Leveraging intrinsic flexibility to engineer enhanced enzyme catalytic activity

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Dynamic motions of enzymes occurring on a broad range of timescales play a pivotal role in all steps of the reaction pathway, including substrate binding, catalysis, and product release. However, it is unknown whether structural information related to conformational flexibility can be exploited for the directed evolution of enzymes with higher catalytic activity. Here, we show that mutagenesis of residues exclusively located at flexible regions distal to the active site of Homo sapiens kynureninase (HsKYNase) resulted in the isolation of a variant (BF-HsKYNase) in which the rate of the chemical step toward kynurenine was increased by 45-fold. Mechanistic pre–steady-state kinetic analysis of the wild type and the evolved enzyme shed light on the underlying effects of distal mutations (>10 Å from the active site) on the rate-limiting step of the catalytic cycle. Hydrogen-deuterium exchange coupled to mass spectrometry and molecular dynamics simulations revealed that the amino acid substitutions in BF-HsKYNase allosterically affect the flexibility of the pyridoxal-5′-phosphate (PLP) binding pocket, thereby impacting the rate of chemistry, presumably by altering the conformational ensemble and sampling states more favorable to the catalyzed reaction.

Conformational dynamics play a key role in enzyme catalysis (1–3). Large conformational changes occurring over a wide range of timescales have been observed during catalysis by a variety of experimental techniques for numerous enzymes, and their role in substrate binding and in modulating the catalytic cycle is being increasingly better understood (4–6). The significance of long-range allosteric networks in enzyme catalysis is underscored by observations showing that mutations remote from the active site can have a profound impact on catalytic properties, affecting both the steady-state turnover number (kcat) and the specificity constant (kcat/KM) through mechanisms that include 1) a shift of the statistical ensemble of conformations arising from the inherent flexible nature of protein structure (7–9) and 2) alteration of the molecular interactions between mutated residues and their nearest neighbors that, in turn, propagate structural changes to remote sites (10–12). The effects of such distal mutations on kcat and kcat/KM have been studied in-depth in the cases of dihydrofolate reductase (13, 14), ribonuclease A (15, 16), voltage-activated K+ channels (17, 18), HIV-1 protease (19), thymidylate synthase (20), and other enzymes (21–25).

Crystallographic B-factors account for the distribution of the atomic electron densities relative to their equilibrium positions. High B-factors are indicative of dynamic and thermal positional disorder (26, 27), and they have been widely used as a measure of intrinsic protein flexibility (28, 29). Even though the relatively low atomic resolution of many crystal structures and crystal packing effects can complicate the relationship between B-factor values and polypeptide flexibility (30), as a rule, residues with high B-factors are located in protein regions with greater flexibility (31–36). Several investigators have reported that site-specific mutagenesis of high B-factor regions guided by phylogenetic analysis (37), Rosetta design (38), molecular dynamics (MD) simulations, or FoldX (39, 40) is a useful approach for improving thermal stability (41–43) or, alternatively, for facilitating crystallization (44). Additionally, random mutagenesis of high B-factor regions and directed evolution have been applied to improve enzyme stability in organic solvents (45, 46). However, targeted mutagenesis of high B-factor regions distal to the active site as a means of increasing catalytic activity toward a desired substrate has not been explored.

Kynureninases (KYNases) are pyridoxal-5′-phosphate (PLP)-dependent enzymes of the aminotransferase superfamily that catalyze the hydrolysis of the C6–C8 bond of L-kynurenine (KYN) or 3′-OH-L-kynurenine (OH-KYN) forming L-alanine (ALA) and either anthranilic acid (AA) or 3′-OH-anthranilic acid (OH-AA), respectively. Similar to all animal KYNases, the Homo sapiens kynureninase (HsKYNase) catalyzes

Significance

Protein flexibility has been recognized as a key contributor to enzyme evolution and catalytic activity. Several studies have illustrated how amino acid substitutions that affect protein flexibility can impact catalysis. However, it is unknown whether structural information regarding conformational flexibility can be exploited for directed evolution of enzymes with higher catalytic activity. Using as a model human kynureninase, an enzyme with important therapeutic implications in cancer immunotherapy, we show that mutagenesis of residues exclusively located within high B-factor regions distal to the active site resulted in a variant with markedly enhanced catalytic activity for its nonpreferred substrate, kynurenine. Our results suggest that modulation of intrinsic flexibility through mutagenesis of remote flexible regions constitutes a promising strategy for directed enzyme evolution.

Competing interest statement: C.S.K., E.M.S., and G.G. are inventors on intellectual property related to this work. This article is a PNAS Direct Submission.

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This article contains supporting information online at http://www.pnas.org/content/119/23/e2118979119.full. This article includes supporting information online at http://www.pnas.org/content/119/23/e2118979119.full.

