Relative Tolerance of an Enzymatic Molten Globule and Its Thermostable Counterpart to Point Mutation†

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ABSTRACT: Enzyme structures reflect the complex interplay between the free energy of unfolding (ΔG) and catalytic efficiency. Consequently, the effects of point mutations on structure, stability, and function are difficult to predict. It has been proposed that the mutational robustness of homologous enzymes correlates with a higher initial ΔG. To examine this issue, we compared the tolerance of a natural thermostable chorismate mutase and an engineered molten globular variant to targeted mutation. These mutases possess similar sequence, structure, and catalytic efficiency but dramatically different ΔG values. We find that analogous point mutations can have widely divergent effects on catalytic activity in these scaffolds. In a set of five rationally designed single-amino acid changes, the thermostable scaffold suffers activity losses ranging from 50-fold smaller, for an aspartate-to-glycine substitution at the active site, to 2-fold greater, for a phenylalanine-to-tryptophan substitution in the hydrophobic core, versus that of the molten globular scaffold. However, biophysical characterization indicates that the variations in catalytic efficiency are not caused by losses of either secondary structural integrity or thermodynamic stability. Rather, the activity differences between variant pairs are very much context-dependent and likely stem from subtle changes in the fine structure of the active site. Thus, in many cases, it may be more productive to focus on changes in local conformation than on global stability when attempting to understand and predict how enzymes respond to point mutations.

The amino acid sequence of a protein encodes its structure and function with high redundancy. This degeneracy permits a wide variety of residue substitutions at many different sequence positions without sacrificing global structural integrity or biological activity (*1*−*4*). Nevertheless, amino acid exchanges can sometimes have profound consequences on folding, stability, or function (*5*−*10*). The search for the rare mutations that provide functional innovation drives protein evolution and often requires maintenance of preexisting activity. In this context, the choice of starting structure can also be crucial for the success of protein engineering efforts. However, the features of a protein scaffold that determine its relative ability to accommodate point mutations remain poorly understood.

One parameter that may influence resilience to mutation is the free energy of unfolding (ΔG).[1] Different proteins can display a wide range of ΔG values, with typical values falling between 3 and 30 kcal/mol, which suggests that this parameter could potentially exert large effects on the response to side chain substitution. Indeed, recent studies using enzyme homologues have shown a correlation between the initial ΔG and the ability of variants to retain activity or gain a new function (*11, 12*). Understanding this phenomenon requires detailed characterization of variant pairs derived from homologues with similar activities that display differing stabilities and dynamic properties.

The enzyme chorismate mutase (CM) is an appealing choice for examining the relationship between the initial ΔG and resilience to point mutation. This enzyme catalyzes the rearrangement of chorismate to prephenate (Figure 1A) and has become an important model system for understanding enzymatic catalysis (*13*). The CM from *Methanococcus jannaschii* (MjCM) is a thermostable dimer (Figure 1B) (*14*). Directed evolution was used to generate a monomeric variant, named mMjCM (Figure 1C), that has near native catalytic activity (*15*). Although the amino acid sequences of MjCM and mMjCM contain only nine amino acid differences (the monomer contains an eight-amino acid insertion and one point mutation relative to the dimer) and adopt α-helical folds, the latter enzyme has a much lower free energy of unfolding ($\Delta\Delta G \sim 25$ kcal/mol). In fact, mMjCM displays all the hallmarks of a molten globule (*16*), a structural state characterized by nativelike secondary structure content and a loosely packed hydrophobic core. Consequently, this catalytically active molten globule possesses greater conformational diversity than traditional native-state enzymes.

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[1] Abbreviations: CD, circular dichroism; CM, chorismate mutase; ΔG, free energy of unfolding; GdmCl, guanidinium chloride; MjCM, *Methanococcus jannaschii* CM; mMjCM, engineered monomeric variant of MjCM; PBS, phosphate-buffered saline; PDB, Protein Data Bank.

