sup> cytochrome P450 enzymes. Here we show that a chimera's thermostability can be predicted from the additive contributions of its sequence fragments. Based on these predictions, we constructed a family of 44 novel thermostable P450s with half-lives of inactivation at 57 °C up to 108 times that of the most stable parent. Although they differ by as many as 99 amino acids from any known P450, the stable sequences are catalytically active. Among the novel functions they exhibit is the ability to produce drug metabolites. This chimeric P450 family provides a unique ensemble for biotechnological applications and for studying sequence-stability-function relationships.**

The versatile cytochrome P450 family of heme-containing redox enzymes hydroxylates a wide range of substrates to generate products of significant medical and industrial importance<sup>5</sup>. A particularly well-studied member of this diverse enzyme family, cytochrome P450 BM3 (CYP102A1, or 'A1') from *Bacillus megaterium*, has been engineered extensively for biotechnological applications<sup>6</sup> that include fine chemical synthesis<sup>7</sup> and production of human metabolites of drugs<sup>7–9</sup>. In an effort to create new biocatalysts for these applications, we used structure-guided SCHEMA recombination of the heme domains of CYP102A1 and its homologs CYP102A2 (A2) and CYP102A3 (A3) to create 620 folded and 335 unfolded chimeric P450 sequences made up of eight fragments, each chosen from one of the three parents<sup>4</sup>. Chimeras are written according to fragment composition: 23121321, for example, represents a protein which inherits the first fragment from parent A2, the second from A3, the third from A1, and so on. A survey of the activities of 14 chimeras demonstrated that the sequence diversity created by SCHEMA recombination also generated functional diversity, including the ability to accept substrates not accepted by any of the parents<sup>10</sup>.

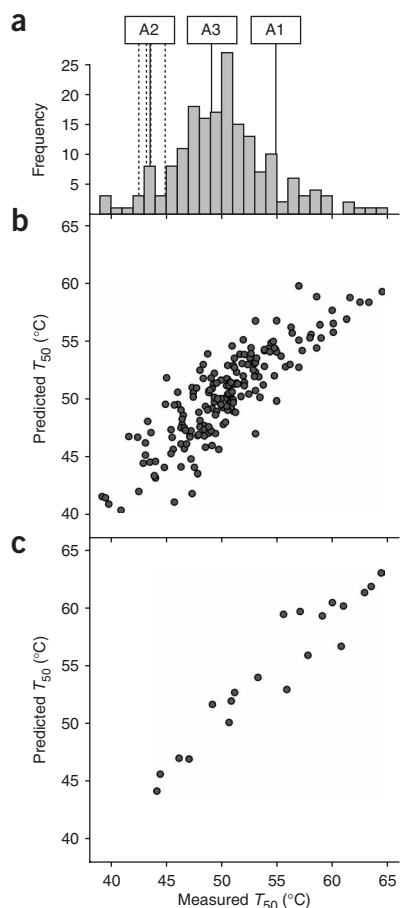
Most mutations (including those made by recombination) are destabilizing; we thus expect that most of the chimeras will be less stable than the most stable parent. Of the thousands of P450s in the library, we would like to choose those with the greatest stability for detailed characterization of activities and specificities. To do so, we first measured the thermostabilities of 184 P450 chimeras (Supplementary Table 1 online) in the form of  $T_{50}$ , the temperature at which 50% of the protein irreversibly denatured after incubation for 10 min. Folded chimeras that were expressed at sufficient levels for the stability analysis and exhibited denaturation curves that could be fit to a two-state denaturation model<sup>3</sup> were selected. The parental proteins have  $T_{50}$  values of 54.9 °C (A1), 43.6 °C (A2) and 49.1 °C (A3) (Fig. 1a). This sample of the folded P450s contains many that are more stable than the most stable parent (A1) (Fig. 1a).

We assessed the contribution of block-additive thermostability effects by analyzing the  $T_{50}$  values of the 184 chimeric P450s with linear regression. Regression of  $T_{50}$  against chimera fragment composition revealed a strong linear correlation between predicted and observed  $T_{50}$  for all 184 chimeras: Pearson  $r = 0.856$  (Fig. 1b and Supplementary Table 2 online).