Published June 3, 2022.
the hydrolysis of OH-KYN but has very low activity toward KYN \((k_{\text{cat}}/K_{\text{M}})^{\text{OH-KYN}} / (k_{\text{cat}}/K_{\text{M}})^{\text{KYN}} > 400)\) (47). In contrast, most bacterial enzymes, such as the well-studied *Pseudomonas fluorescens* kynureninase (PKYNase), strongly prefer KYN over OH-KYN (48). KYNases are of great interest as therapeutic agents for the systemic depletion of kynurenine, which exerts immunosuppressive effects in cancer and whose levels are elevated in numerous neurodegenerative diseases (49).

Recently, we showed that administration of bacterial, highly active KYN-hydrolyzing enzymes (PKYNase and *Mucidiaginibacter paladism* kynureninase) that had been conjugated to poly-ethylene-glycol to confer long circulation persistence can completely deplete circulating and tumoral KYN, which in turn, reversed immunosuppression in the tumor microenvironment in preclinical models (47). Unfortunately, bacterial enzymes are recognized as foreign by the human immune system, thereby eliciting adverse immune responses (50, 51).

However, the low catalytic activity of the human KYNase in the hydrolysis of KYN (and especially its low \(k_{\text{cat}}\) of \(\sim 0.1 \text{s}^{-1}\)) precludes its use for clinical development and necessitates its improvement through engineering methodologies.

Here, we used focused combinatorial saturation mutagenesis of high B-factor regions in HsKYNase to obtain enzyme variants with high catalytic activity against its nonpreferred substrate, KYN. Functional interrogation of saturation mutagenesis libraries targeting high B-factor regions remote from the active site was carried out by using an *Escherichia coli* genetic selection screen, leading to the isolation of an enzyme variant (BF-HsKYNase) which displays \(\sim 20X\) higher \((k_{\text{cat}}/K_{\text{M}})^{\text{OH-KYN}}\) and \(\sim 10X\) lower \((k_{\text{cat}}/K_{\text{M}})^{\text{KYN}}\) than the parental, wild-type enzyme (HsKYNase). These changes in KYN catalytic activity and substrate specificity arose without the introduction of any amino acid substitution in the active site. Detailed pre-steady-state kinetic analysis, together with hydrogen-deuterium exchange (HDX) monitored by mass spectrometry (HDX-MS) and MD simulations provided mechanistic insights regarding the effects of distal mutations on the rate-determining steps in the reactions toward KYN and OH-KYN. Our work demonstrates that mutagenesis of high B-factor regions may be a productive and efficient strategy for the directed evolution of higher catalytic activity, and it highlights the role of remote residues in enzyme catalysis.

**Results**

**B-Factor Mutagenesis and Engineering of HsKYNase KYN Activity.** We analyzed the B-factor profile of HsKYNase (Protein Data Bank accession no. 3E9K) using the B-FITTER program (52) (https://www.kofo.mp.de/en/research/biocatalysis) and focused on regions with B-factors higher than the mean value for the entire protein. A nine-residue loop (K378-P386) on the surface of HsKYNase is missing from the crystal structure (SI Appendix, Fig. S1A) and was modeled using the program YASARA Structure (53) (Fig. 1A). To experimentally examine the flexibility of regions identified by the B-factor analysis, we used HDX-MS which monitors the deuterium \((D_2O)\) exchange of backbone amide hydrogens and thus provides information on intrinsic structural flexibility (54, 55). B-factor and HDX-MS analyses of HsKYNase showed similar flexibility profiles for HsKYNase, with HDX-MS suggesting larger flexibility than the respective B-factor analysis only for an active site loop (residues A95-N127) covered by the peptide range 42 to 47 (Fig. 1A and B and SI Appendix, Fig. S1D).

We also observed a high HDX rate for residues within the nine-residue disordered loop that is missing from the crystal structure.

Two high B-factor regions remote from the active site were of particular interest. Region A consists of an \(\alpha\)-helix followed by a loop domain (residues H179-I201), whereas region B includes domains comprising residues N375-A436 which are interconnected through an \(\alpha\)-helix and four antiparallel \(\beta\)-sheets (Fig. 1A and SI Appendix, Fig. S1 B and C). To select residues for combinatorial saturation mutagenesis within regions A and B, first a phylogenetic analysis was performed using the amino acid sequence of HsKYNase (SI Appendix, Fig. S2). Residues with a low degree of sequence conservation are less likely to have a negative impact on enzyme stability and were therefore selected for mutagenesis. Of note, structural comparison of HsKYNase and bacterial ortholog PKYNase revealed that the regions A and B chosen for mutagenesis exhibit large structural diversity, with HsKYNase’s loops being considerably longer than the respective loops of PKYNase (SI Appendix, Fig. S3).

