Catalytic versus Inhibitory Promiscuity in Cytochrome P450s: Implications for Evolution of New Function

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S *Supporting Information*

ABSTRACT: Catalytically promiscuous enzymes are intermediates in the evolution of new function from an existing pool of protein scaffolds. However, promiscuity will only confer an evolutionary advantage if other useful properties are not compromised or if there is no "negative trade-off" induced by the mutations that yield promiscuity. Therefore, identification and characterization of negative trade-offs incurred during the emergence of promiscuity are required to further develop the evolutionary models and to optimize in vitro evolution. One potential negative trade-off of catalytic promiscuity is increased susceptibility to inhibition, or inhibitory promiscuity. Here we exploit cytochrome P450s (CYPs) as a model protein scaffold that spans a vast range of catalytic promiscuity and apply a quantitative index to determine the relationship between promiscuity of catalysis and promiscuity of inhibition for a series of homologues. The aim of these studies is to begin to identify properties that, in general, correlate with catalytic promiscuity, hypothetically such as inhibitory promiscuity. Interestingly, the data indicate that the potential negative trade-off of inhibitory promiscuity is nearly insignificant because even highly substrate specific CYPs have high inhibitory promiscuity, with little incremental increase in susceptibility to inhibitory interactions as the substrate promiscuity increases across the series of enzymes. In the context of evolution, inhibitory promiscuity is not an obligate negative trade-off for catalytic promiscuity.
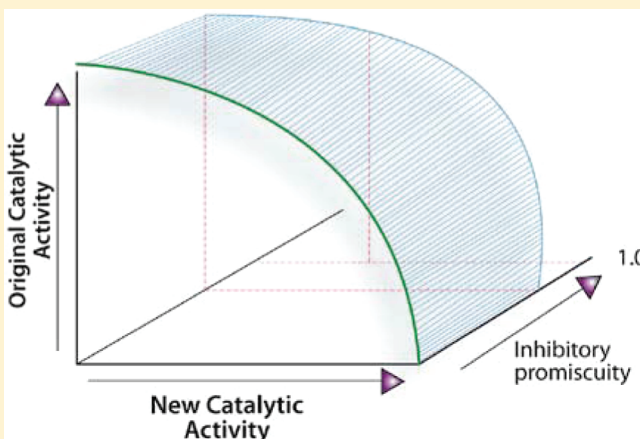
P romiscuity at the molecular level plays a critical role in several cellular processes, including signal transduction, immunity, and detoxification.[1−9] More broadly, the evolution of new enzymatic activities from a pool of existing protein scaffolds, whether in nature or in vitro directed evolution, may proceed through "promiscuous" intermediates that catalyze multiple reactions.[10−19] A common description of this possible evolutionary process and relevant factors is schematized in Figure 1, wherein a specific enzyme undergoes mutation to yield a promiscuous variant that catalyzes a new reaction, without forfeiting its original activity.[11,20] At early stages along the evolutionary trajectory, the mutation is neutral with respect to the original function. If this second enzymatic activity provides any survival advantage, then it is retained, and after gene duplication, one copy of the gene encoding the promiscuous enzyme can undergo further refinement to optimize the new function, without loss of the original function. It may be useful to distinguish between promiscuous enzymes with catalytic activities unrelated to their presumed biological function and "multifunctional" enzymes with a clear functional advantage in accepting multiple substrates, as suggested.[20] Here we use the term "promiscuity" for both cases, because we aim to understand the physical and chemical properties of enzymes that process multiple substrates, regardless of the functional purpose. Recent studies have suggested that promiscuous intermediates easily evolve into efficient and specific enzymes.[11,20] While this model provides an extremely useful framework for conceptualizing evolutionary structure−function relationships, promiscuity is not a well-understood property, and it is difficult to quantify, although progress has been made.[17,19]

