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Abstract

An engineered monomeric chorismate mutase (mMjCM) has been found to combine high catalytic activity with the characteristics of a molten globule. To gain insight into the dramatic structural changes that accompany binding of a transition-state analog, we examined mMjCM by isothermal calorimetry and compared it with its dimeric parent protein, MjCM (CM from Methanococcus jannaschii), a thermostable and conventionally folded enzyme. As expected for a ligand-induced ordering process, there is a large entropic penalty for binding to the monomer relative to the dimer (− TΔΔS = 5.1 ± 0.5 kcal/mol, at 20 °C). However, this unfavorable entropy term is largely offset by enthalpic gains (ΔΔH = − 3.5 ± 0.4 kcal/mol), presumably arising from tightening of non-covalent interactions throughout the monomeric complex. Stopped-flow kinetic measurements further reveal that the catalytic molten globule binds and releases ligands significantly faster than its natural counterpart, demonstrating that partial structural disorder can speed up molecular recognition. These results illustrate how structural plasticity may strongly perturb the thermodynamics and kinetics of transition-state recognition while negligibly affecting catalytic efficiency.
Kinetics and thermodynamics of ligand binding to a molten globular enzyme and its native counterpart

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An engineered monomeric chorismate mutase (mMjCM) has been found to combine high catalytic activity with the characteristics of a molten globule. To gain insight into the dramatic structural changes that accompany binding of a transition state analog, we examined mMjCM by isothermal calorimetry and compared it to its dimeric parent protein, MjCM, a thermostable and conventionally folded enzyme. As expected for a ligand-induced ordering process, there is a large entropic penalty for binding to the monomer relative to the dimer (\( -T\Delta S = 5.1 \pm 0.5 \text{ kcal/mol, at } 20^\circ\text{C} \)). However, this unfavorable entropy term is largely offset by enthalpic gains (\( \Delta H = -3.5 \pm 0.4 \text{ kcal/mol} \)), presumably arising from tightening of non-covalent interactions throughout the monomeric complex. Stopped flow kinetic measurements further reveal that the catalytic molten globule binds and releases ligands significantly faster than its natural counterpart, demonstrating that partial structural disorder can speed up molecular recognition. These results illustrate how structural plasticity may strongly perturb the thermodynamics and kinetics of transition state recognition while negligibly affecting catalytic efficiency.
**Keywords:** chorismate mutase; molten globule; isothermal titration calorimetry; entropy-enthalpy compensation; fly-casting mechanism

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**Running title:** effects of enzyme (dis)order on ligand binding

Abbreviations used: CM, chorismate mutase; MjCM, CM from *M. jannaschii*; mMjCM, engineered CM monomer; EcCM, CM from *E. coli*; ΔG, Gibbs free energy change; ΔH, enthalpic change; ΔS, entropic change; ΔC_p, heat capacity change; ITC, isothermal titration calorimetry; K_d, dissociation constant; K_i, inhibition constant; H/D exchange, hydrogen/deuterium exchange.
**Introduction**

Protein function is dictated by three-dimensional structure. While conventional polypeptides generally adopt well-defined folded structures as their native states, an increasingly large number of proteins have been found to be intrinsically unstructured, folding only upon binding their biological target molecules. Disordered proteins, which can range from fully denatured conformational ensembles to molten globules and segmentally mobile species, play important roles in various biological processes, including transcriptional regulation, translation, and cellular signal transduction.

Intrinsic disorder has also been recently shown to be compatible with high enzymatic activity. We converted a homodimeric chorismate mutase (CM) from *Methanococcus jannaschii* (MjCM) into a monomer (mMjCM) by inserting a hinge-loop sequence into the middle of the long N-terminal helix that spans the dimer (Fig. 1a). The topologically redesigned protein catalyzes the conversion of chorismate to prephenate (Fig. 1b) with a $k_{cat}$ value (3.2 s$^{-1}$) that is identical to that of MjCM and a $K_m$ value (170 µM) that is only three-fold higher. Unexpectedly, however, and in contrast to its thermostable parent, mMjCM displays all the properties of a molten globule. It undergoes non-cooperative thermal denaturation, shows poor NMR signal dispersion, exhibits rapid hydrogen/deuterium (H/D) exchange, and binds hydrophobic dye molecules like 1-anilinonaphthalene 8-sulfonate. Upon binding the transition state analog 1 (Fig. 1c), this highly dynamic ensemble gains structure, although it retains unprecedented flexibility on the millisecond timescale across its entire length. These observations challenge the long-held view that structural preorganization is required for efficient catalysis.