To examine whether the results allow generalization from one data subset to another and to address the possibility of over-fitting, we randomly divided the data into a training set (139 data points) and a test set (45 data points). The s.d. of regression ( $\sigma_R$ ) and measurement ( $\sigma_M = 1.0$  °C) were used to guide the data training. After each training cycle, every data point was weighted in terms of its role in determining the regression line. If the prediction error (the temperature difference between the predicted  $T_{50}$  and the measured one) of a data point was more than  $2\sigma_R$ , it was removed. When  $\sigma_R$  was  $< 2\sigma_M$  (2.0 °C), the training process stopped. After two training cycles, a  $\sigma_R$  of 1.9 °C was achieved. After removing only eight outliers,  $r$  for the training set was improved from 0.847 to 0.892 (Supplementary Fig. 1a online). When the trained regression parameters (Supplementary Table 2 online) were used to predict thermostabilities of proteins in the test data set, the correlation was  $r = 0.857$ , validating the regression model (Supplementary Fig. 1b online). The linear regression model was further confirmed by tenfold cross-validation<sup>11</sup> (data not shown).

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**Figure 1** Thermostabilities of parental and chimeric cytochromes P450 vary widely and are well predicted by an additive model. **(a)** The distribution of  $T_{50}$  values for 184 chimeric cytochrome P450s are shown, with  $T_{50}$ s for parents A1, A2 and A3 indicated (solid lines), including four experimental replicate measurements for A2 to examine measurement variability (dotted lines, s.d. of 1.0 °C). Some chimeras are more stable than the most stable parent. **(b)** Predicted  $T_{50}$  from a simple linear model correlates with the measured  $T_{50}$  for 184 P450 chimeras, with  $r = 0.856$ . **(c)** Linear model derived from data in **b** accurately predicts stabilities of 20 new chimeras, including the most thermostable P450 (MTP) (top right-most point).

We then used the linear regression model parameters obtained from the 204  $T_{50}$  measurements (**Supplementary Table 2** online) to predict  $T_{50}$  values for all 6,561 chimeras in the library (**Supplementary Fig. 3** online). A significant number ( $\sim 300$ ) of chimeras are predicted to be more stable than A1. We selected all those with predicted  $T_{50}$  values  $\geq 60$  °C (total of 30) for construction and further characterization. Five were already generated in our previous work<sup>4</sup>; the remaining 25 were constructed. All 30 predicted stable chimeras were stable, with  $T_{50}$  between 58.5 °C and 64.4 °C (**Table 1**). The stability predictions were quite accurate, with root mean square deviations between the predicted and measured  $T_{50}$  values of 1.6 °C, close to the measurement error (1.0 °C).

We also wished to determine whether the multiple sequence alignment of the folded chimeras could be used to predict the stable sequences, similar to ‘consensus stabilization’ methods based on natural sequence alignments<sup>13–15</sup>. We estimated the stability of each chimera from the collection of folded chimeras as described<sup>15</sup>. We observed that lower consensus energies are associated with higher  $T_{50}$  values (**Fig. 2a**; Pearson  $r = -0.58$ ,  $P < 10^{-9}$ ). Furthermore, folded proteins tend to have lower consensus energies than unfolded ones (**Fig. 2b**; Wilcoxon signed rank test  $P < 10^{-9}$ ).

We explored the tradeoff between the number of chimera sequences used to calculate the energies and the statistical error associated with ranking chimeras by consensus. First, we selected random subsets, each containing from 5 to 300 sequences (in increments of 5), from the 613 folded chimeras. Second, we calculated consensus energies for the three parents and 204 chimeras with known  $T_{50}$ s. Third, we calculated the Spearman rank correlation coefficient ( $r_s$ ) between the consensus energy predictions and the measured  $T_{50}$  values. We repeated this entire process ten times, and calculated the average  $r_s$  and s.d. for each sample size (**Supplementary Fig. 4** online). The average rank-order correlation coefficient is reliably  $>0.5$  (with s.d. values  $<0.1$ ) when 85 or more chimera sequences are used.