For example, natural evolution of new function does not happen in isolation. Rather, each evolutionary intermediate must function in the context of the existing niche that includes other
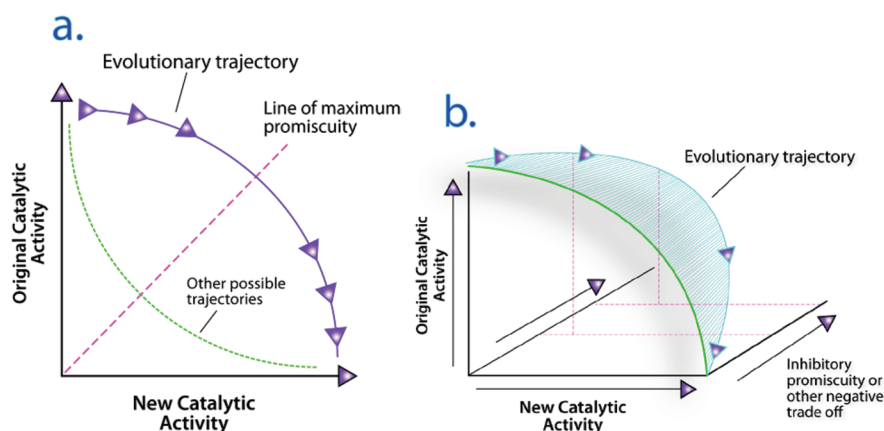
Figure 1. (a) Promiscuous intermediate model for evolution of new enzymatic function. Enzymes with high catalytic specificity for one reaction (original catalytic activity) move along the indicated trajectory. At early stages, they become promiscuous, gaining new catalytic activity without forfeiting the original activity. The dotted line indicates that other evolutionary trajectories are possible. (b) Three-dimensional evolutionary trajectory relating catalytic function toward original and new substrates with susceptibility to inhibition. Susceptibility to inhibition could be a significant negative trade-off that increases the evolutionary distance from any intermediate to the desired evolutionary end point on the new activity axis. Each incremental increase in catalytic promiscuity has an associated cost of potential new inhibitory interaction with other ligands; the negative trade-off is revealed as an increase in the distance between the green two-dimensional curve and the blue trajectory as the catalytic promiscuity increases. The greater the increase in distance between the blue and green curves, the greater the negative trade-off. The detailed shape of the new trajectory is not known for any enzyme, but it ultimately restricts the possible evolutionary trajectories by defining the extent of negative trade-off.

enzymes and a "background" of potential ligands. Within a niche, a loss due to mutation in any useful trait is termed a "negative trade-off".[20] The evolutionary trajectory of an enzyme is unlikely to be a two-dimensional process as depicted in the model in Figure 1a, but it will trace a multidimensional surface that includes the relationships between promiscuity and other properties that comprise the overall fitness of the enzyme. That is, there will be a third collective coordinate for negative trade-offs, as in Figure 1b. In this case, promiscuity per se is not an obvious advantage in the evolutionary process unless it is achieved without significant negative trade-offs.[20] Whereas a highly promiscuous enzyme would provide a very efficient template from which to evolve many new functions from a single protein sequence, such an intermediate would unlikely be neutral with respect to all of the original properties. If the magnitude of any negative trade-off is coupled exactly to the magnitude of promiscuity, then the mutational neutrality will not be maintained in all dimensions of fitness space and the path toward new function will have a different shape than if the putative negative trade-off is not correlated with the promiscuity in any dimension. Ultimately, the relationship between any potential negative trade-off and catalytic promiscuity will determine whether evolution will follow that path. These concepts underscore the need to define the relationship between catalytic promiscuity and other protein properties.

Speculatively, for example, a new promiscuous enzyme would have increased susceptibility to inhibition by ligands that do not inhibit the "original" enzyme, and this would be a negative trade-off. This is based on the observation that structurally diverse drugs can bind to and inhibit a single enzyme, without yielding products (consider antibiotics, statins, etc.). In contrast, catalysis requires proper alignment of multiple reactive functional groups. Each incremental step toward promiscuity, i.e., toward the diagonal of Figure 1a, will be advantageous only if the new catalytic activity is not accompanied by an equal or greater increase in susceptibility to "new" inhibitory interactions that, at ambient ligand concentrations, limit the original enzyme

activity. For the example here, inhibitory promiscuity is a potential negative trade-off that adds a new dimension to yield the scheme in Figure 1b and can be substituted with any parameter hypothesized to be a negative trade-off.

We emphasize that the current studies are not intended to establish specific evolutionary mechanisms, which may best be accomplished by successive rounds of random mutagenesis and relevant selection protocols. Rather, we aim to understand more generally the relationships between catalytic promiscuity and other properties of enzymes that could limit or define evolutionary pathways. An understanding of these relationships is necessary to understand the role of catalytic promiscuity in evolution and to advance the incomplete model of Figure 1. The cytochrome P450s provide an excellent test case for improving our understanding of these relationships because, as a family with a highly conserved scaffold, they span a very wide range of catalytic promiscuity. Human hepatic CYPs dominate drug metabolism and have very broad substrate selectivities.[19,21] Here we use these multifunctional CYPs as a model for physicochemical traits of enzymes near the limits of catalytic promiscuity. Other CYPs, including bacterial and human isoforms used here, contribute to specific biosynthetic pathways and are very substrate specific.[22,23] Collectively, the CYP superfamily provides insight into the relationship between catalytic and inhibitory promiscuity. The results indicate a surprising level of inhibitory promiscuity even for substrate specific CYPs, and they further suggest that because inhibitory promiscuity is already present in highly specific enzymes, there is little cost associated with evolutionary steps toward catalytic promiscuity.

■ MATERIALS AND METHODS

Materials. Recombinant CYP Supersomes were purchased from Becton Dickinson (San Jose, CA). NADPH was obtained from EMD Chemicals (Gibbstown, NJ). HPLC solvents were from J. T. Baker (Phillipsburg, NJ). All other chemicals were

purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest grade available.