Subsequently, MD simulations using the structure of HsKYNase (3E9K, including the modeled missing surface loop) were performed to calculate root-mean-square fluctuation (rmsf) values for these residues. The rmsf values from the MD-simulation analysis were in good agreement with both the B-factor analysis and the HDX-MS data (Fig. 1C and SI Appendix, Fig. S4). B-factor values of the residues chosen for mutagenesis, as well as their respective distances from the active site (\(\varepsilon\) to internal aldimine which is formed between catalytic K276 and the PLP), are shown in Fig. 1D.

Saturation mutagenesis libraries comprising two to five non-conserved residues adjacent in the crystal structure and such that each mutagenized position was represented within two libraries (one that included upstream randomized residues and a second with downstream residues) were constructed (SI Appendix, Tables S1 and S2), and *E. coli* transformants were screened for increased KYN hydrolysis activity using a genetic selection screen (Methods and SI Appendix, Fig. S5).

Briefly, *E. coli* cells deficient in anthranilate synthetase (ATrpB) cannot grow in minimal media without anthranilate; therefore, they are auxotrophic for Trp. Cells expressing KYNases with sufficient KYN hydrolysis activity provide the necessary levels of anthranilate to support growth in minimal media supplemented with KYN. This selection enables the enrichment of variants with as little as threefold difference in catalytic activity to be enriched after \(\sim 60\) generations in liquid media. Individual clones were picked, and the catalytic activity of HsKYNase was analyzed in 96-well plates (SI Appendix, Fig. S5). Clones that showed \(>20\)% higher specific activity relative to the parental, wild-type HsKYNase were sequenced; expressed in a preparative scale; purified by immobilized metal affinity chromatography (purity > 95%, as shown in SI Appendix, Fig. S6); and kinetically characterized. All isolated variants showed higher \(k_{\text{cat}}/K_{\text{M}}\) for KYN, as expected (SI Appendix, Tables S1 and S2). Interestingly, unlike HsKYNase, a subset of the improved variants (six and four variants from regions A and B, respectively, accounting for less than 30% of total characterized variants) displayed kinetic cooperativity, as evidenced by sigmoidal steady-state kinetics (with Hill coefficient values in the range of 1.3 to 2.4), suggesting that certain mutations may possibly modulate KYN binding at the second active site of homodimeric HsKYNase (the active site is formed at the interface between the two monomers).

Mutations from variants from the saturation libraries of regions A and B having the highest enzyme activity were combined (combination variants) (SI Appendix, Table S3) to engineer a variant with the highest activity, termed BF-HsKYNase.
Table 1. Steady-state kinetic parameters of HsKYNase and BF-HsKYNase against KYN and OH-KYN measured in PBS, pH 7.4, at 37°C

| Enzyme         | KYN             | OH-KYN          | Specificity constant ratio |
|----------------|-----------------|-----------------|---------------------------|
|                | $k_{cat}$ (s$^{-1}$) | $k_m$ (μM) | $k_{cat}/k_m$ (M$^{-1}$ s$^{-1}$) | Fold | $k_{cat}$ (s$^{-1}$) | $k_m$ (μM) | $k_{cat}/k_m$ (M$^{-1}$ s$^{-1}$) | Fold | (k_{cat}/k_m)^{OH-KYN/}
| HsKYNase†      | 0.12 ± 0.02    | 1200 ± 120     | 100 ± 26                    | 1     | 1.7 ± 0.1 | 29 ± 5       | (6 ± 1.3) $\times$ 10^4 | 1 | 600 |
| BF-HsKYNase    | 1.1 ± 0.05     | 600 ± 70       | 1835 ± 295                  | 18.35 | 0.44 ± 0.01 | 55 ± 3       | 8 (± 0.63) $\times$ 10^3 | 0.13 | 4 |

Parameters are shown as the best fit value ± SE upon fitting the experimental data to the Michaelis-Menten model (SI Appendix, Methods, Eq. 1).

†The reported $k_{cat}$ values of HsKYNase and BF-HsKYNase against KYN do not take into account the significant substrate inhibition observed at high concentrations (see main text for more information).

Steady-state kinetics of HsKYNase against OH-KYN are from Karamitros et al. (56).