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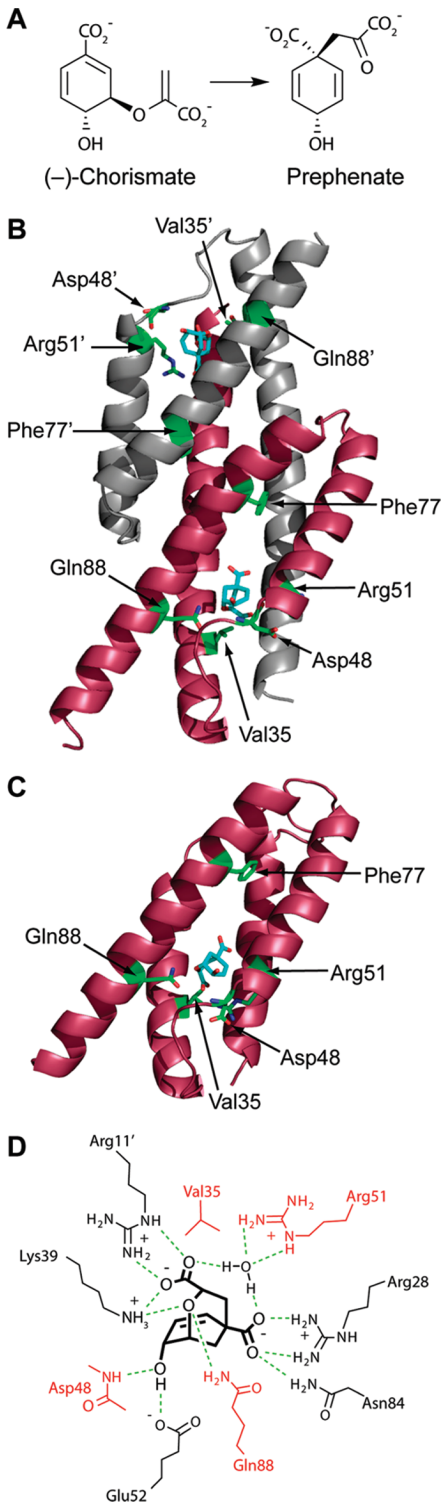

FIGURE 1: Chorismate mutases. (A) CMs catalyze the conversion of chorismate to prephenate. (B) MjCM adopts a homodimeric α-helical fold. Both subunits (colored burgundy and gray) contribute to each active site. The high-resolution structure of MjCM has not been determined but is represented here by the related enzyme from *Escherichia coli* with a bound transition-state analogue (PDB entry 1ecm) (*48*). Residues targeted for mutation are indicated. (C) The NMR structure of mMjCM bound to the transition-state analogue (PDB entry 2gtv) (*17*) shows an α-helical bundle with the active site completely contained within a single subunit. Residues targeted for mutation are indicated. (D) A schematic of the mMjCM and MjCM active sites illustrates the variety of electrostatic and hydrophobic interactions involved in binding to the transition-state analogue (bold). Four of the five residues targeted for mutation are colored red. Phenylalanine 77, in the hydrophobic core, is not shown.

To compensate for its relative lack of preorganization, mMjCM undergoes substantial structural ordering upon substrate binding, which generates the same organized array of polar active site residues (Figure 1D) necessary for efficient catalysis as MjCM (*17*). The ligand-induced ordering of mMjCM results in substantial entropy−enthalpy compensation as well as elevated rates of ligand binding compared to that of MjCM (*18*). A recent computational study of dimeric and monomeric CMs found that, even in the ligand-bound form, the ensemble of catalytically competent conformations is broader for the molten globular enzyme than for the native-state enzyme (*19*). Thus, we anticipated that equivalent structural perturbations in MjCM and mMjCM might have different consequences for catalysis, despite their similar sequences, folds, and activities.

Here, we survey the influence of point mutations on structural integrity, conformational stability, and catalytic activity in both scaffolds. Specific substitutions were chosen to induce a variety of chemical or structural perturbations. We find that, for the variants examined here, analogous sequence changes cause catalytic efficiency to change differently in the two scaffolds and that most, but not all, of the substitutions cause the less stable scaffold to lose more activity. Furthermore, the mutations to which the activity of mMjCM was found to be more sensitive do not disrupt its overall structural integrity, and this scaffold displays much smaller changes in ΔG for these sequence changes than MjCM does. We conclude that the initial ΔG exerts an important, yet highly context-dependent, influence on the abilities of these scaffolds to tolerate point mutations and that the less stable scaffold is not particularly sensitive to inactivation via mutation-induced misfolding or destabilization of the folded state.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. The desired mutations were introduced into the DNA sequences encoding mMjCM and MjCM within the pET-mMjCM-pATCH (*15*) and pET-MjCM-pATCH (*14*) plasmids, respectively, by PCR with primers, purchased from Microsynth (Balgach, Switzerland), containing the specific nucleotide changes. Oligonucleotide sequences and reaction conditions are provided in the Supporting Information.

Protein Production and Purification. All of the mMjCM and MjCM variants, fused to C-terminal His_6 tags, were produced in KA13 cells and purified as previously described (*14, 15*). All proteins were analyzed by ESI mass spectrometry to confirm primary structure. Calculated and observed masses are provided in the Supporting Information.

Enzymatic Activity. To assess the effects of the mutations on CM activity, kinetic assays were performed by monitoring the reaction spectrophotometrically at 275 nm according to a published procedure (*15*). Measurements were performed in PBS buffer at 20 °C. The steady-state parameters k_{cat} and K_m were calculated from duplicate determinations of background-corrected initial rates using a minimum of five substrate concentrations ranging from at least 2-fold below K_m to at least 2-fold above K_m (except for mMjCM R51Q). Enzyme concentrations ranged from 70 nM (for the most active variants) to 10 μM (for the least active catalysts).