Determination of Catalytic and Inhibitory Parameters. Kinetic constants for CYP-catalyzed turnover were determined using a substrate depletion approach.[25,26] Briefly, incubations consisted of 1 pmol of recombinant CYP enzyme, 3 mM magnesium chloride, and substrate (0.001−25 μM, final concentration) in 100 mM potassium phosphate buffer (pH 7.4) in a volume of 250 μL. Reactions (performed in duplicate) were initiated with the addition of 1 mM NADPH (final concentration) and allowed to proceed for 0, 5, 10, or 20 min before being quenched with 2 volumes of acetonitrile containing 0.1 μM tolbutamide as an internal standard. To determine IC$_{50}$ values, a similar incubation procedure was used. Probe substrates were incubated at their previously determined K_M values (data not shown), and inhibitor concentrations ranged from 0 to 25 μM. Reactions ($n = 3$) were quenched after 10 min as described above. Samples were vortex-mixed and centrifuged prior to liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis.

Liquid Chromatography−Tandem Mass Spectrometry Analysis. All samples were analyzed on an Applied Biosystems API4000 Q-trap mass spectrometer (Applied Biosystems, Foster City, CA) that was operated in triple-quadrupole mode. The LC−MS/MS system was comprised of an electrospray ionization source coupled to a binary pump with in-line solvent degasser (Shimadzu, Columbia, MD) and a LEAP CTC HTS PAL autosampler (CTC Analytics, Carrboro, NC). Chromatographic separation was achieved using a mobile phase system consisting of 0.1% formic acid in acetonitrile and 0.1% formic acid in 5 mM ammonium formate with a Gemini C18 2.0 mm × 30 mm, 5 μm column (Phenomenex, Torrance, CA). MS/MS conditions were optimized for each compound prior to analysis.

Statistical Analysis. Substrate depletion rate constants were determined by fitting the percentage of substrate remaining versus time to first-order decay functions, as [substrate] versus ln time. Data points that resulted in the substrate depletion plot deviating from linearity were not included in determining the depletion rate constants. Michaelis constants (K_M) were obtained by plotting the substrate depletion rate constants as a function of the substrate concentration using the following equation (where k_{dep} is the substrate depletion rate constant, K_M is the Michaelis constant, [S] is the substrate concentration, and $k_{dep:[S]=0}$ represents the maximal theoretical rate of substrate depletion):

$$k_{dep} = k_{dep:[S]=0} \left(1 - \frac{[S]}{[S] + K_M} \right)$$

IC$_{50}$ values were determined by fitting inhibition data to the following equation, using the algorithms contained in GraphPad Prism 7.0 (Graphpad Software Inc., San Diego, CA):

$$\% \text{ activity} = \min + \frac{\max - \min}{1 + 10^{\log[I] - \log IC_{50}}}$$

where % activity refers to the percent activity remaining relative to a control with no inhibitor present, max and min are the maximal and minimal percent activities remaining in the presence of inhibitor, respectively, and [I] is the inhibitor concentration.

Calculation of catalytic promiscuity, J_{cat}, and inhibitory promiscuity, J_{inh}, was based on the previously described method that

quantifies the probability that any enzyme will metabolize a specific substrate from a set of substrates, wherein the probability is normalized to account for chemical dissimilarity among substrates in the set.[19,24] Substrates that are chemically very different from others in the set are weighted more. For each of the 65 compounds studied here, 161 chemical properties were assigned a score of "1" if the compound contained that property or "0" if it did not. For each of the 161 compounds, its chemical similarity to others in the set was calculated as described below. Briefly, the J_{cat} is defined, as in ref 19, by

$$J = \frac{N}{(\sum_{i=1}^{N} \langle \delta \rangle_i) \times \log N} \times \sum_{i=1}^{N} \langle \delta \rangle_i \frac{e_i}{\sum_{j=1}^{N} e_j} \times \ln \frac{e_i}{\sum_{j=1}^{N} e_j}$$

where e_i is the k_{cat}/K_M for the ith substrate in a basis set with N substrates, $\langle \delta \rangle_i = \overline{\delta_{ij}}/\delta_{set}$ is the average distance in chemical space of the ith substrate from the other members of the set, and δ_{set} is a measure of the chemical space covered by the set, with a theoretical maximum of 1. More specifically, for a pair of chemicals A and B, where a is the number of features present only in A, b is the number of features present only in B, and c is the number of features present in both A and B, the Tanimoto distance is $\delta_{AB} = a + b/a + b + c$. For substrates in a set, we can also define $\overline{\delta_{ij}}$ as the mean Tanimoto distance from member i to all the other members in the set. For the calculations here, there are 161 chemical properties used to determine the $\langle \delta \rangle_i$ for each of the 65 substrates. The overall set dissimilarity δ_{set} serves as an upper bound for $\overline{\delta_{ij}}$: if k is the number of features present in at least one but not all of the members of the set and l is the number of features present in all members of the set, then $\delta_{set} = k/(k+l)$. Finally, $\langle \delta \rangle_i = \overline{\delta_{ij}}/\delta_{set}$ yields the normalized mean distance for each substrate i. With this measure of distance in chemical space between substrates, the substrates that are farthest from others in the chemical space are weighted more heavily in the calculation of promiscuity. If many structurally similar substrates are metabolized with the same efficiency, then the resulting J_{cat} value will not be as great as when the substrates are far from each other in chemical space.