Pre-Steady-State Kinetic Characterization of BF-HsKYNase against KYN and OH-KYN Substrates. We employed pre-steady-state stopped-flow fluorescence spectroscopy to examine the kinetics of BF-HsKYNase with KYN or OH-KYN, using fluorescence to monitor formation of anthranilate or OH-anthranilate.
respectively. Previously, we showed that the rate-determining step in the reaction of HsKYNase with KYN is the chemical step that leads to the formation of anthranilate (AA) (56). As with HsKYNase (Fig. 2A), in the reaction of BF-HsKYNase with KYN, AA formation is rate-limiting, as evidenced by the lag in the AA fluorescence signal (Fig. 2F and steps 2 to 4 in SI Appendix, Scheme S1). To enable a quantitative assessment of the intrinsic rate constants that govern the rate-limiting step for the reaction of HsKYNase and BF-HsKYNase with KYN, we performed substrate concentration-dependence experiments and globally fit the data with the KinTek Explorer program (57) using a four-step minimal model (Fig. 2D and E), where 1) the enzyme [E] binds to its substrate [S] and forms the complex E=S, 2) E=S leads to the formation of products E=P~AA, 3) E=P~AA releases AA, and 4) E=P releases P (where P is the second product alanine). The substrate concentration-dependence of the lag phase in the reaction of BF-HsKYNase with KYN afforded estimates of substrate binding and dissociation rate constants, in addition to $k_0 = k_{cat}$. Because we observed significant substrate inhibition at high KYN concentrations for both HsKYNase (56) and BF-HsKYNase (SI Appendix, Fig. S10), we restricted our pre-steady-state analysis to KYN concentrations that are not inhibitory (up to 500 and 300 μM for HsKYNase and BF-HsKYNase, respectively). We note that while in the steady-state kinetic analyses $k_{cat}/K_m$ is well defined by the data at low concentrations, the estimations of $k_{cat}$ and $K_m$ are based on the extrapolation of data collected at low concentrations to the limit of infinite KYN concentrations. Accordingly, the values of $k_{cat}$ and $K_m$ shown in Table 1 do not account for the substrate inhibition seen at higher substrate concentrations.

For the substrate KYN, the global fit of the pre-steady-state data to a realistic four-step model resolved accurately both $k_{cat}/K_m$ and $k_{cat}$. From this analysis, we found that the observed $k_{cat}$ defines the rate of the chemical step (formation of AA) for BF-HsKYNase, as is the case with the parental HsKYNase enzyme (Fig. 2D and E). Remarkably, the $k_{cat}$ of BF-HsKYNase ($k_0 = 0.0445 \text{s}^{-1}$ in Fig. 2E) is enhanced by ~45-fold relative to HsKYNase ($k_0 = 0.0011 \text{s}^{-1}$ in Fig. 2D and Table 2). The $k_{cat}/K_m$ of BF-HsKYNase ($k_{cat}K_m$ of ~350 M$^{-1}$s$^{-1}$) was found to be ~17-fold higher than that of HsKYNase ($k_{cat}K_m$ of ~20 M$^{-1}$s$^{-1}$), while the calculated second-order rate constant for KYN binding ($k_0$) appears to be ~30-fold slower for BF-HsKYNase relative to HsKYNase. The latter finding agrees with the more pronounced lag phase observed with BF-HsKYNase (Fig. 2B). The free-energy profile analysis (SI Appendix, Fig. S11) further supports these findings, showing a slower substrate binding step followed by faster catalysis of the BF-HsKYNase relative to HsKYNase. In addition, confidence contour analysis (58) (SI Appendix, Fig. S12) showed that the calculated rate constants (Fig. 2 D and E and SI Appendix, Tables S5 and S6) derived by global data fitting are well constrained by the data.

When BF-HsKYNase was mixed with OH-KYN, we observed a minor lag phase during the first 50 ms of the reaction due to slow substrate binding (59) (Fig. 2C). Global fitting of these data show that for the reaction of BF-HsKYNase with OH-KYN, the rate-determining step was product (ALA) release, as is the case for the parental HsKYNase. To further resolve the intrinsic rate constants of OH-KYN binding, as well as for the formation and release of OH-AA, we analyzed the substrate concentration-dependence (concentration range of 0 to 3x$K_m$) (Fig. 2C) and fit the data globally using the KinTek Explorer program, using a four-step minimal kinetic model shown in Fig. 2F. Fitting the data globally, rate constants of ~1.6 and ~4.5 s$^{-1}$ for OH-AA formation and release, respectively, were observed (SI Appendix, Table S7). For comparison, the respective rate constants for HsKYNase were 7.85 and 20 s$^{-1}$ (the apparent second-order rate constant for OH-KYN binding to BF-HsKYNase ($k_0 = 0.27 \text{μM}^{-1}\text{s}^{-1}$) was about fourfold slower than the respective value for HsKYNase (1 μM$^{-1}$s$^{-1}$) (56). The OH-AA formation and release steps are 3.5- and 10-fold faster than the apparent steady-state turnover value for OH-KYN by BF-HsKYNase (0.44 s$^{-1}$) (Table 1), consistent with the conclusion that ALA release is rate limiting. The $k_{cat}/K_m$ of HsKYNase [defined by $k_0\times(k_{cat}/(k_{cat}+k_{-1}))$] for
BF-HsKYNase was calculated to be $7,800 \text{ M}^{-1}\text{s}^{-1}$ which is about eightfold lower than the respective value for HsKYNase, in agreement with the steady-state data (Table 1). The confidence contour analysis (SI Appendix, Fig. S13) afforded an estimate of errors for the calculated rate constants reported in Fig. 3F.