Having demonstrated that sequence and folding status alone can be used to make nontrivial predictions of relative stability, we proceeded to predict the most stable chimeras. First, we calculated the consensus energy for each chimera fragment (**Supplementary Table 3** online). Then we calculated the total consensus energies of all 6,561 chimeras in the library; the 20 with the lowest consensus energies are listed in **Supplementary Table 4** online. We constructed a total of 17 of these top 20 (8 of which had already been constructed based on linear regression prediction). Five additional chimeras that were predicted to be stable and were constructed are also included in **Table 1**. All 44 chimeras that were constructed for this study are more stable than the most stable parent, have predicted  $T_{50}$ s above measured  $T_{50}$  of the most stable parent and are also predicted to be more stable based on consensus energy.

The sequence with the highest-frequency fragments at all eight positions, chimera 21312333, is called the consensus sequence. It has the lowest consensus energy and is predicted to be the most stable. In

The most thermostable P450 chimera predicted by the model parameters obtained from the training set would have a  $T_{50}$  of 63.8 °C and fragment composition 21312333. This sequence was constructed, expressed and characterized; its  $T_{50}$  of 64.4 °C, within measurement error of the predicted value, made it 9.5 °C more stable than the most thermostable parent, A1. It was, in fact, the most stable of all the more than 230 chimeras that have been characterized to date. To further test the model predictions, we measured the  $T_{50}$  values of 19 additional chimeras from the 620 folded chimeras previously reported<sup>4</sup>, seven predicted to be highly thermostable and twelve picked at random (**Supplementary Table 1** online). Predicted and measured  $T_{50}$  values for all 20 new P450s, including the most thermostable P450, correlated extremely well ( $r = 0.956$ ) (**Fig. 1c**).

In the absence of noise, one may fully determine an  $N$ -parameter regression model using only  $N$  specific measurements. In the presence of noise, additional measurements will tend to increase the accuracy of the predictions<sup>12</sup>. We randomly selected a certain number of sequences from the 204 chimeras with measured  $T_{50}$ s and tested the ability of regression models based on these sequences to predict the  $T_{50}$ s of the remaining chimeras. By using a large randomized training set we reduced the effect of experimental noise. Equally important, by training on chimeras scattered throughout the sequence space, we avoided biasing the resulting regression model to a single reference state. We found that just 35–40 measurements were sufficient for accurate predictions of chimera stability, although slight improvements in prediction accuracy could be seen with more data points (**Supplementary Fig. 2** online).

**Table 1 Parent cytochrome P450 heme domains and 44 stabilized chimeras constructed by recombination of stabilizing fragments**

Sequence	Predicted $T_{50}$ (°C)	Measured $T_{50}$ (°C)	Consensus energy	Relative activity <sup>a</sup>	Sequence	Predicted $T_{50}$ (°C)	Measured $T_{50}$ (°C)	Consensus energy	Relative activity <sup>a</sup>
11111111	44.8	54.9	0.000	1.0	22312313	60.6	61.0	-2.324	2.5
22222222	N/A	43.6	N/A	0.5	21313313	60.6	64.4	-2.324	4.7
33333333	45.1	49.1	-1.013	0.2	21312133	60.5	60.1	-2.832	2.8
21312333	63.8	64.4	-3.247	1.0	22311331	60.4	58.9	-1.603	5.1
21312331	62.8	60.6	-3.057	3.1	22312231	60.3	61.4	-2.790	2.3
21311333	62.8	59.2	-1.994	2.5	21313231	60.3	61.0	-2.791	1.8
21312233	62.7	63.1	-3.181	0.6	22311233	60.3	60.9	-1.727	3.1
22312333	62.4	63.5	-3.045	1.9	21311311	60.0	61.0	-1.083	3.2
21313333	62.4	62.9	-3.046	3.8	22313331	60.0	58.5	-2.655	7.2
21312313	62.0	62.2	-2.525	2.8	21312211	59.9	59.3	-2.270	2.8
21311331	61.8	62.9	-1.805	1.0	22313233	59.9	60.4	-2.779	5.7
21312231	61.7	62.8	-2.991	1.0	21212333	59.6	63.2	-3.120	0.4
21311233	61.7	62.7	-1.928	0.7	21112333	59.5	61.6	-3.202	1.1
21313331	61.4	62.2	-2.856	5.5	22313231	58.9	59.0	-2.589	6.3
22312331	61.4	59.3	-2.856	5.1	21212233	58.5	60.0	-3.055	1.3
22311333	61.4	60.1	-1.793	4.7	21112331	58.5	61.6	-3.013	0.6
22312233	61.3	61.0	-2.980	2.7	21111333	58.5	62.4	-1.950	2.6
21313233	61.3	60.0	-2.980	3.3	21112233	58.4	58.7	-3.137	0.7
21312311	61.0	59.1	-2.336	3.0	22212333	58.2	58.2	-2.919	3.2
22313333	61.0	64.3	-2.845	9.0	22112333	58.1	58.0	-3.001	4.2
21311313	61.0	61.2	-1.273	2.7	21113333	58.1	61.0	-3.002	4.1
21312213	60.9	60.6	-2.459	1.1	23313333	57.1	61.2	-2.433	2.0
21312332	60.8	59.9	-2.739	1.3	22112233	57.0	58.7	-2.935	5.2
21311231	60.7	63.2	-1.739	0.8					