For inhibition, the J_{inh} value is the susceptibility to inhibition and is

$$J_{susc} = -\frac{M}{\log M \times \sum_{i=1}^{M} \langle \delta \rangle_i} \sum_{i=1}^{M} \langle \delta \rangle_i \frac{x_i}{\sum_{j=1}^{M} x_j} \log \frac{x_i}{\sum_{j=1}^{M} x_j}$$

where x_i is the inhibitory potency of an inhibitor from a set containing M inhibitors and $\langle \delta \rangle_i$ is the average distance in chemical space from other inhibitors. As with the catalytic parameters, inhibitors that are chemically very different from others in the set are weighted more heavily in the determination of inhibitory promiscuity.

RESULTS

Promiscuity Parameters. Eight hepatic human CYP isoforms, typically considered to be detoxification enzymes, and two CYPs with high catalytic activity toward defined endogenous substrates were compared directly for their ability to metabolize 65 substrates. These substrates collectively span a wide range of the 166-dimensional chemical space, with a δ_{set} of 0.99, as

Table 1. Promiscuity Parameters

CYP isoform	J_{cat}	J_{inh}
detoxification CYPs		
CYP3A4	0.83	0.78
CYP3A5	0.82	
CYP2D6	0.81	0.82
CYP2E1	0.77	
CYP2C8	0.69	0.85
CYP2C9	0.65	0.80
CYP2C19	0.70	0.84
CYP1A2	0.81	0.79
substrate specific CYPs		
CYP26A1	0.30	0.58
CYP4F12	0.16	0.65

defined in Materials and Methods. This value of δ_{set}, which has a theoretical maximum of 1.0, confirms that the substrates and inhibitors used here are extrememly diverse with respect to chemical space. The catalytic parameters k_{cat}, K_M, and k_{cat}/K_M for each CYP isoform and its probe substrate are summarized in the Supporting Information. The promiscuity values (J values) obtained for each CYP, calculated as described in Materials and Methods, are summarized in Table 1. Briefly, the J value is a normalized distribution of k_{cat}/K_M values for the substrates in the basis set. A wide distribution implies a promiscuous enzyme, and a narrow distribution reflects high specificity (see Materials and Methods). The scale ranges from 1, the upper limit of promiscuity, to 0, which describes a completely substrate specific enzyme. Promiscuity values (J_{cat}) for some of these isoforms were previously reported on the basis of a smaller basis set of substrates, and the values reported here with a significantly expanded basis set further validate and extend our previous approach.[19] J values obtained from basis sets that sample more chemical space will be more predictive than J values obtained from a basis set of chemically similar substrates. Notably, the hepatic CYP enzymes not previously studied, CYP2E1, CYP3A5, CYP2C8, and CYP1A2, are highly promiscuous, as expected. The promiscuity parameters for the biosynthetic CYP26A1 and the fatty acid-metabolizing CYP4F12, which are usually considered to be substrate specific, are much lower. The hepatic drug-metabolizing CYPs, with J values of >0.7, are clearly segregated from the biosynthetic CYPs, with J values of ≤0.30.

To validate the approach and this basis set of substrates, we used statistical "jackknife" methods; for each data set, each substrate was removed from the basis set and the J_{cat} value was recalculated. If the basis set is adequately unbiased, then removal of any individual substrate has no effect of the recovered J value, if the enzyme is promiscuous. In contrast, for substrate specific enzymes, removal of the "cognate substrate" from the calculation results in a large increase or decrease in the J value and removal of other substrates has a negligible effect. The results of these jackknife calculations are summarized in Figure 2. Clearly, the detoxification CYPs are highly promiscuous, whereas CYP26A1 and CYP4F12 are highly specific.