The pH dependence of $k_{cat}$ and $(k_{cat}/K_m)_{KYN}$ for BF-HsKYNase and HsKYNase, respectively, along with details for the data fitting, are shown in SI Appendix, Figs. S14 A and B and Methods. In the catalytic mechanism of KYNase enzymes, base-assisted addition of water (H$_2$O) to the γ-carbonyl group of the substrate (red H$_2$O molecule labeled in KYN ketimine species in SI Appendix, Scheme S1) gives rise to a gem-diolate intermediate. Subsequently, this gem-diolate intermediate undergoes retro-Claisen cleavage to give anthranilate (first product of the reaction, labeled red in step 4 of SI Appendix, Scheme S1) and enamine, followed by the final release of alanine. We confirmed the role of this H$_2$O molecule, as there is a twofold solvent isotope effect in the alaninol release in the catalysis of KYN by HsKYNase, as shown in SI Appendix, Fig. S14C (rates of linear phases in H$_2$O versus D$_2$O of $\sim 0.038$ and 0.018 $\text{s}^{-1}$, respectively). Interestingly, the pH dependence of $k_{cat}$ (indicative of ionizations of the enzyme-substrate complex) yielded apparent pK$_a$ (negative base 10 logarithm of the acid dissociation constant) values of 7.22 $\pm$ 0.48 and 6.7 $\pm$ 0.44 for HsKYNase and BF-HsKYNase, respectively, at the acidic ascending limb. Considering the catalytic mechanism of KYNase enzymes, it seems plausible to suggest that the pH dependence of $k_{cat}$, which is defined by the chemical step, reflects the pK$_a$ of the catalytic K276 (SI Appendix, Scheme S1, step 3). This hypothesis would indicate that at pH 7.4, the catalytic K276 of BF-HsKYNase (pK$_a$ = 6.67) is slightly more prone to nucleophilic attack and activation of a H$_2$O molecule compared to the K276 in HsKYNase (pK$_a$ = 7.2) and thus likely more readily promotes gem-diolate formation. Of note, the respective pH dependence of $k_{cat}$ of the bacterial PfKYNase, which preferentially catalyzes the hydrolysis of KYN with high activity, yielded a pK$_a$ of 6.43 (48), close to that of BF-HsKYNase.

**HDX-MS Analysis of BF-HsKYNase in the Presence of Either KYN or OH-KYN.** We used next HDX-MS to monitor the dynamics of BF-KYNase during steady-state catalysis of either KYN or OH-KYN. We recovered $\sim 200$ overlapping peptides that could be followed throughout 1-, 10-, or 100-min exposure to D$_2$O (SI Appendix, Table S8 and Dataset S1). The difference in average HDX in the presence and absence of substrate was calculated for each peptide at each timepoint, and significance was defined as a difference $\geq 0.5 \%$ Da, with $P < 0.01$ (Welch’s t test with $n = 3$). Steady-state parameters for BF-HsKYNase determined in D$_2$O-PBS (PBS: phosphate buffered saline) ($k_{KYN}^{\text{cat}} = 0.27 \pm 0.03 \text{ s}^{-1}$, $k_{OH-KYN}^{\text{cat}} = 0.20 \pm 0.03 \text{ s}^{-1}$, $K_m^{\text{KYN}} = 2,000 \pm 300 \text{ M}$, and $K_m^{\text{OH-KYN}} = 0.145 \pm 0.012 \text{ s}^{-1}$, $K_m^{\text{OH-KYN}} = 72 \pm 18 \text{ M}$) suggest that the reaction was under steady-state conditions for the first two timepoints (1 and 10 min).

In the absence of substrate, we observed similar HDX for HsKYNase and BF-HsKYNase, suggesting that the mutations in the latter enzyme did not result in significant changes in the conformational flexibility of the enzyme in its resting state. We also observed very similar HDX for the peptides spanning the mutated regions of BF-HsKYNase in the presence of either KYN or OH-KYN (SI Appendix, Fig. S15). One exception is region M427-H445 that contains four amino acid substitutions (K427M-N429E-G432A-A436T). This region showed a small decrease in exchange with KYN or OH-KYN at 10 min that may reflect the role of R434 in substrate orientation (SI Appendix, Fig. S17). Collectively, HDX in the mutated regions appears indistinguishable from the parental HsKYNase (56). The mutations in BF-HsKYNase are thus not generally influencing the dynamics of their backbone atoms in either the resting or the catalytic state. Thus, unexpectedly, even though the amino acid substitutions in BF-HsKYNase have a dramatic effect in the catalytic rate with KYN, they do not appear to affect the flexibility of the respective mutated regions in the enzyme.