<sup>a</sup>Relative activity on 2-phenoxyethanol, reported as total turnover number normalized to that of the most active parent (A1). N/A: Due to library construction bias, we cannot predict  $T_{50}$  or calculate the consensus energy for heme domains containing fragment A2 at position 4.

fact, 21312333 has the highest measured stability among all 238 chimeras with known  $T_{50}$  and is also the most thermostable P450 predicted by the linear regression model. The consensus sequence obtained by analyzing the alignment of multiple folded chimeras differs substantially from that obtained by simply examining the three parental sequences and designating the consensus fragment as that which differs the least from the other two parents (21221332).

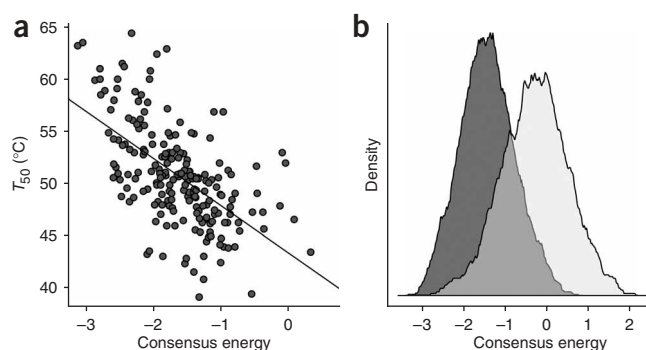
We found that the stability predictions were sufficiently accurate to identify both sequencing errors and point mutations in the chimeras. The sequences of P450 chimeras were originally determined by DNA probe hybridization, which has an ~3% error rate<sup>4</sup>; small numbers of point mutations during library construction are also expected. We resequenced 13 chimeras with prediction error >4 °C from the original set of 189 chimeras whose  $T_{50}$ s were measured and analyzed by linear regression. We found that five either had incorrect sequences or contained point mutations (**Supplementary Table 5** online); they were eliminated from the subsequent analyses.

Further work also showed that both the regression and consensus models perform well enough to substantially increase the odds of identifying sequencing errors and mutations. The chimeras 22313333, 21311311 and 22311333 were predicted to be highly stable while they had previously been reported to be unfolded<sup>4</sup>. Full sequencing showed that the original 22313333 construct was incomplete and missing some fragments; the original 21311311 construct had an insertion; 22311333 had two point mutations leading to two amino acid substitutions. After correction, all three chimeras were very stable (**Table 1**).

The newly constructed thermostable chimeras and corrected sequences were added to the previously published sequence-folding status data (**Supplementary Table 6** online). We performed the consensus analysis again using the corrected sequence-folding data

(of 644 folded chimeras) versus 238 chimeras with measured  $T_{50}$ s. The correlation  $r$  between consensus energy and measured thermostability improved significantly, from -0.58 to -0.67.

An enzyme's half-life of (irreversible) inactivation ( $t_{1/2}$ ) is commonly used to describe stability<sup>16</sup>. We measured  $t_{1/2}$  at 57 °C for 13 stable chimeras and the three parents (**Supplementary Table 7** online). The results show that the increased stability can have a profound effect on half-life: whereas the most stable parent, A1, lost its ability to bind CO with a  $t_{1/2}$  of 15 min at this temperature,