Inhibitory Promiscuity. The calculation of inhibitory promiscuity has been done previously for CYP2D6, -1A2, -3A4, -2C9, -2C8, and -2C19 using a basis set of 65 inhibitors.[24] Here we have calculated the inhibitory J_{inh} values for CYP26A1 and CYP4F12, using the basis set of compounds listed in the
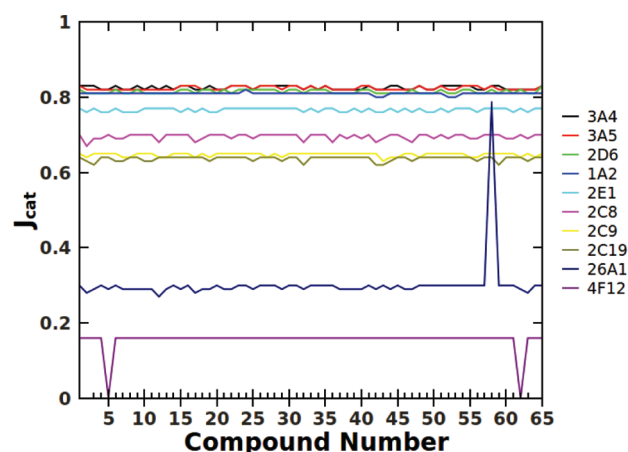

Figure 2. Jackknife analysis of J_{cat} values for all CYPs listed in Table 1. Each compound is removed from the basis set and the J_{cat} recalculated. The analysis validates that the basis set of substrates does not bias the calculation of J_{cat} and further differentiates promiscuous enzymes from specific enzymes. See the text for further explanation.
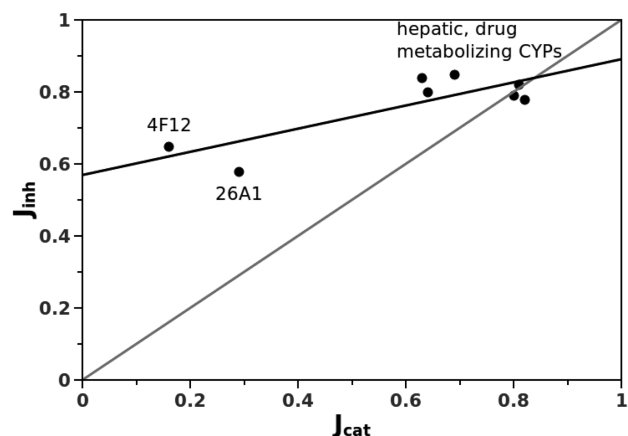

Figure 3. Plot of J_{inh} vs J_{cat} for CYP3A4, -2D6, -2C8, -2C9, -2C19, -1A2, -26A1, and -4F12. The gray diagonal line is for visualization only and represents a perfect correlation between J_{cat} and J_{inh}. The fitted line ($r^2 = 0.68$, and slope = 0.32) indicates that the negative trade-off of J_{inh} is nearly insignificant as the CYPs become more catalytically promiscuous. The shallow slope of the line is due to an inherently high J_{inh} even for enzymes with a low J_{cat}.

Supporting Information, in each isoform specific probe reaction. These inhibitors span a wide range of structural space, as did the substrates. A plot of J_{inh} versus J_{cat} is shown in Figure 3. The diagonal line in Figure 3 represents a line with a slope of 1 and therefore is the expected line along which the data would lie if catalytic promiscuity and inhibitory promiscuity were exactly correlated. This line represents the negative trade-off of inhibitory promiscuity that evolving organisms would "pay" if each incremental increase in catalytic promiscuity were accompanied by an equal incremental increase in inhibitory promiscuity. The relationship would not, a priori, be linear, but if it is, the slope could in principle be 1, <1, or >1. A slope much greater than 1 would, for example, constitute a significant negative trade-off, and a slope much less than 1 would constitute a minimal negative trade-off. As one can see, the data for the hepatic CYPs are clustered near this diagonal at the top right, suggesting that these
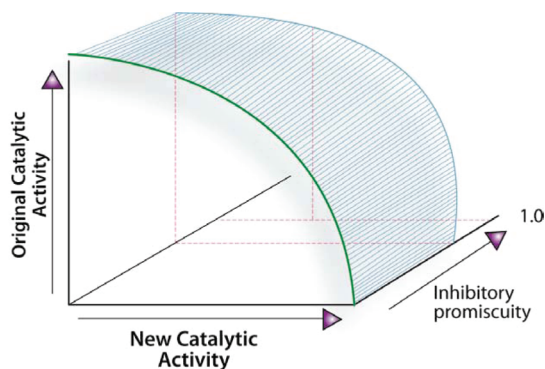
Figure 4. Schematized summary of the relationship between J_{cat} and J_{inh} for CYPs. The inhibitory promiscuity remains nearly constant, starting at a high J_{inh}, with only a marginal further increase as the enzymes progress toward higher J_{cat} values. As a result, promiscuity of inhibition is unlikely to be a significant negative trade-off as enzymes progress from specificity for the original activity toward promiscuity.

enzymes are near the functional limit of both catalytic and inhibitory promiscuity. In contrast, the CYP26A1 and CYP4F12 lie near the lower limits of catalytic promiscuity but are clearly above the diagonal. Although the J_{inh} values of CYP26A1 and CYP4F12 are relatively low compared to those of the detoxification CYPs, they are clearly higher than their catalytic promiscuity. In effect, the location of J_{inh} values for the catalytically specific CYPs well above the diagonal in Figure 3 indicates that it is much "easier" to inhibit a substrate selective enzyme, or one with intermediate promiscuity, than it is to provide an alternative substrate. Although it is intuitively expected that inhibition would be easier than catalysis, the magnitudes of J_{inh} values for the substrate specific enzymes are interestingly high. Because this ligand basis set spans a substantial fraction of chemical space, these results further suggest that this is likely to be a general behavior for CYPs.