Here we exploit isothermal titration calorimetry to investigate binding of the transition state analog to the molten globular mMjCM enzyme and to its conventionally folded dimeric parent, MjCM. Our findings provide insight into the distinct energetic origins of molecular recognition in the two scaffolds. The thermodynamic data are further complemented by comparative kinetic measurements with the transition state analog and with prephenate, the product of the CM reaction. These results show that conformational flexibility can speed ligand recognition substantially and highlight a potential advantage of intrinsic disorder for enzyme catalysis.
Results
Isothermal titration calorimetry

Calorimetric measurements are useful for gaining insight into the relative entropic and enthalpic contributions in protein folding transitions and protein-ligand binding. The conversion of molten globules to native states represents a final step in the folding of many proteins, and the corresponding enthalpic and entropic changes reflect the extent to which the intermediates are disordered.

Calorimetric titrations of wild-type MjCM and the molten globular mMjCM with the oxabicyclic transition state analog 1 (Fig. 2) were performed over a range of temperatures (10-45°C and 10-30°C, respectively). Previous calorimetric studies on the evolutionarily related E. coli enzyme (EcCM), which has essentially the same active site architecture as MjCM and mMjCM, have shown that solvent reorganization and hydrophobic interactions are the major driving forces for inhibitor binding. As in the case of EcCM, formation of a complex with 1 is enthalpically and entropically favorable for MjCM and mMjCM between 10°C and 30°C at 1 M standard states (Table 1). Although the two proteins have similar free energies of binding, the relative entropic and enthalpic contributions differ significantly. Ligand binding to the monomeric molten globule is entropically less favorable (-TΔΔS = 5.1 ± 0.5 kcal/mol at 20°C) but enthalpically more favorable (ΔΔH = -3.5 ± 0.4 kcal/mol at 20°C) than binding to the preorganized wild-type dimer (Fig. 3). While analogous measurements with prephenate would have been interesting, both MjCM and mMjCM bind this molecule >100-times less tightly than 1, making ITC experiments impractical.

Changes in heat capacity (ΔC_p) associated with the ligand binding process are believed to be proportional to the burial of molecular surface, to motion restriction, and to changes in vibrational content on complex formation, providing a thermodynamic signature for protein ordering. The ΔC_p values (dΔH/dT) for transition state analog binding to MjCM and mMjCM are -0.059 ± 0.005 kcal mol⁻¹ K⁻¹ and -0.25 ± 0.03 kcal mol⁻¹ K⁻¹, respectively (Fig. 3a, slopes of the lines fitting the data in the top plot). The 4-fold larger ΔC_p for the molten globule is in line with an ordering transition. Similar values have been obtained for the conversion of other molten globules to native-like states.

Ligand binding kinetics
We recently reported pre-steady state kinetic data of ligand binding to mMjCM. Different concentrations of 1 were rapidly mixed with the molten globular enzyme and changes in protein fluorescence were recorded. The data fit well to a double exponential (Fig. 4a): the fast phase represents the bimolecular binding process (\( k_1 = 4 \pm 1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}, k_{-1} = 8 \pm 2 \text{ s}^{-1} \), Fig. 4b) and the slow phase is consistent with an ‘induced fit’ mechanism (\( k_2 = 5.4 \pm 0.2 \text{ s}^{-1} \), \( k_{-2} = 0.2 \pm 0.1 \text{ s}^{-1} \)).\(^6,19,20\) Initially, a ‘loose’ mMjCM•1 complex (\( K_d = 200 \mu\text{M} \)) is formed that subsequently rearranges into a ‘tight’ mMjCM’•1 (\( K_d = 7 \mu\text{M} \)) structure:

\[
\text{mMjCM} + 1 \xrightarrow{k_1} \text{mMjCM•1} \xrightarrow{k_2} \text{mMjCM’•1}
\]

The overall dissociation constant for the high affinity mMjCM’•1 complex is in excellent agreement with the equilibrium value determined by ITC (\( K_d = 5.0 \mu\text{M} \)) and by competitive inhibition of enzyme activity (\( K_i = 5.0 \mu\text{M} \)).