Circular Dichroism (CD) Spectroscopy. Far-UV CD spectra were recorded for each protein (16 μM) in PBS buffer by averaging five scans from 260 to 200 nm (1 nm steps, 2 s averaging time, 2 mm path length, 20 °C). Buffer background was then subtracted from the spectra.

Chemical Denaturation. Chemical denaturation curves were recorded on the basis of previously reported procedures for both MjCM (*14*) and mMjCM (*15*). The denaturation of mMjCM and MjCM variants was monitored by fluorescence spectroscopy. Tyrosine fluorescence was assessed by excitation at 278 nm (2 nm bandwidth) and emission at 320 nm (4 nm bandwidth). Measurements were performed in PBS using a 1.0 cm path length quartz cuvette at 20 °C with constant stirring. The initial protein concentration was 10 μM (MjCMs) or 4 μM (mMjCMs), each of which is in the concentration-independent region of ΔG for these proteins. An additional slit (1 mm × 6 mm) was placed between the excitation beam and the sample for the F77W mutants because of their enhanced fluorescence properties. Denaturant titrations were performed by removing an appropriate volume of the protein solution and adding the same volume of GdmCl (~8 M in PBS) or urea (~10 M in PBS) stock solutions. The use of different denaturants for the two scaffolds was dictated by the insensitivity of MjCM to urea-induced unfolding and the high sensitivity of mMjCM to GdmCl, but both sets of ΔG values represent the stability of the folded form in the absence of denaturant. Following each addition of denaturant, the solution was equilibrated for 4 min, and then the fluorescence signal was recorded and averaged over 60 s. This value was corrected for PBS and denaturant background and then normalized to account for the dilution of the proteins. To derive values for the free energy of unfolding at 0 M denaturant (ΔG), cooperativity (m), and transition midpoint, the data were fit to a two-state model (*15, 20*).

RESULTS

Design, Production, and Purification of mMjCM and MjCM Variants. In enzymes, point mutations are frequently detrimental to catalytic activity (*21*−*24*). However, the extent of activity loss upon mutation of homologous enzymes can differ considerably (*11, 12*). To investigate the relationship between initial scaffold stability and tolerance to amino acid substitution, we chose a set of diverse point mutations to perturb both MjCM and mMjCM at different positions throughout their structures (Figure 1B−D). These amino acid substitutions were designed to impact activity and/or stability based on a variety of structural and functional rationales. For instance, the F77W[2] mutation was intended to perturb hydrophobic core packing by introducing a residue with a different size and shape. Changing valine 35 to alanine should also lead to alterations in hydrophobic packing, this time within the active site. The introduction of a glycine at position 48 removes the solvent-exposed, α-helix-capping side chain of an aspartic acid whose backbone makes an important hydrogen bond with the substrate. The R51Q substitution should relieve Coulombic repulsion in the highly cationic active site while possibly maintaining the ability to interact with a structurally important water molecule. Finally, the Q88N mutation was designed to reposition a residue that forms a crucial hydrogen bond with the transition state. A comparison of the structural and functional consequences of these diverse substitutions in each scaffold should illuminate the influence of initial stability on mutational tolerance.

[2] The nomenclature used for numbering MjCM and mMjCM residues corresponds to the *E. coli* CM numbering throughout this text.

Table 1: Kinetic Parameters of the MjCM and mMjCM Variants[a]

	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
MjCM	3.0 ± 0.1	54 ± 3	56000
F77W MjCM	0.41 ± 0.01	73 ± 5	5600
Q88N MjCM	0.042 ± 0.001	1200 ± 100	35
V35A MjCM	0.50 ± 0.01	30 ± 2	17000
R51Q MjCM	2.6 ± 0.1	1300 ± 100	2000
D48G MjCM	1.3 ± 0.1	59 ± 3	22000
mMjCM	2.8 ± 0.1	150 ± 20	19000
F77W mMjCM	0.81 ± 0.03	220 ± 20	3700
Q88N mMjCM	0.0095 ± 0.0006	1500 ± 200	6.3
V35A mMjCM	1.1 ± 0.1	1500 ± 200	700
R51Q mMjCM	≥0.27	≥4100	65
D48G mMjCM	0.28 ± 0.01	1900 ± 200	150

[a] Assays for chorismate mutase activity were carried out in PBS buffer (pH 7.5) at 20 °C by monitoring the change in absorbance at 275 nm over time.

All the variants were overproduced in *E. coli* and purified to homogeneity by sequential Ni^{2+} affinity and gel filtration chromatography steps. The high yields obtained for these variants (ranging from 9 to 27 mg/L of culture) enabled their detailed kinetic and biophysical characterization.

Catalytic Properties. To determine the effects of the mutations on CM activity, kinetic assays were performed using a continuous UV spectrophotometric assay according to an established procedure (*15*). As expected, all mutations caused catalytic efficiency to decrease, although the magnitude of these decreases (in both scaffolds) ranged from just a few-fold to more than 1000-fold (Table 1). However, the relative losses in activity were not the same for equivalent mutations in the thermostable and molten globular scaffolds.