This, in turn, has very significant implications for the developing paradigm concerning the role of functional promiscuity in the evolution of new function from the existing pool of protein scaffolds. The line that is fit to the data, with an r^2 of 0.68 and a slope of 0.32, indicates that the negative trade-off of J_{inh} is much less than the line of equal negative trade-off (slope of 1). Each incremental increase in J_{cat} is accompanied by an only small increase in J_{inh}. However, more important than the shallow slope in this case is the fact that the y-intercept is well above the origin. In fact, the J_{inh} is high even for catalytically specific CYPs, and J_{inh} is relatively insensitive to J_{cat} as greater catalytic promiscuity is observed.

These results can be cast in the context of Figure 1. For the CYPs, the three-dimensional plot of original activity versus new activity versus inhibitory promiscuity (J_{inh}) would include a curve that starts well-displaced along the J_{inh} axis. This curve deviates only slightly from a parallel trajectory as the J_{cat} increased and decreased again toward the new function. The initially high J_{inh} values change only slightly as the J_{cat} values span the range from 0.16 to 0.89.

◼ DISCUSSION

Functional promiscuity of proteins has become increasingly recognized in the past few years, but the relationship between catalytic promiscuity and other physicochemical traits is not yet

understood. Here we utilize a quantitative index of the functional promiscuity of cytochrome P450s to advance our understanding of the relationship of promiscuity to other protein traits. We also consider the utility of CYPs as a model for examining the widespread suggestion that promiscuity is a useful property in the evolution of new protein function. For an extensive set of compounds with sufficient structural diversity, this index provides a useful tool for comparing catalytic promiscuity within a set of enzymes with a common structural fold. Such an index facilitates testing of meaningful hypotheses to further advance our understanding of enzymatic promiscuity.

The results quantitatively support the well-known, but "intuitive", notion that hepatic detoxification CYPs are more promiscuous than CYPs involved in specific biosynthetic pathways with defined substrates. The segregation of detoxification CYPs from biosynthetic CYPs along the diagonal of Figure 3 is striking. Equally apparent is the high degree of susceptibility toward inhibition, even for highly substrate specific isoforms. In fact, while the J_{cat} values span nearly the entire range of promiscuity, with CYP4F12 and CYP3A4 defining the lower and upper limits for this set of CYPs, the J_{inh} values vary only between 0.6 and 0.85. This supports the intuition that fewer chemical constraints must be satisfied to achieve inhibition than catalysis.

Regardless of whether CYPs reflect the general properties of other protein folds, one implication of the results is that inhibitory promiscuity is not an obligate negative trade-off in the progression of specific enzymes to promiscuous intermediates and further toward specific enzymes with new functions. This general conclusion is true despite the fact that more compounds in chemical space are likely to be inhibtors of an enzyme than a substate for that enzyme, as the data demonstrate. Inhibitory promiscuity may be a liability already for specific enzymes, prior to the development of catalytic promiscuity, so relatively little additional cost is incurred, in terms of J_{inh}, when J_{cat} increases. If one considers catalytic promiscuity to be an evolutionary advantage for CYPs and inhibitory promiscuity to be the associated cost, then all mutations that confer catalytic promiscuity are potentially advantageous, because of the inherently high starting point of CYP inhibitory promiscuity. In other words, any increase in P450 J_{inh} in a series of variants is nearly inconsequential when compared to the high initial value of J_{inh}. Although the positive correlation we observe in Figure 3 suggests a greater likelihood that catalytically promiscuous intermediates will exhibit inhibitory promiscuity, the results also suggest that inhibitory promiscuity does not necessarily represent a new limit on the utility of such intermediates, because its incremental increase among intermediates may be small compared to the beneficial catalytic promiscuity. Further studies of the type described here could reveal other parameters or properties that are negative trade-offs correlated with catalytic promiscuity, which do limit the evolutionary trajectory. Such properties could include thermal stability, susceptibility to proteases, aggregation, increased activity toward important metabolites, or others properties that may result from structural plasticity.