Outside of the mutated regions, however, BF-HsKYNase did show different HDX with KYN and OH-KYN relative to HsKYNase. More specifically, significant differences were observed in regions surrounding the PLP cofactor. In the catalysis of KYN we observed reduced exchange at 1 min and increased exchange at 10 min in M134-L137 and K163-A171 (Fig. 3A–E, example peptides 134 to 141 and 161 to 175). At 100 min, this increased exchange extended further beyond these PLP binding regions (SI Appendix, Fig. S16 A and B). An increase in exchange during the catalytic cycle was not seen for HsKYNase with KYN or OH-KYN, revealing that BF-HsKYNase has increased structural flexibility in regions that harbor critical PLP-interacting residues, such as A136, L137, F165, and D168 (Fig. 3E). Flexibility of the PLP pocket likely contributes to the enhanced chemical and/or product release steps for KYN when compared to HsKYNase (60–62). Increased solvent accessibility of these regions can also modulate the electrostatic environment of the active site (63–65), impacting ionization states of critical catalytic residues such as K276 discussed above.

In contrast to the increased HDX of peptides in the PLP pocket of BF-HsKYNase, a reduction in exchange was observed in these regions during catalysis of OH-KYN. This was the case for M134-L137, K163-A171, D250-L260, and V303-V339 (Fig. 4A–E, example peptides 250 to 259 and 303 to 310; Fig. 3E, example peptides 134 to 141, 161 to 175 and 329 to 338; and SI Appendix, Fig. S16 C and D). Key PLP-interacting residues in the latter two regions are D250, W305, and N333. Notably, the reduced exchange of K163-A171 and the region containing N333 observed for BF-HsKYNase were not observed for HsKYNase, suggesting that BF-HsKYNase

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**Table 2. Rate constant of chemical step $k_2$ derived from the global fit analysis of the reaction of HsKYNase and BF-HsKYNase with noninhibitory concentrations of KYN**

| Enzyme        | Rate constant (defines $k_{cat}$) (s$^{-1}$) | Lower limit* | Upper limit† | % Range‡ | Best fit |
|---------------|---------------------------------------------|--------------|--------------|----------|----------|
| HsKYNase      | $k_2$                                       | 0.000987     | 0.00101      | 1.15     | 0.001    |
| BF-HsKYNase   | $k_2$                                       | 0.044        | 0.045        | 1.1      | 0.0445   |

Data are shown in Fig. 2A, D, B, and E in the main text.

*Lower limit represents a threshold of 2% deviation from the minimal sum square error (SSE) in the confidence contours.
†Upper limit represents a threshold of 2% deviation from the minimal sum square error (SSE) in the confidence contours.
‡The percentage range was calculated by dividing the mean of the range by the best fit value as follows: (upper – lower)/(2 x best fit). This represents the allowable variation of each best fit value as a percentage.

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becomes more stabilized during catalysis of OH-KYN than does HsKYNase. This increased stability and rigidity of key PLP-interacting regions during the catalytic cycle are likely the cause of compromised OH-KYN turnover by BF-HsKYNase when compared to HsKYNase.

Alongside changes in the PLP pocket, we also observed reduced exchange in the region surrounding the critical H102 (Y100-S115), which is involved in substrate binding (66). This region has reduced exchange at 1 and 10 min for both KYN and OH-KYN, with the reduction being larger for OH-KYN than for KYN (Fig. 3E, example peptide 100 to 116). Interestingly, we also detected reduced exchange in I218-Y226 and G281-F289 in BF-HsKYNase in the catalysis of OH-KYN whereas in HsKYNase there was no exchange at those respective regions (Fig. 4 A–E, example peptide 281 to 289; and SI Appendix, Fig. S17, example peptide 217 to 225). G281-F289 is located behind the ligand binding pocket, while I218-Y226 is at the rear of the active site cavity. Collectively, the HDX-MS data for BF-HsKYNase suggest that the amino acid substitutions in high B-factor regions of BF-HsKYNase modulate the flexibility of critical regions in the active site, especially those relevant for PLP and substrate recognition. BF-HsKYNase appears to have increased flexibility in these key active site regions during turnover of KYN. Conversely, BF-HsKYNase shows increased stability in several active site and third-shell (~15 Å away from K276) regions during turnover of OH-KYN.