**Figure 2** Relative chimera thermostabilities and folding status can be predicted from sequence element frequencies in a multiple sequence alignment of folded proteins. **(a)** Consensus energies computed from fragment frequencies of folded chimeras correlate with measured thermostabilities ( $T_{50}$ s) of 204 chimeric proteins. **(b)** The distribution of consensus energies of 613 folded chimeras and 334 unfolded chimeras (all chimeras reported previously<sup>6</sup>, minus chimeras having A2 at position 4). Folded chimeras (dark gray) have lower consensus energies than unfolded chimeras (light gray).

chimera 21312231 had a  $t_{1/2}$  of 1,600 min, or more than 108 times greater. The most thermostable P450 and the consensus chimera 21312333 similarly has a very long half-life of 1,550 min.  $T_{50}$  has also been shown to correlate linearly with urea concentrations required for half-maximal denaturation for variants of CYP102A1 (ref. 3). Therefore, we expect that the stable P450 chimeras are also more tolerant to inactivation by chemical denaturants.

All 44 stable chimeras were verified by full sequencing to eliminate any possibility that the enhanced thermostabilities were due to mutations, insertions or deletions. The stable chimeras comprise a diverse family of sequences, differing from one another at 7–99 amino acid positions (46 on average) (Supplementary Fig. 5 online). The distance from the closest parent is as high as 99 amino acids. We also noted that the expression levels of most of the thermostable chimeras were higher than those of the parent proteins. Most thermostable chimeras expressed well even without the inducing agent isopropyl- $\beta$ -D-thiogalactopyranoside (data not shown).

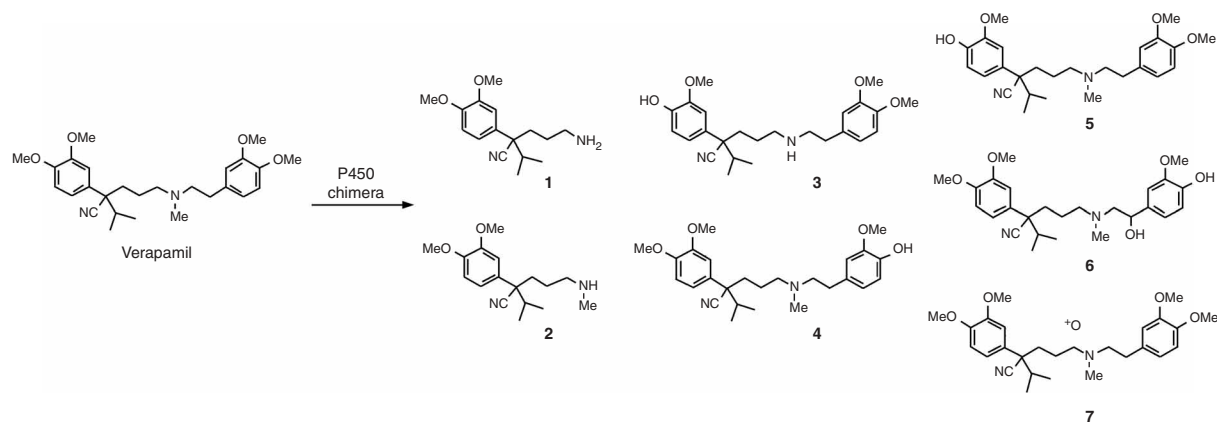
To determine whether the stable chimeras retained catalytic activity and, more importantly, whether they acquired activities of

biotechnological importance, we measured the peroxygenase activities of the thermostable chimeras on 2-phenoxyethanol, a substrate on which all three parent enzymes are active, and found that all 44 chimeras are active (Table 1). Furthermore, many of them were more active than the most active parent (A1). We also tested the thermostable chimeras for activity on two drugs, verapamil (Calan Covera, Isoptin, Verelan) and astemizole, and measured the extent of metabolite formation by high-performance liquid chromatography (HPLC)/mass spectrometry (MS) with higher order MS analysis. Although none of the parents showed activity on either drug, three chimeras produced significant quantities of metabolites for verapamil, and two chimeras produced metabolites from both verapamil and astemizole. Products 2, 4, 5, 8 and 10 (Table 2) are known human metabolites and are the products of reactions with the human CYP3A4, 1A2, 2C and 2D6 enzymes<sup>17–20</sup>.