Other groups have examined the intriguing relationship between catalytic and inhibitory promiscuity and also emphasize that evolution from promiscuous intermediates is dependent on the "ligand matrix" (i.e., the collective interactions with potential inhibitors and alternative substrates) that such enzymes encounter.[27] In that work, DNA shuffling was used to generate a library of variants from two parental enzymes. Clusters of variants with sufficiently similar catalytic properties were defined

as a "quasi-species". Individual proteins within a quasi-species may display a preference for one substrate over another or susceptibility toward a particular inhibitor but have different amino acid sequences and hence can exhibit differences in other biophysical properties. Evolution from a quasi-species, rather than from a single protein sequence, ensures efficient sampling of sequence and functional space. Our results expand upon and complement the quasi-species concept in several ways. (1) Just as with individual member proteins, we predict that the quasi-species that have the greatest catalytic promiscuity will also be those with the greatest inhibitory promiscuity. (2) Nonetheless, all quasi-species will tend to inherit significant inhibitory promiscuity, so that a low J_{inh} is unlikely to strongly distinguish one quasi-species from another. (3) J_{cat} and J_{inh} provide global, model-free measures of evolutionarily relevant functional properties, which could, for example, be used to select first-generation variants for subsequent rounds of laboratory evolution. Clustering enzyme variants into quasi-species is analytically challenging in high-dimensional spaces, especially in the context of larger ligand matrices (e.g., the 65×65 matrix of this work, compared to the 2×3 matrix of ref 27).

The results summarized here provide potentially useful quantitative benchmarks for evolutionary models that are based on either quasi-species or individual proteins. We speculate that very low J_{inh}/J_{cat} ratios may be rare among members of any quasi-speices or individual intermediates if they derive from substrate specific enzymes; our results provide the first benchmarks for this ratio and suggest that the upper limit is ~2 (CYP26A1), which differs only modestly from the lower limit of 1.2 (CYP2C8). These results suggest the critical point that, to obtain a low J_{inh}/J_{cat} ratio, very high values of J_{cat} are required (~0.8). Such high levels of J_{cat} would require multiple mutations in a highly substrate specific enzyme and could require a long evolutionary trajectory. Possibly, evolution would be more efficient by utilizing intermediates with lower J_{cat} values, at the expense of higher J_{inh}/J_{cat} values.

Although these data refute the intuitive hypothesis that inhibitory promiscuity necessarily will be a negative trade-off for all inceases in catalytic promiscuity, it remains to be determined whether this is generally true of all enzyme families. We chose CYPs as a model because, as a family, they are assumed to span a wide range of J_{cat} values, which enhances our understanding of promiscuity at the extremes of this behavior. It is interesting to consider that the CYP scaffold is unique among protein folds and is especially prone to high J_{inh} values, thus making it an ideal scaffold for evolution of promiscuous detoxification enzymes. The CYP scaffold, in principle, could be uniquely biased toward promiscuity, with high J_{inh}/J_{cat} ratios even for isoforms with low J_{cat} values. This trait may have been selected evolutionarily to achieve the evolutionary end point for detoxification enzymes, with high J_{cat} values. If this speculation is true, then our results cannot be generalized to substrate specific enzymes that have lower starting J_{inh} values. Clearly, more studies of this type, with additional protein scaffolds, are required to understand the role of promiscuity in evolution.

Finally, the hepatic CYPs studied here collectively account for the majority of human drug metabolism. Examples of CYP-dependent drug—drug interactions are numerous, wherein one drug inhibits the metabolism of a second. In many cases, this severely restricts the utility of a drug or at least limits its therapeutic application. Our results confirm that the susceptibility of CYPs to metabolic drug interactions is an inherent property of their structural scaffold and is likely unavoidable.

■ ASSOCIATED CONTENT

ⓢ Supporting Information. k_{cat}, K_M, k_{cat}/K_M, and $k \rightarrow 0$ values for the 65 substrates used for each CYP (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Wilhelm, T., Nasheuer, H. P., and Huang, S. (2003) Physical and functional modularity of the protein network in yeast. *Mol. Cell. Proteomics 2*, 292–298.

(2) Dunker, A. K., Cortese, M. S., Romero, P., Iakoucheva, L. M., and Uversky, V. N. (2005) Flexible nets. The roles of intrinsic disorder in protein interaction networks. *FEBS J. 272*, 5129–5148.

(3) Sethi, D. K., Agarwal, A., Manivel, V., Rao, K. V., and Salunke, D. M. (2006) Differential epitope positioning within the germline antibody paratope enhances promiscuity in the primary immune response. *Immunity 24*, 429–438.

(4) Grubor, N. M., Hayes, J., Small, G. J., and Jankowiak, R. (2005) Cross-reactivity and conformational multiplicity of an anti-polycyclic aromatic hydrocarbon mAb. *Proc. Natl. Acad. Sci. U.S.A. 102*, 7453–7458.

(5) Dimitrov, J. D., Lacroix-Desmazes, S., Kaveri, S. V., and Vassilev, T. L. (2007) Transition towards antigen-binding promiscuity of a monospecific antibody. *Mol. Immunol. 44*, 1864–1873.