Fig. 3. HDX-MS of BF-HsKYNase with and without KYN. (A and C) BF-HsKYNase homology model colored by the difference in fractional D-uptake (as a percentage) between no substrate and KYN after 1 min (A) and 10 min (C). Panels were prepared using DynamX per residue output without statistics and PyMOL. Residues without coverage are shown in gray, and PLP is shown in yellow sticks. (B and D) Volcano plots showing the average ΔHDX calculated by subtracting either BF-HsKYNase with KYN from no substrate after 1 min (B) and 10 min (D). P values were calculated using Welch’s t test. Significance cutoffs are P < 0.01 and an average ΔHDX > 0.5 Da. Boundaries of significant peptides are labeled on the plot and listed in SI Appendix, Table S9. Panels were created using HD-eXplosion (78). (E) Example peptides with significant ΔHDX in the active site and PLP binding pocket of BF-HsKYNase. D-uptake plot traces for no substrate (black), with KYN (orange), or with OH-KYN (gray). Error bars are ±2σ from three technical replicates. The y axis is scaled to 70% of the theoretical maximum uptake. BF-HsKYNase is colored as in C, and PLP is shown in yellow sticks (nitrogen, blue; phosphate, orange; and oxygen, red). Interactions between residues and PLP and the respective distances are shown in yellow dashed lines.
MD Simulations of BF-HsKYNase in the Presence of Either KYN or OH-KYN. We performed MD simulations of BF-HsKYNase and HsKYNase with the external aldimine KYN- and OH-KYN–quinonoid intermediates docked in the active site (Methods and SI Appendix, Figs. S18 and S19). Quinonoid intermediates have been shown to form transiently during the reaction of KYNase with KYN (67) (SI Appendix, Scheme S1, step 3), as well as in many other PLP-dependent enzyme-catalyzed reactions (68). Thus, 50-ns MD simulations applying the AMBER14 force field integrated into the YASARA Structure program were performed (69, 70). Three independent simulation runs were performed using different initial velocities in each run, leading to a total MD-simulation time of 1 μs (initial testing runs were performed under identical velocity conditions to ensure reproducibility of the trajectories). Additionally, simulations were performed for both BF-HsKYNase and HsKYNase in complex with the OH-KYN–quinonoid intermediate (Fig. 5B). Thus, the MD calculations are

Fig. 4. HDX-MS of BF-HsKYNase with and without OH-KYN. (A and C) BF-HsKYNase homology model colored by the difference in fractional D-uptake (%) between no substrate and OH-KYN after 1 min (A) and 10 min (C). Panels were prepared using DynamX per residue output without statistics and PyMOL. Residues without coverage are shown in gray, and PLP is shown in yellow sticks. (B and D) Volcano plots showing the average ΔHDX calculated by subtracting HDX for BF-HsKYNase with OH-KYN from HDX with no substrate after 1 min (B) and 10 min (D) of reaction. P values were calculated using Welch’s t test. Significance cutoffs are \( P < 0.01 \) and an average \( \Delta \text{HDX} > 0.5 \) Da. Boundaries of significant peptides are labeled on the plot and listed in SI Appendix, Table S9. Panels were created using HD-eXplosion. (E) Example peptides with significant \( \Delta \text{HDX} \) in the active site and PLP binding pocket of BF-HsKYNase. D-uptake plot traces for no substrate (black), with KYN (orange), or with OH-KYN (gray). Data have not been corrected for back exchange. Errors bars are ±2σ from three technical replicates. The y axis is scaled to 70% of the theoretical maximum uptake. BF-HsKYNase is colored as in C, and PLP is shown in yellow sticks following an atom-based coloring format (nitrogen, blue; phosphate, orange; and oxygen, red). Interactions between residues and PLP and the respective distances are shown in yellow dashed lines.
limiting step and catalytic activity, resulted in 45-fold acceleration of the rate and by HDX-MS and MD simulations and selection for high conformations.

We found that mutagenesis of residues in protein regions with high conformational flexibility, as evidenced by high B-factors and by HDX-MS and MD simulations and selection for high catalytic activity, resulted in 45-fold acceleration of the rate-limiting step and ~20-fold increase in \( k_{cat}/K_m \) of human KYNase. This pronounced effect in catalysis resulted from amino acid substitutions that are located 10 Å or more away from the active site. Interestingly, out of 22 HsKYNase high B-factor residues subjected to saturation mutagenesis, 10 substitutions found in BF-HsKYNase converged to either similar or identical residue relative to the consensus amino acid found in bacterial KYNase, which unlike the human enzyme, have high catalytic activity toward KYN (SI Appendix, Fig. S21 and Table S10).

Our detailed pre-steady-state kinetic analysis of BF-HsKYNase with either KYN or OH-KYN showed that the enhancement in catalytic activity toward KYN did not alter the identity of the rate-determining step for either reaction. As mentioned above, with KYN, the rate-determining chemical step was increased by 45-fold, whereas for OH-KYN, the rate constant for the equivalent step (i.e., OH-AA formation) decreased by 12-fold relative to the parental HsKYNase enzyme. Of note, the pH dependence of the \( k_{cat} \) for the KYN reaction suggested that the pK\textsubscript{a} of the catalytic base in BF-HsKYNase may have become more acidic and thus similar to the respective pK\textsubscript{a} value of the bacterial KYN-preferred PKYNase. Taken together, these data underscore how remote mutations can impart profound changes in catalysis through rather subtle changes in protein conformation.