The k_{cat} values of MjCM and mMjCM are initially identical, but the relative effects that point mutations have on this parameter differ considerably (Figure 2A,B). When polar interactions are disrupted by mutations, the resulting mMjCM variants have k_{cat} values that are 4−10 times lower than those of their MjCM analogues. In contrast, the V35A and F77W mMjCM variants, which change hydrophobic packing, both display k_{cat} values that are ~2-fold higher than those of their MjCM counterparts. Thus, in terms of maximum velocity (turnover number), the more stable scaffold does not always retain more catalytic power.

For the set of mutations examined here, the rank order of catalytic efficiency differs between the two scaffolds (Table 1). For example, F77W mMjCM is the most active monomeric variant, but D48G MjCM has the highest k_{cat}/K_m value of the dimeric variants. Both k_{cat} and K_m effects contribute to the observed decreases in k_{cat}/K_m seen within the mMjCM scaffold in all cases. In contrast, a loss of efficiency in MjCM can sometimes be attributed solely to a decreased k_{cat} value (for the V35A variant) or an increased K_m value (for the R51Q variant). In terms of the relative k_{cat}/K_m, F77W was the only substitution to which mMjCM was less sensitive than MjCM (Figure 2C,D). This mutation caused a 2-fold larger drop in k_{cat}/K_m for MjCM than for mMjCM, narrowing the initial 3-fold difference between these scaffolds to near

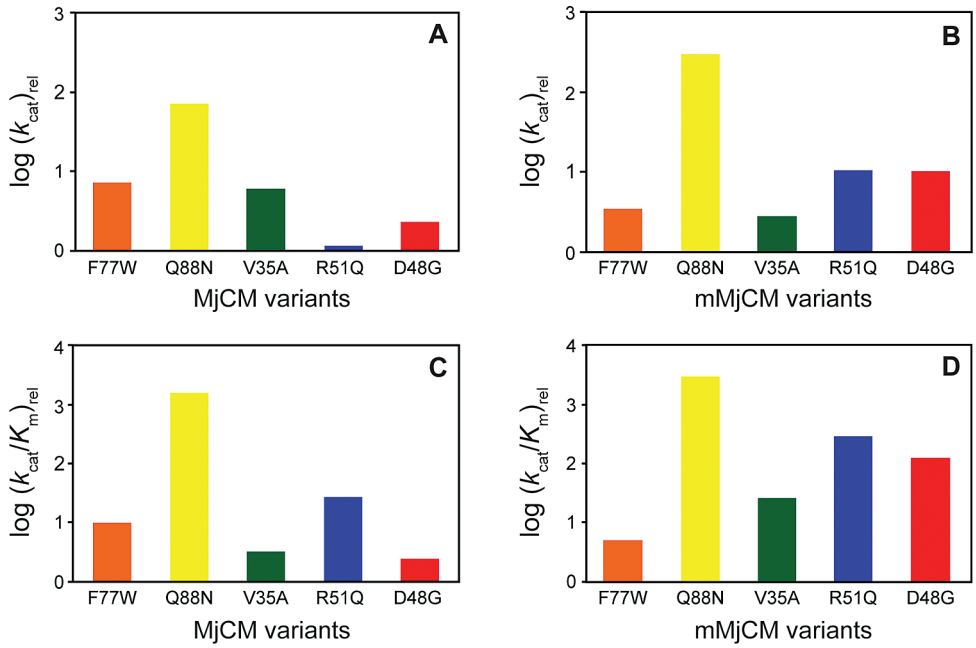

FIGURE 2: Effect of mutation on the catalytic parameters of mMjCM and MjCM. The log of the ratio of the k_{cat} value of the unmutated enzyme to that of the variant for each mutation is plotted as a histogram for MjCM (A) and mMjCM (B). The log of the ratio of the k_{cat}/K_m value of the unmutated enzyme to that of the variant for each mutation is similarly shown for MjCM (C) and mMjCM (D).

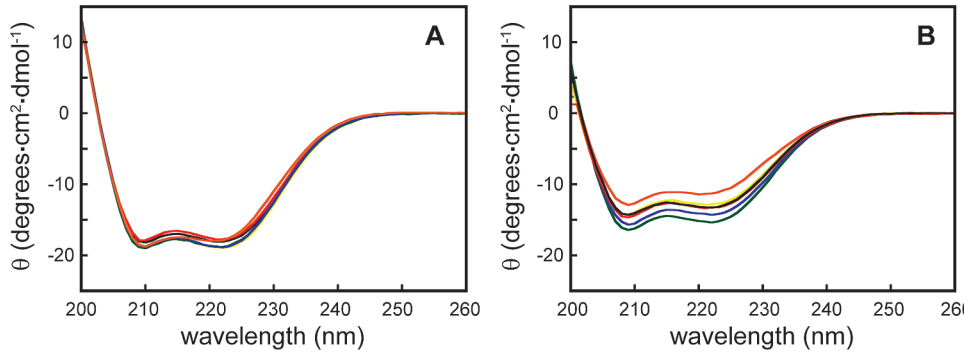

FIGURE 3: Far-UV CD spectra of the MjCM (A) and mMjCM (B) variants. Spectra are shown for unmutated mMjCM and MjCM (black), as well as the F77W (orange), Q88N (yellow), D48G (red), R51Q (blue), and V35A (green) variants.