Analogous experiments have now been carried out with wild-type MjCM. The data for inhibitor 1 binding to the dimer fit well to a single exponential (Fig. 4a), giving an on rate of \( 1.6 \pm 0.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) and an off rate of \( 0.21 \pm 0.06 \text{ s}^{-1} \) (Fig. 4b). The elevated dissociation constant for the MjCM•1 complex derived from these rate constants (\( K_d = 13 \mu\text{M} \)), compared to the values obtained from calorimetry (\( K_d = 0.3 \mu\text{M} \)) and enzyme inhibition measurements (\( K_i = 0.2 \mu\text{M} \)), suggests that binding of 1 to the wild-type enzyme may also proceed via an induced fit mechanism. However, in contrast to the molten globule, tightening of the rather rigid MjCM scaffold leads to much smaller overall fluorescence changes, consistent with the lack of tryptophan residues and more subtle ligand-induced structural changes. As a consequence, the second phase leading to the tighter complex is not detected spectroscopically in this case.

The binding kinetics for prephenate, the product of the enzymatic reaction, were also examined. All observed curves fit well to single exponential functions. MjCM was found to bind and release prephenate (\( k_{on} = 3.3 \pm 0.2 \times 10^5 \text{ M}^{-1} \text{s}^{-1}, k_{off} = 30 \pm 16 \text{ s}^{-1} \), at 20°C, Fig. 4c) significantly less rapidly than mMjCM.\(^8\) In fact, product binding to the molten globule is too

\(^8\) The rate constants for prephenate binding to MjCM are very similar to those determined previously for EcCM at 20°C (\( k_{on} = 8 \times 10^5 \text{ M}^{-1} \text{s}^{-1}, k_{off} = 32 \pm 13 \text{ s}^{-1} \), Gray et al.,

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fast to be detected by stopped-flow in aqueous buffer at 20°C and was monitored in 20-50% glycerol at 7.5°C instead. The rate constants were obtained by extrapolating the data to 0% glycerol ($k_{on} = 1.4 \pm 0.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 250 \pm 80 \text{ s}^{-1}$). For both proteins, the calculated dissociation constants agree well with independently determined $K_i$ values (Table 2).
Discussion

Desolvation and hydrophobic interactions are believed to be the main driving forces for ligand binding to the mesostable CM from *E. coli* (EcCM). Our calorimetric data support a similar mechanism for the thermostable MjCM. Since the configurational entropy of the rigid transition state analog will not change significantly upon binding to the protein, the large and favorable entropic contribution to binding (at 1 M standard state) can be largely ascribed to release of ordered water molecules from the charged active site and ligand upon complexation.

Ligand binding to the molten globular mMjCM is entropically much less favorable than binding to MjCM (-TΔS = 5.0 kcal/mol, at 20°C), consistent with extensive biochemical and biophysical characterization showing that mMjCM undergoes dramatic structural ordering upon ligand binding. Nevertheless, this unfavorable entropy term is largely offset by enthalpic gains (ΔΔH = -3.5 kcal/mol). Given that mMjCM and MjCM have identical active site residues, the binding interactions with the transition state analog 1 (Fig. 1c) might have been expected to afford similar enthalpic contributions. However, binding to mMjCM is enthalpically more favorable because tightening of the molten ensemble in response to the ligand leads to improved packing interactions throughout the protein. In contrast, most stabilizing interactions pre-exist in the parent dimer, so less structural tightening occurs.

The partial enthalpy-entropy compensation observed for ligand binding to mMjCM relative to MjCM is a general property of weak intermolecular interactions governing biological systems and has been noted previously for other intrinsically disordered proteins. Upon complex formation, motion restriction at one interaction site propagates cooperatively to strengthen multiple weak interactions across the entire protein structure. Strong interaction between the binding partners thus leads to a tighter binary complex (gain in enthalpy) and greater order in the system (loss in entropy).

A disorder-to-order transition generally involves formation of a more cooperative set of interactions within the protein that replaces a less cooperative set of interactions between the protein and the solvent, resulting in negative heat capacity changes. For ligand binding processes, such ΔC_p values generally correlate with a decrease in solvent accessible hydrophobic surface area and with changes in the dynamics of both the protein and the protein-bound water molecules. The difference in ΔC_p values for mMjCM and MjCM (ΔΔC_p^mmjCM-MjCM = -0.19 kcal mol^{-1} K^{-1}) can consequently be ascribed to the different extents.
of structural ordering in these two proteins. Specifically, the significantly more negative $\Delta C_p$ value exhibited by the mMjCM monomer likely reflects its substantially more dynamic apo structure. In fact, using the heat capacity for folding a polypeptide chain composed of $n$ residues ($\Delta C_p^{U-N} = -14n$ cal mol$^{-1}$ K$^{-1}$ residue$^{-1}$), the 109 amino acid molten globule can be estimated to be 13\% less ordered in the absence of ligand than when complexed with 1

$$\left[\Delta \Delta C_p^{\text{mMjCM-MjCM}} / \Delta C_p^{U-N}\right] = (-0.19)/(-1.5) \times 100\% = 13\%.$$  