We have demonstrated two approaches to predicting protein stability using different data. One is performed by linear regression of sequence-stability data, and the other is based on consensus analysis of the multiple sequence alignment. The best prediction approach

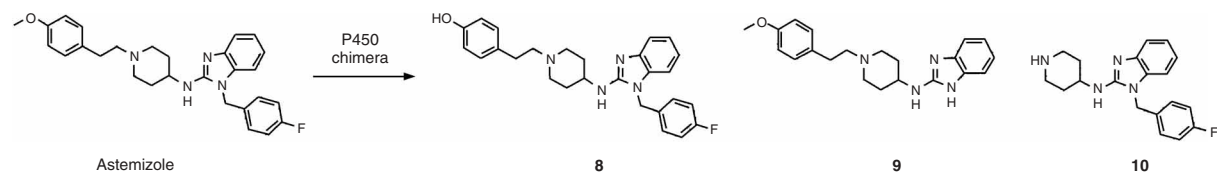
**Table 2** Thermostable chimeras are active on drugs not accepted by the parent enzymes

(a) Products of biotransformations on verapamil



Chimera	% conversion <sup>a</sup>	% 1	% 2	% 3	% 4	% 5	% 6	% 7
21312332	6		33	17	17			33
21313331	5		20	20	20		20	20
21113333	5		20		40		20	20
22313231	43		32			47	5	16
22313333	34	15	20			41	9	15

(b) Products of biotransformations on astemizole



Chimera	% conversion <sup>a</sup>	% 8	% 9	% 10
22313231	9	45	33	22
22313333	9	56	22	22

<sup>a</sup>200  $\mu$ l reactions were run at 25 °C for 2 h using clarified lysate containing 2.5  $\mu$ M P450 chimera, 250  $\mu$ M drug and 1 mM hydrogen peroxide.

depends on the target protein and the relative ease with which folding status and stability are measured. The linear regression model uses stability data, which are often more difficult to obtain than a simple determination of folding status. The linear regression model, however, also requires fewer measurements and always predicted more true positives with fewer false positives than the consensus approach based on folding status (**Supplementary Fig. 6** online).

Consensus stabilization is based on the idea that the frequencies of sequence elements correlate with their corresponding stability contributions. This correlation is typically assumed to follow a Boltzmann-like exponential relationship<sup>15</sup>. Such a relationship is most sensible if, in analogy to statistical mechanics, the sequences are randomly sampled from the ensemble of all possible folded P450s. Natural sequences are related by divergent evolution and may not comprise such a sample. Our chimeric protein data set, in contrast, represents a large and nearly random sample of all the 6,561 possible chimeras. We find support for the fundamental assumptions underlying consensus stabilization approaches: sequence elements contribute additively to stability, stabilizing fragments occur at higher frequencies among folded sequences, and the consensus sequence is the most stable in the ensemble. These results demonstrate the tolerance of the consensus stabilization idea to different ensembles (chimeric libraries versus evolved families) and sequence changes (recombination versus stepwise mutation). Unlike previous implementations of consensus stabilization, however, the approach described here generated dozens of stable proteins, and these proteins differ from each other and from the parents at many amino acid residues.

The high degree of additivity that we observe may appear surprising, considering the cooperative nature of protein folding and the many tertiary contacts in the native structure. The additivity of stability changes to proteins has long been known<sup>21</sup>. Nonadditive effects are expected when sequence changes are coupled or result in significant structural changes<sup>21</sup>. Structural disruption is less likely in chimeras than with random mutants because all sequence elements are believed to fold to a similar structure in at least one context, that of the parental sequence. Furthermore, we anticipate that such block additivity is maximized by the library design, which reduces coupling. SCHEMA identifies sequence fragments that minimize the number of contacts, or interactions, that can be broken upon recombination. Two residues in a chimera are defined to have a contact if any heavy atoms are within 4.5 Å; the contact is broken if they do not appear together in any parent at the same positions. Among a total of ~500 contacts for a P450 chimera, an average of fewer than 30 were broken for the sequences in the SCHEMA library. The SCHEMA fragments that were swapped in this library have many intrafragment contacts; the interfragment contacts are either few or are conserved among the parents. As a result, the fragments function as pseudo-independent structural modules that make roughly additive contributions to stability. The additivity was strong enough to enable detection of sequencing errors based on deviations from additivity, prediction of thermostabilities for uncharacterized chimeras with high accuracy and prediction of the  $T_{50}$  of the most stable chimera to within measurement error. Because SCHEMA effectively identifies functional chimeras with other protein scaffolds, such as  $\beta$ -lactamases<sup>22</sup>, this approach should allow one to identify novel stable, functional sequences for other protein families.