equality. At the other extreme, the gap in k_{cat}/K_m widens to 150-fold for the variants containing the D48G mutation. The greater loss in catalytic efficiency for D48G mMjCM resulted from both a larger increase in K_m and a greater decrease in k_{cat}. Differential effects on both parameters were also seen for the Q88N mutation, which cripples catalytic efficiency in mMjCM twice as much as in MjCM. Overall, the lack of consistent trends in the relative k_{cat}/K_m values for these variant pairs reflects a partitioning between k_{cat} and K_m effects that differs between the two scaffolds and depends on the particular substitution.

Structural and Stability Properties. The five mutations perturb the structures of MjCM and mMjCM differently, as indicated by the variations in activity seen for the mutant pairs. To assess the relative secondary structure content of the MjCM and mMjCM variants, CD spectroscopy measurements were performed. The far-UV CD spectra of all the variants are consistent with the α-helical fold of these CMs (Figure 3). The nearly superimposable spectra of the MjCMs indicate that they possess identical secondary structures. The lower average signal intensities seen for the mMjCMs are consistent with previous measurements (*16*). However, unlike the MjCM variants, the spectra of the mMjCM variants show a small amount of scatter to higher or lower mean residue ellipticity compared to mMjCM itself (±10%), indicating that some of the mutations may induce a bit more restructuring in the less stable scaffold. Nevertheless, the larger activity losses seen for most of the mMjCM variants cannot be ascribed to a greater susceptibility to defects in the α-helical fold.

How do the mutations affect structural stability in these scaffolds? To address this question, we turned to chemical denaturation. Equilibrium unfolding titrations were performed according to previously published procedures by monitoring intrinsic protein fluorescence as a probe for tertiary structural integrity and using guanidinium chloride (GdmCl) as the denaturant for the MjCMs (*14*) or urea for the mMjCMs (*15*). The ΔG values for MjCM and mMjCM (Table 2)[3] report

[3] For most of the proteins examined here, the plots of the fraction of unfolded protein (f_U) vs denaturant concentration showed small deviations from an ideal two-state model (see the Supporting Information). Such deviations indicate the presence of an unfolding intermediate, as previously seen for MjCM. Because the fluorescence properties of the unfolding intermediates are similar to those of the unfolded state, data points indicating accumulation of the unfolding intermediate were excluded from the analysis, as in the previous determination of ΔG for MjCM (*14*).

Table 2: Conformational Stability Parameters of the MjCM and mMjCM Variants

	ΔG (kcal/mol)	M (kcal mol^{-1} M^{-1})	midpoint (M)[a]
MjCM[b]	28 ± 1	5.0 ± 0.2	4.2
F77W MjCM	21 ± 1	3.4 ± 0.1	3.9
Q88N MjCM	28 ± 1	5.8 ± 0.4	3.6
V35A MjCM	24 ± 1	4.3 ± 0.1	3.8
R51Q MjCM	25 ± 1	4.9 ± 0.3	3.6
D48G MjCM	26 ± 1	5.7 ± 0.3	3.4
mMjCM[c]	3.4 ± 0.1	0.70 ± 0.03	4.8
F77W mMjCM	3.9 ± 0.1	0.94 ± 0.14	4.2
Q88N mMjCM	3.2 ± 0.1	0.63 ± 0.02	5
V35A mMjCM	nd[d]	nd[d]	nd[d]
R51Q mMjCM	2.9 ± 0.2	0.66 ± 0.04	4.5
D48G mMjCM	2.7 ± 0.1	0.60 ± 0.02	4.5

[a] Midpoint values represent the denaturant concentration at the halfway point of the unfolding transition. [b] Conformational stability determinations for MjCM and its variants were carried out using GdmCl as the denaturant. [c] Conformational stability determinations for mMjCM and its variants were carried out using urea as the denaturant. [d] Not determined.

on the native-state versus unfolded-state equilibria and molten globule-state versus unfolded-state equilibria, respectively, and are in good agreement with the previously reported values (*14, 15*). The largest changes in stability were seen for F77W MjCM and V35A MjCM, which displayed ΔG values for unfolding that were 7.8 and 4.7 kcal/mol lower than that of wild-type MjCM, respectively. Conversely, the F77W mutation had a stabilizing influence on mMjCM, increasing ΔG by 0.5 kcal/mol. For V35A mMjCM, the issue of conformational stability is complicated by deviations from two-state behavior that are too large to permit an estimation of ΔG. The first transition apparent in the urea titration of V35A mMjCM appears earlier than the main transition for unmutated mMjCM, which indicates that this mutation might be slightly destabilizing, but in constrast to mMjCM and most other monomeric mutants, modestly cooperative two-state thermal unfolding (see the Supporting Information) suggests the opposite. None of the other mutations increased conformational stability, although their destabilizing effects were much more modest in the mMjCM scaffold than in the MjCM scaffold.