Conformational flexibility is critical for enzymatic catalysis, enabling the protein to sample multiple and catalytically competent conformations (72). Here we show that flexibility plays an important role in the modulation of the enzymatic activity against its natural substrates, and notably, this property is substrate dependent. The HDX-MS analysis showed that in BF-HsKYNase, regions around the PLP binding pocket exhibit a significant increase in flexibility during the turnover of KYN. This flexibility increase may allow the PLP to sample multiple conformations upon KYN binding (which is not as efficient as the respective OH-KYN binding due to the lack of the aromatic hydroxyl group) and, in turn, facilitate the energy transition state stabilization for the chemical reaction and facilitate product release (9, 73).

In contrast, BF-HsKYNase did not show any increase in flexibility during OH-KYN turnover. In fact, key active site and PLP-interacting regions showed stabilization in the case of the BF-HsKYNase/OH-KYN reaction but no change in wild-type HsKYNase with OH-KYN. Along these lines, several new regions showed significant stabilization in the case of BF-HsKYNase but not of HsKYNase (56). These findings illustrate that unlike in the case of the KYN reaction, BF-HsKYNase exhibits a high degree of active site stabilization during OH-KYN hydrolysis, and this protection extends to several additional structural domains distal to the active site. This extensive stabilization of BF-HsKYNase in the presence of OH-KYN (which had not been observed with HsKYNase) may in part explain the overall catalytic activity decrease against this substrate, as such a rigidification could impair the dynamic mobility of important active site regions involved in substrate binding and catalysis. MD-simulation analysis further corroborated the HDX-MS findings, illustrating that the engineered BF-HsKYNase showed significantly larger destabilization than HsKYNase in complex with KYN, whereas both species

Discussion

We found that mutagenesis of residues in protein regions with high conformational flexibility, as evidenced by high B-factors and by HDX-MS and MD simulations and selection for high catalytic activity, resulted in 45-fold acceleration of the rate-limiting step and ~20-fold increase in \( k_{cat}/K_m \) of human KYNase. This pronounced effect in catalysis resulted from amino acid substitutions that are located 10 Å or more away from the active site. Interestingly, out of 22 HsKYNase high B-factor residues subjected to saturation mutagenesis, 10 substitutions found in BF-HsKYNase converged to either similar or identical residue relative to the consensus amino acid found in bacterial KYNases, which unlike the human enzyme, have high catalytic activity toward KYN (SI Appendix, Fig. S21 and Table S10).

Our detailed pre-steady-state kinetic analysis of BF-HsKYNase with either KYN or OH-KYN showed that the enhancement in catalytic activity toward KYN did not alter the identity of the rate-determining step for either reaction. As mentioned above, with KYN, the rate-determining chemical step was increased by
demonstrated a similarly stable Ca-rmsd profile in the presence of OH-KYN. The observed differences in the flexibility of BF-HsKYNase in the reaction with KYN and OH-KYN are in line with other reports showing that the conformational dynamics of enzymes can be strongly substrate specific (74, 75) and that remote mutations can significantly modulate and shift the dynamic conformational ensemble toward different states, depending upon the substrate.

In summary, in the present study, we provide extensive experimental evidence that by targeting residues remotely located at flexible sites of an enzyme, it is possible to significantly improve its catalytic activity against a substrate for which the enzyme has not evolved to efficiently catalyze. Despite many advances in understanding the fundamental aspects of enzyme conformational flexibility and its link to catalysis and evolution, the practical implementations of such knowledge in enzyme engineering remain limited. Our experimental findings underscore the utility of mutagenesis of distal, high-B-factor regions in directed enzyme evolution. Equally importantly, the current strategy can be further exploited in a wide variety of genetic selections, including yeast and other facile expression systems coupled to synthetic biology tools, as well as high-throughput screening platforms for the functional interrogation of large mutant libraries (76, 77).

Methods
Detailed descriptions on protein expression, purification, and characterization; B-factor analysis; library design, construction, and screening; steady-state kinetic analysis and study of the pH effect on the enzymes’ catalytic activity; pre-steady-state kinetic analysis, model development, and global fitting of the data by numerical integration of the rate equations; HDX-MS experiments and data analysis; and computational methods are provided in Supporting Information.

Data Availability. All study data are included in the article and/or supporting information.

ACKNOWLEDGMENTS. We are grateful to F. Bartozka for comments on the manuscript. This work was supported by funding from the Cancer Prevention and Research Institute of Texas (Grant DP150061) and Ikena Oncology.

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