Both approaches demonstrated here identify highly stable sequences; recombination ensures that they also retain biological function and exhibit high sequence diversity by conserving important functional residues while exchanging tolerant ones. This sequence diversity can give rise to useful functional diversity. Assembly of the

stable P450 chimeras was motivated in part by a desire to generate new or improved P450 activities in a stable catalyst framework. This study demonstrated improvements in activity (on 2-phenoxyethanol) as well as acquisition of entirely new activities (on verapamil and astemizole) in the stabilized enzymes. That the P450 chimeras can produce authentic human metabolites of drugs opens the door to rapid drug metabolic profiling and lead diversification using soluble enzymes that are produced efficiently in *Escherichia coli*.

In summary, we show that chimeric proteins exhibit a broad range of stabilities, and that stability of a given folded sequence can be predicted based on data (either stability or folding status) from a limited sampling of the chimeric library. By assembling predicted stable sequences, we generated 44 stabilized P450s that differ significantly from their parent proteins, are expressed at high levels and are catalytically active. Individual members of the stable P450 family exhibit activity on biotechnologically relevant substrates. This approach allows the creation of whole families of stabilized proteins that retain existing functions and also explore functions of human P450s.

## METHODS

**Thermostability measurements.** Cell extracts were prepared and P450 concentrations were determined as reported previously<sup>4</sup>. Cell extract samples containing 4  $\mu$ M of P450 were heated in a thermocycler over a range of temperatures (36 °C–75 °C) for 10 min followed by rapid cooling to 4 °C for 1 min. The precipitate was removed by centrifugation. The P450 remaining in the supernatant was measured by CO-difference spectroscopy<sup>4</sup>.  $T_{50}$  was determined by fitting the data to a two-state denaturation model<sup>8</sup>. To check the variability and reproducibility of the measurement, four parallel independent experiments (from cell culture to  $T_{50}$  measurement) were conducted on A2, which yielded an average  $T_{50}$  of 43.6 °C and a s.d. ( $\sigma_M$ ) of 1.0 °C. For some sequences,  $T_{50}$ s were measured twice, and the average of all the measurements was used in the analysis.

**Linear regression.** The linear model  $T_{50} = a_0 + \sum_i \sum_j a_{ij} x_{ij}$  was used for regression, where  $T_{50}$  is the dependent variable and fragments  $x_{ij}$  (from the  $i^{\text{th}}$  position and  $j^{\text{th}}$  parent, where  $i = 1, 2, \dots, 8$  and  $j = 2$  or 3) are the independent variables. The  $x_{ij}$  were dummy coded, such that if a chimera has fragment 1 from parent 2,  $x_{12} = 1$  and  $x_{13} = 0$ . Parent A1 was used as the reference for all eight positions, so the constant term ( $a_0$ ) is the predicted  $T_{50}$  of A1 and the regression coefficients  $a_{ij}$  represent the thermostability contributions of fragments  $x_{ij}$  relative to the corresponding reference (A1) fragments. In general, the reference fragment at each of the eight positions can be chosen arbitrarily. Due to construction bias, the fragment from parent A2 at position 4 is almost completely missing from the data set. We therefore deleted the few chimeras having this fragment from all analyses, including consensus analysis. Regression was performed using SPSS (SPSS for Windows, Rel. 11.0.1. 2001. SPSS Inc.).

**Consensus energy calculation.** Assuming the frequency of a fragment at position  $i$  is exponentially related to its stability contribution and that these fragment contributions are additive, total chimera consensus energy relative to a reference sequence can be calculated from  $\Delta e_{\text{total}} \propto \sum -\ln f_i/f_{i,\text{ref}}$ , where  $f_{i,\text{ref}}$  is the ensemble frequency of the fragment at  $i$  in a reference sequence. We again used A1 as the reference, so that A1 has consensus energy of zero; the choice of reference sequence is arbitrary and does not influence the results. Note that the values reported are actually proportional to energy differences from the reference; we refer to them as consensus energies for brevity. The raw frequencies  $f_{ij}^{\text{raw}}$  of fragment  $i$  from parent  $j$  in the folded ensemble may reflect biases in the assembly of chimeras from their constituent fragments. Bias can be assessed by measuring the frequencies  $f_{ij}^{\text{unselected}}$  in an unselected set of sequences to determine the biases  $b_{ij} = n_{\text{parents}} f_{ij}^{\text{unselected}}$ , which in an unbiased ensemble will be equal to 1. For the P450 ensemble the  $f_{ij}^{\text{unselected}}$  are known (**Supplementary Table 3** online). Construction bias can be corrected directly by dividing the  $f_{ij}^{\text{raw}}$  by the  $b_{ij}$ , and we used bias-corrected frequencies in all analyses.