DISCUSSION

Understanding how enzymes respond to mutations can provide insight into structure−function relationships (*9, 10*) and can also illuminate plausible evolutionary trajectories (*25*−*27*). Homologous enzymes with similar structures and activities present a potentially stringent test of this understanding. The degree to which the activities of homologous enzymes are affected by similar mutations can vary widely, and the basis for this variability is not well understood. This high context dependence of mutational effects is often ignored in mechanistic studies of enzymes, which often focus on a single, arbitrarily chosen member of a whole family. Among the few studies in which homologous enzymes have been mutagenized and characterized in parallel, the identity of the parent enzyme has been found to have a significant influence on the functional properties of the resulting variants, and initial thermostability seems to correlate with higher final activity (*11, 12*). Consistent with these findings, the thermostable MjCM scaffold retained more activity than the marginally stable mMjCM scaffold for most of the mutations examined here, an advantage that ranged up to 50-fold for the D48G mutation. However, this trend is not absolute, and the less stable mMjCM can better accommodate some mutations. Given the disproportionate and context-dependent effects of the mutations examined here, it is informative to examine the kinetic and biophysical properties of the variant pairs in light of the original design rationale.

The greatest divergence of catalytic activities was seen for the D48G mutation, which caused k_{cat}/K_m to decrease by 120-fold in mMjCM but only 3-fold in MjCM. In the unmutated enzymes, aspartate 48 is a helix-capping residue located on the exterior surface whose main chain makes a hydrogen bond with the substrate/transition state. The positioning of the backbone may be reinforced by the rigidity of the MjCM scaffold, largely preserving the catalytically important hydrogen bond in this case. On the other hand, the intrinsic flexibility of the mMjCM scaffold and the high degree of conformational mobility provided by glycine may combine to increase the energetic cost of fixing the backbone NH group. Additionally, removal of the helix-capping interaction in mMjCM could have indirect effects on the positioning of active site residues located within the uncapped helix. However, the α-helical contents of both scaffolds are unaffected by this mutation, suggesting that these variants do not misfold. Both scaffolds do exhibit decreases in conformational stability upon loss of this helix capping interaction (0.7 kcal/mol in mMjCM and 1.9 kcal/mol in MjCM), but the large difference in catalytic efficiency for the D48G variant pair is not a consequence of a thermodynamic preference for the unfolded state on the part of the mMjCM mutant. Interestingly, an in vivo complementation experiment indicated that the D48G mutation causes a ≥20-fold decrease in the activity of the homologous, but mesostable, CM from *E. coli*, suggesting that the mutational tolerance of this scaffold might be intermediate between the two extremes examined in this study (*28*).

Greater initial scaffold stability also corresponded with greater mutational tolerance for the other two substitutions of polar active site residues. The higher activities of R51Q MjCM and Q88N MjCM relative to their mMjCM counterparts demonstrate that greater conformational flexibility does not necessarily help enzymes adjust to mutations that change the positional ordering of catalytically important hydrogen bond donors. However, the activity losses seen for these variants cannot be attributed to global restructuring or instability. As electrostatic interactions have stringent geometric requirements and require precise positioning of the residues involved, these contacts might be easier for more stable scaffolds to maintain upon mutation.

Nevertheless, thermostability does not always confer enhanced resilience to change, as seen for the substitutions of nonpolar residues. The F77W mutation induces structural alterations in the hydrophobic cores of MjCM and mMjCM that propagate to the active site, causing catalytic activity decreases in both scaffolds. However, the more stable scaffold is less tolerant of this mutation, suffering a 2-fold greater drop in catalytic efficiency. While F77W MjCM remains much more stable than F77W mMjCM overall, this mutation enhances the stability of the monomer but causes a large drop in stability for the dimer. Apparently, extra hydrophobic surface area is welcomed in the loosely packed hydrophobic core of mMjCM, and the loose packing better

insulates the active site from this mutation. In contrast, the extra bulk introduced by this substitution is more disruptive with respect to the tightly packed hydrophobic core of MjCM, and this destabilizing effect may be doubled by the 2-fold symmetry of the dimer. The properties of V35A mMjCM (smaller drop in k_{cat} compared to V35A MjCM, more cooperative thermal denaturation compared to parent mMjCM) also suggest that less stable scaffolds may often adjust relatively well to mutations that alter hydrophobic packing.