(6) Jimenez, R., Salazar, G., Yin, J., Joo, T., and Romesberg, F. E. (2004) Protein dynamics and the immunological evolution of molecular recognition. *Proc. Natl. Acad. Sci. U.S.A. 101*, 3803–3808.

(7) Ekroos, M., and Sjogren, T. (2006) Structural basis for ligand promiscuity in cytochrome P40 3A4. *Proc. Natl. Acad. Sci. U.S.A. 103*, 13682–13687.

(8) Redinbo, M. R. (2004) Promiscuity: What protects us perplexes us. *Drug Discovery Today 9*, 431–432.

(9) Lewinson, O., Adler, J., Sigal, N., and Bibi, E. (2006) Promiscuity in multidrug recognition and transport: The bacterial MFS Mdr transporters. *Mol. Microbiol. 61*, 277–284.

(10) O'Brien, P. J., and Herschlag, D. (1999) Catalytic promiscuity and the evolution of new enzymatic activities. *Chem. Biol. 6*, R91–R105.

(11) Khersonsky, O., Roodveldt, C., and Tawfik, D. S. (2006) Enzyme promiscuity: Evolutionary and mechanistic aspects. *Curr. Opin. Chem. Biol. 10*, 498–508.

(12) James, L. C., and Tawfik, D. S. (2003) Conformational diversity and protein evolution: A 60-year-old hypothesis revisited. *Trends Biochem. Sci. 28*, 361–368.

(13) Poelarends, G. J., Veetil, V. P., and Whitman, C. P. (2008) The chemical versatility of the β-α-β fold: Catalytic promiscuity and divergent evolution in the tautomerase superfamily. *Cell. Mol. Life Sci. 65*, 3606–3618.

(14) Yew, W. S., Fedorov, A. A., Fedorov, E. V., Rakus, J. F., Pierce, R. W., Almo, S. C., and Gerlt, J. A. (2006) Evolution of enzymatic activities in the enolase superfamily: L-Fuconate dehydratase from *Xanthomonas campestris*. *Biochemistry 45*, 14598–14608.

(15) Glasner, M. E., Gerlt, J. A., and Babbitt, P. C. (2007) Mechanisms of protein evolution and their application to protein engineering. *Adv. Enzymol. Relat. Areas Mol. Biol. 75*, 193–239.

(16) Lairson, L. L., Watts, A. G., Wakarchuk, W. W., and Withers, S. G. (2006) Using substrate engineering to harness enzymatic promiscuity and expand biological catalysis. *Nat. Chem. Biol. 2*, 724–728.

(17) Mannervik, B., Runarsdottir, A., and Kurtovic, S. (2009) Multi-substrate-activity space and quasi-species in enzyme evolution: Ohno's dilemma, promiscuity and functional orthogonality. *Biochem. Soc. Trans. 37* (Part 4), 740–744.

(18) Glasner, M. E., Gerlt, J. A., and Babbitt, P. C. (2006) Evolution of enzyme superfamilies. *Curr. Opin. Chem. Biol. 10*, 492–497.

(19) Nath, A., and Atkins, W. M. (2008) A quantitative index of substrate promiscuity. *Biochemistry 47*, 157–166.

(20) Khersonsky, O., and Tawfik, D. S. (2010) Enzyme Promiscuity: A mechanistic and evolutionary perspective. *Annu. Rev. Biochem. 79*, 471–505.

(21) Guengerich, F. P. (2008) Cytochrome p450 and chemical toxicology. *Chem. Res. Toxicol. 21*, 70–83.

(22) Pikuleva, I. A. (2008) Cholesterol-metabolizing cytochromes P450: Implications for cholesterol lowering. *Expert Opin. Drug Metab. Toxicol. 4*, 1403–1414.

(23) Karlgren, M., Miura, S., and Ingelman-Sundberg, M. (2005) Novel extrahepatic cytochrome P450s. *Toxicol. Appl. Pharmacol. 207* (Suppl. 2), 57–61.

(24) Nath, A., Zientek, M. A., Burke, B. J., Jiang, Y., and Atkins, W. M. (2010) Specificity and Promiscuity in Small-Molecule Inhibition of Cytochrome P450 Isoforms. *Drug Metab. Dispos. 38*, 2195–2203.

(25) Obach, R. S., and Reed-Hagen, A. E. (2002) Measurements of Michaelis constants for cytochrome P450-mediated biotransformation reactions using a substrate depletion approach. *Drug Metab. Dispos. 30*, 831–837.

(26) Nath, A., and Atkins, W. M. (2006) A theoretical validation of the substrate depletion approach to determining kinetic parameters. *Drug Metab. Dispos. 34*, 1433–1435.

(27) Mannervik, B., and Runarsdottir, A. (2010) The quest for molecular quasi-species in ligand-activity space and its application to directed enzyme evolution. *FEBS Lett. 584* (12), 2565–2571.