The latter calculation agrees well with changes in the solvent accessibility of backbone amide hydrogen bonds, as assessed by H/D exchange experiments.$^4$ Ligand binding shields 40 labile hydrogen atoms (17%) from water in mMjCM shortly after initiation of exchange (10 s), compared with only 7 hydrogen atoms (3%) in MjCM. Assuming that the additional protection observed for the mMjCM•1 complex arises from tightening of the overall protein structure, the molten globule is 14\% (17\% - 3\%) less ordered than the ligand-bound state. Since disorder is spread throughout the unliganded enzyme, as judged by NMR spectroscopy,$^4,6$ these results provide a rough estimate of the degree of global — as opposed to local — disorder in the molten globule. They indicate that the extent of preorganization required for efficient catalysis is not as stringent as commonly supposed, and provide some idea as to how far this requirement may be relaxed.

Striking differences between the molten monomer and the thermostable dimer are evident not only in the thermodynamics but also in the kinetics of ligand binding. The high flexibility of mMjCM speeds up recognition of the transition state analog 1 about 3-fold. The prephenate association/dissociation rates are also significantly faster for mMjCM than for MjCM, even though they were measured at substantially lower temperature (Table 2). The greater active site accessibility of the molten globule probably facilitates formation of weak long-range protein-ligand interactions, which strengthen as the enzyme accommodates its target, thereby accelerating molecular recognition. This behavior is reminiscent of the ‘fly-casting’ mechanism that has been invoked to explain ligand binding to natively unstructured proteins.$^{24}$ The large capture radius of an unfolded polypeptide chain enhances the process of molecular recognition by enabling weak binding at large distances, followed by an increase in order as the protein wraps around its target. Our results suggest that the associated kinetic advantage is not restricted to completely unstructured proteins, but may constitute an inherent functional advantage of many intrinsically disordered proteins — including molten globules — over their well-folded counterparts.
Although the reduced dynamic behavior of mMjCM upon transition state analog binding is nominally in line with the proposal of Williams et al. that improved bonding within enzyme transition state structures can promote catalysis, both CMs achieve identical \(10^6\)-fold rate accelerations (i.e. \(k_{cat}/k_{uncat}\)), while exhibiting strikingly different extents of ligand-induced ordering. In light of the induced fit binding mechanism observed for the transition state analog (but not product) and the extensive millisecond dynamics seen for the mMjCM\(\cdot\)I complex (but not MjCM\(\cdot\)I), the structural rearrangements that ensue upon substrate binding are unlikely to be complete prior to the catalytic step. Indeed, structural tightening appears to be integral to both molecular recognition and catalysis by the molten globular mMjCM, but less important for the function of the well-folded MjCM. Reduced enzyme dynamics may certainly be one source of ligand binding energy, but not always the major contributor. Its importance will depend on details of binding pocket architecture and the extent of structural tightening/reorganization.

This study shows that the similar catalytic efficiencies exhibited by a conventionally folded natural enzyme (MjCM) and an engineered molten globular catalyst (mMjCM) have very different kinetic and thermodynamic origins. Although the need for thermal and proteolytic stability may have favored rigid over molten enzyme scaffolds in the course of natural evolution, structural plasticity is not necessarily deleterious for biological function. As a consequence of extensive entropy-enthalpy compensation, transition state recognition by an intrinsically disordered catalyst can be efficiently coupled to the folding of the protein; the kinetics of substrate binding can even be significantly enhanced compared to the rates observed for a more preorganized binding pocket. Deciphering the ‘codes’ of protein-ligand recognition in such systems can be expected to aid the design of enzymes with tailored properties and, more generally, improve our understanding of the role intrinsically disordered proteins play in biology.

**Materials and Methods**

**Isothermal titration calorimetry**

mMjCM and MjCM were produced and purified as previously described. The proteins were extensively dialyzed in PBS buffer (10 mM sodium phosphate, 160 mM NaCl, pH = 6.5) prior to ITC experiments. Their concentrations were determined by amino acid analysis, averaging data from three independent measurements. The concentration of a ligand stock
solution, prepared by dissolving of a racemic mixture of 1 in buffer, was determined by $^1$H-NMR spectroscopy using 1,4-dioxane as an internal standard. As previously observed for EcCM, both enantiomers of the ligand appear to bind to mMjCM and to MjCM with similar affinities. In each experiment, the ligand stock was appropriately diluted with dialysis buffer to final concentrations of 0.6-2.0 mM. Titrations were carried out with a Microcal VP-ITC microcalorimeter (Northampton, MA) at different temperatures (10-30°C for mMjCM and 10-45°C for MjCM). The stirred cell contained 1.4 ml of enzyme solution (50-120 µM). Following an initial injection of 2 µl (not used in data analysis), 27 injections of 10 µl each were performed at 300 s intervals. The heat released at each titration step was calculated by integrating the differential power measured upon each addition of ligand (units of µcal s$^{-1}$) with respect to time within a 120 s window. The small heats observed under saturating conditions (last injections) arise from dilution and mixing effects and were subtracted from the raw data. The data were fitted to a single site binding model using the program Origin (version 5.0, Micro Cal, Inc.).