The properties of these MjCM and mMjCM variants can be contrasted with those of cytochrome P450 variants obtained by directed evolution of homologous scaffolds (*12*). Screening of error-prone PCR libraries revealed that mutants derived from the more stable starting enzyme bound the heme cofactor roughly twice as frequently as those derived from the less stable enzyme. Variants based on the more stable scaffold also exhibited activity improvements with alternative substrates 6 times more frequently. Further, functional gains often coincided with substantial decreases in the melting temperature. Two different point mutations were identified that were functionally beneficial in the thermostable scaffold but resulted in an inactive enzyme when transplanted into the less stable scaffold. In contrast, a point mutation found to be functionally beneficial in the less stable scaffold had similar effects when introduced into the more stable scaffold.

On the basis of lattice model simulations, ligand binding properties, and previous theoretical studies, these results were explained in terms of a generally greater resistance to mutation-induced unfolding for more thermostable starting scaffolds (*12, 29*−*33*). According to this view, for homologous enzymes that differ in initial stability, analogous mutations should cause similar changes in activity. As mutations accumulate, the less stable homologue is more prone to crossing a global stability threshold ($\Delta G = 0$), at which point the unfolded state becomes thermodynamically favored, and this homologue consequently starts to lose much more activity than its fully folded counterpart. Apparently, a small number of mutations can sometimes be sufficient to force the marginally stable cytochrome P450 over this threshold.

The detailed biophysical characterization of the mMjCM and MjCM variants presented here indicates that the differing abilities of these scaffolds to tolerate point mutations (Figure 4A,B) are related neither to crossing a thermodynamic stability threshold (Figure 4C) nor to alterations in the α-helical fold (Figure 3) on the part of the point mutants. It could be argued that the generally lower resilience of mMjCM might stem from destabilization of an unobserved nativelike state in the molten globular ensemble of the free enzyme (i.e., a rare but catalytically competent conformation that most closely resembles the structure of MjCM and is required to bind substrate). Such an explanation would be consistent with both our results and the model developed in the cytochrome P450 study. However, this scenario is unlikely because mMjCM utilizes an induced fit mechanism during catalysis (*17*). Free mMjCM need not pass through a nativelike conformation to bind substrate since many different members of the molten globular ensemble are catalytically competent (*19*). Instead, our results may be rationalized by differences within the equilibria of the folded ensembles between the MjCM and mMjCM variants, likely involving

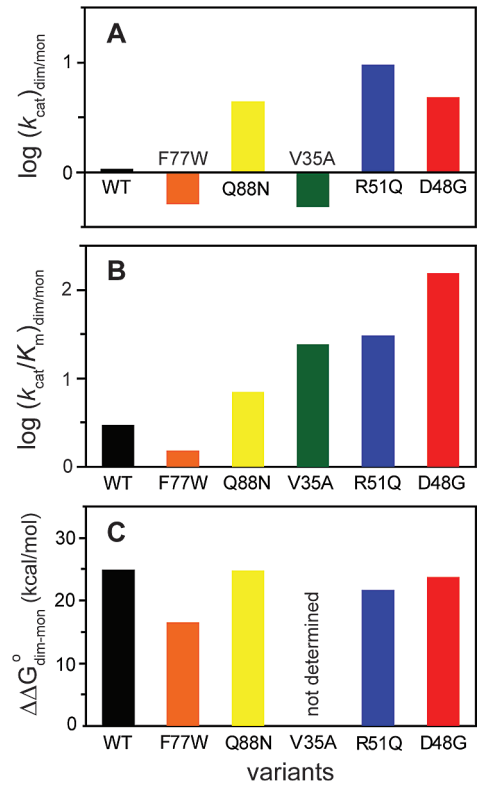

FIGURE 4: Relative sensitivity of the mMjCM and MjCM scaffolds to the different point mutations. (A) The log of the ratio of the k_{cat} value of an MjCM variant to that of its corresponding mMjCM variant is given for each enzyme pair. (B) The log of the ratio of the k_{cat}/K_m value of an MjCM variant to that of its corresponding mMjCM variant is given for each enzyme pair. (C) $\Delta\Delta G$ values are shown for each enzyme pair and correspond to the difference in conformational stability between an MjCM variant and its mMjCM counterpart. WT, unmutated MjCM and mMjCM parent scaffolds.

alternative positioning of individual side chains. While the definition of a protein's "native structure" could be extended to the residue level, we believe that such a detailed view of the native state is not particularly productive because proteins are dynamic and stochastic molecules.