**Construction of thermostable chimeric cytochrome P450s.** To construct a given stable chimera, two chimeras having parts of the targeted gene (e.g., 21311212 and 11312333 for the target chimera 21312333) were selected as templates. The target gene was constructed by overlap extension PCR, cloned into the pCWori expression vector, and transformed into the catalase-free *E. coli* strain SN0037 (ref. 4). All constructs were confirmed by complete sequencing.

**Enzyme activity assays.** Activity on 2-phenoxyethanol was measured as reported previously with slight modifications<sup>10</sup>. We mixed 80  $\mu$ l of cell lysate containing 4  $\mu$ M P450 chimera with 20  $\mu$ l of 2-phenoxyethanol solution (60 mM) in each well of a 96-well plate. The reaction was initiated by adding 20  $\mu$ l of hydrogen peroxide (120 mM). Final concentrations were: 2-phenoxyethanol, 10 mM; hydrogen peroxide, 20 mM. After 1.5 h, the reactions were quenched with 120  $\mu$ l urea (8M in 200 mM NaOH) before adding 36  $\mu$ l 4-aminoantipyrine (0.6%). Mixtures were blanked on the plate reader at 500 nm before adding 36  $\mu$ l potassium peroxodisulfate (0.6%). After 10 min of color development, the solutions were remeasured for absorbance. Absorbances were normalized to the most active parent A1.

**Biotransformations with verapamil and astemizole.** We mixed 60  $\mu$ l of cell lysate containing  $\sim$ 8.3  $\mu$ M P450 chimera with 90  $\mu$ l of EPPS buffer (0.1M, pH 8.2) and 10  $\mu$ l drug (5 mM). The reaction was initiated by addition of 40  $\mu$ l hydrogen peroxide (5 mM). Final concentrations were: drug, 250  $\mu$ M; hydrogen peroxide, 1 mM. After 1.5 h, the reaction was quenched with 200  $\mu$ l acetonitrile and the mixtures centrifuged 10 min at 18,000g. We analyzed 25  $\mu$ l supernatant by HPLC. Conditions used with solvent A (0.2% formic acid (vol/vol) in H<sub>2</sub>O) and solvent B (acetonitrile) to elute the products of metabolism at 200  $\mu$ l/min were: 0–3 min, A:B 90:10; 3–25 min, linear gradient to A:B 30:70; 25–30 min, linear gradient to A:B 10:90. Samples whose chromatograms contained more than the parent drug peak were further analyzed by liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS). Identical conditions to the HPLC method detailed above were used for the LC portion of the analysis followed by MS operation in positive electrospray ionization mode. MS/MS spectra were acquired in a data-dependent manner for the most intense ions. Product identification was accomplished by comparison of retention times and tandem MS spectra against controls from rat liver microsomes<sup>17–20</sup>. HPLC separations were performed using a Supelco Discovery C18 column (2.1  $\times$  150 mm, 5 $\mu$ ) on a Waters 2690 Separation module in conjunction with a Waters 996 PDA detector. LC-MS and MS/MS spectra were obtained using the ThermoFinnigan LCQ classic at the Caltech MS facility.

Note: Supplementary information is available on the Nature Biotechnology website.

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#### AUTHOR CONTRIBUTIONS

Y.L., A.M.S. and F.H.A. designed the research; Y.L. and A.M.S. performed the research; Y.L., D.A.D., A.M.S., C.D.S. and J.D.B. contributed to the analytical tools; Y.L., D.A.D., A.M.S., C.D.S., J.D.B. and F.H.A. analyzed data; Y.L., D.A.D., A.M.S., C.D.S. and F.H.A. wrote the paper.

#### COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturebiotechnology/>.

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