Ligand binding kinetics

Pre-steady state kinetics were monitored with an Applied Photophysics SX.18MV stopped-flow instrument at 20°C. Samples were excited at 275 nm and fluorescence emission was monitored through a 305 nm cut-off filter. Each trace was fitted to a single exponential decay function ($F_t = F_1 \exp(-k_{obs}t) + F_2$), where $F_t$ is the fluorescence at time $t$, $k_{obs}$ is the observed rate constant. The final MjCM concentration was 2 µM. Each measurement was repeated at least 5 times and the data were averaged.

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References


Figure legends

**Fig. 1.** (m)MjCM•1 model structures, enzymatic reaction, and active site-ligand interactions. (a) The dimer structure (left) is based on the x-ray structure of the homologous EcCM bound to a transition state analog.\(^{21}\) The two identical subunits are depicted in pink and cyan for clarity. The engineered loop in the monomer (right) is shown in red. The oxabicyclic dicarboxylic acid 1 is bound at each active site. (b) (m)MjCM efficiently catalyze the conversion of chorismate to prephenate. The transition state analog 1 mimics the geometry of the transition state. (c) Network of electrostatic and hydrogen bonding interactions between active site residues and 1. The amino acid numbering is based on the EcCM sequence.\(^{21}\) The hydrophobic active site residues, which form van der Waals contacts with the apolar portions of the ligand, are not shown.

**Fig. 2.** Isothermal calorimetric titrations of (m)MjCM with 1 at 20°C. The raw data are shown on the top. A single spike corresponds to the heat that is released upon addition of 10 µl ligand stock solution (1.0 mM and 1.2 mM for MjCM and mMjCM, respectively) to 120 µM protein (1.4 ml) in PBS, pH 6.5. The heats released upon consecutive ligand additions were calculated by integration of the corresponding injection peaks (top), and are plotted against the molar ratio of transition state analog to protein active site (bottom). The data fit well to a single-site binding model, from which the molar enthalpy (\(\Delta H\)), the dissociation constant (\(K_d\)), and stoichiometry (\(n\)) of the binding reaction were calculated by non-linear regression analysis.\(^{27,28}\)

**Fig. 3.** Entropy-enthalpy balance. (a) Thermodynamic parameters as a function of temperature for MjCM (filled circles) and mMjCM (open circles). The enthalpic gain offsets the entropic cost in both cases, resulting in a temperature independence of the Gibbs free energy \(\Delta G\). (b) Differences in thermodynamic parameters between ligand binding to mMjCM and MjCM at 20°C (\(\Delta_{\text{mMjCM}} - \Delta_{\text{MjCM}}\)), calculated from the results in Table 1.

**Fig. 4.** Pre-steady state ligand binding kinetics. (a) Fluorescence intensity changes upon addition of 1 (160 µM) to mMjCM (2 µM, top) and MjCM (2 µM, bottom) fitted to a double and single exponential function, respectively. More pronounced structural changes around tyrosine and tryptophan residues in mMjCM account for the larger fluorescence increase
upon ligand binding. (b) Observed rate constants for transition state analog binding to MjCM (filled circles) plotted against ligand concentration. The recently reported data for mMjCM (open circles) are shown for comparison.\textsuperscript{6} (c) Observed rate constants for prephenate binding to MjCM (filled circles) as a function of ligand concentration. The published kinetic data for mMjCM (open circles), extrapolated to 0% glycerol, at 7.5°C,\textsuperscript{6} are shown for comparison.

The rate constants for transition state analog binding (b) and prephenate binding (c) increase linearly with ligand concentration: $k_{obs} = k_{on}[1] + k_{off}$. Both the slope ($k_{on}$) and intercept ($k_{off}$) are smaller for MjCM (filled circles) than for mMjCM (open circles). The differences are, however, much more dramatic in the case of prephenate (c).
Figure 1
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Figure 2
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Figure 4

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