Rather than global stability effects, the stringent structural requirements for transition-state stabilization by an active site likely underlie the tendency of a less stable enzyme to lose more activity upon mutation relative to a more stable enzyme.[4] Small movements of catalytic residues at the transition state can have profound impacts on activity without affecting the fold, and it is well-known that point mutations can have large effects on enzyme activity due to subtle changes in structure (*10, 34*). In a less stable enzyme, a newly introduced side chain may have greater freedom to acquire alternative interactions that change the fine structure but not

[4] The topology of a protein fold can also influence the effects of point mutations. The cytochrome P450 fold might be instrinsically more prone to mutation-induced misfolding or unfolding than the MjCM/mMjCM fold. However, for the P450 variant pairs described by Bloom et al. (*12*), the extents of restructuring and of changes in ΔG caused by equivalent mutations have not been directly examined. The properties of the MjCM and mMjCM variants demonstrate that a disproportionate loss of catalytic efficiency is not necessarily linked to global misfolding or to changes in ΔG. Given the stringent requirements for binding a transition-state at an active site, it seems likely that pathways for mutation-induced inactivation that do not involve misfolding or unfolding could be common.

the fold. Such subtle rearrangements can sometimes have large ramifications for activity (*35*) and may also dampen the destabilizing effects of mutations (*36, 37*).

It is important to keep in mind that the small set of mutations examined here is biased in terms of both their location and the type of residue substitution. The apparently greater functional resilience of the thermostable MjCM might be somewhat exaggerated by the fact that most of the residues we targeted lie close to the active site, where small, localized structural changes tend to have the greatest impact (*38*). Although residues throughout the fold tend to contribute to protein stability, core residues are particularly important in this respect (*39*). While we introduced only one mutation into the core, the F77W variants show that structural alterations remote from the active site may propagate in different ways, causing larger decreases in both k_{cat} and ΔG for the thermostable parent while leaving the overall fold of both scaffolds intact. Tight packing of the core seems to potentiate the ramifications of residue substitutions, at least in this case.

In contrast to hydrophobic core positions, mutation of active site residues often involves activity−stability trade-offs (*40,41*). Indeed, evolutionary studies have identified nonfunctional, but stabilizing, mutations remote from the active site that compensate for functionally innovative, but destabilizing, active site changes (*42, 43*). We did not observe any such trade-offs in the active sites of MjCM and mMjCM, which provides further evidence that functionally important residues are not always destabilizing (*44*). As in the core, the less stable scaffold seems to be better insulated from the destabilizing influence of active site mutations, which likely also holds true for those that confer new functions.

An additional factor that likely influences mutational tolerance is the chemical nature of a residue exchange, which in our designs tended to be relatively conservative. More drastic changes in terms of size or polarity might have different consequences for the activity, stability, and structure of these scaffolds. Nevertheless, it is remarkable that such a limited set of substitutions was sufficient for identifying mutations that cause significant deviations (in both directions) in the relative activities of these highly homologous enzymes. A broader sampling of variants might uncover mutations that lead to even more pronounced divergence.

This relatively rapid rate of functional divergence per mutation has evolutionary implications, as it relates to the concept of mutational robustness (*29, 45*). In Nature, proteins are constantly subjected to (random) mutation and selection for function. Because beneficial mutations are rare, mutational robustness, the ability of a protein to accumulate sequence changes and maintain activity (and provide a growth advantage to the host organism), is an important aspect of its evolvability. The differing abilities of MjCM and mMjCM to maintain catalytic efficiency upon the introduction of point mutations is consistent with the ideas that mutational robustness is a function of both the nature of the mutation(s) and the identity of the starting scaffold and that more stable starting structures more often exhibit greater robustness.

The basis of this robustness advantage has previously been explained in terms of thermostable proteins being less prone to falling below a stability threshold, as mentioned above. Our results show that global unfolding effects cannot always explain differences in mutational tolerance and subtle structural rearrangements can be important even at low mutation loads. Higher mutation loads may very well cause unfolding effects eventually, but our single mutations are sufficient to demonstrate that catalytic efficiency can be lost disproportionately between closely related enzymes before unfolding effects arise. Given the additive effects of most mutations on catalytic activity, increasing the mutation load (adding a second mutation and a third and so on) is most likely to make this functional divergence even more pronounced before the unfolded state becomes thermodynamically favored. In addition to catalytic efficiency, the biological activity of an enzyme undoubtedly involves other parameters, such as susceptibility to degradation, that are related to stability. From a structural perspective, however, our results reinforce the view that it may be more productive to focus on conformational specificity, rather than stability, in attempting to predict the functional consequences of point mutations (*7, 46*).

The behavior of the mMjCM variants observed here supports the notion that less stable structures, such as molten globules, might prove to be better starting points for developing new catalytic activities (and mechanisms). Such an evolutionary leap likely requires the active site to adopt an alternative conformation that could be more easily accessed from a less stable starting point (*31*). This potential advantage may be particularly useful for the directed evolution of new enzymes in the laboratory using selection or screening systems that do not depend on maintaining the original catalytic activity. Regardless, our results substantiate the idea that, upon mutation, enzymes can lose their activities more easily than their folds (*47*), which has important implications for their evolution and engineering.

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SUPPORTING INFORMATION AVAILABLE

Additional denaturation and enzyme kinetic data for the experiments described here as well as a more detailed description of the site-directed mutagenesis procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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