Dynamic allostery can drive cold adaptation in enzymes

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Adaptation of organisms to environmental niches is a hallmark of evolution. One prevalent example is that of thermal adaptation, in which two descendants evolve at different temperature extremes. Underlying the physiological differences between such organisms are changes in enzymes that catalyse essential reactions, with orthologues from each organism undergoing adaptive mutations that preserve similar catalytic rates at their respective physiological temperatures. The sequence changes responsible for these adaptive differences, however, are often at surface-exposed sites distant from the substrate-binding site, leaving the active site of the enzyme structurally unperturbed. How such changes are allosterically propagated to the active site, to modulate activity, is not known. Here we show that entropy-tuning changes can be engineered into distal sites of *Escherichia coli* adenylate kinase, allowing us to quantitatively assess the role of dynamics in determining affinity, turnover and the role in driving adaptation. The results not only reveal a dynamics-based allosteric tuning mechanism, but also uncover a spatial separation of the control of key enzymatic parameters. Fluctuations in one mobile domain (the LID) control substrate affinity, whereas dynamic attenuation in the other domain (the AMP-binding domain) affects rate-limiting conformational changes that govern enzyme turnover. Dynamics-based regulation may thus represent an elegant, widespread and previously unrealized evolutionary adaptation mechanism that fine-tunes biological function without altering the ground state structure. Furthermore, because rigid-body conformational changes in both domains were thought to be rate limiting for turnover, these adaptation studies reveal a new model for understanding the relationship between dynamics and turnover in adenylate kinase.

Adenylate kinase catalyses the reversible Mg$^{2+}$-dependent phosphoroly transfer reaction ATP + AMP ↔ 2ADP, which is essential for *E. coli* survival. Adenylate kinase contains three domains, canonically named CORE (residues 1–29, 74–121 and 160–214, Fig. 1a), AMP-binding domain (AMPbd; residues 30–73), and LID (122–159). The CORE domain, which positions the catalytic residues that are crucial for phosphoryl transfer, is the most stable domain and forms a majority of the binding site at which the transfer occurs. Consistent with numerous structures in the Protein Data Bank (PDB), in which isolated domains exhibit conformational changes sufficient to limit access to and from the active site in the bound complex (Extended Data Figs. 1, 2 and Supplementary Tables 6,7), flexible LID and AMPbd domains in adenylate kinase undergo fluctuations that become spatially restricted during binding, remaining dampened until product release. Although these large conformational changes are often modelled as rigid-body motions, recent studies reveal that the LID undergoes uncorrelated sub-global motions for the entire group of residues that comprise the LID domain, a process akin to ‘local unfolding’.

To isolate the functional effects of local unfolding, allosteric mutation sites were selected to increase the probability of local unfolding, while preserving the ground state structure (Extended Data Fig. 3). Using strict criteria (see Methods), solvent-exposed sites distal to the active site were targeted for mutation to Gly. Positions Ala37 and Ala55 were identified in AMPbd, and Val135 and Val142 were identified in LID (Fig. 1a). If the conformational fluctuations involving those regions were indeed local unfolding, Gly mutations would increase the number of backbone conformations in those states. The maximum expected increase is approximately 3.4–4.5-fold for positions that are Ala and Val, respectively, stabilizing the locally unfolded states by approximately 730 and 910 cal mol$^{-1}$ and increasing their probability (Fig. 1b). Alternatively, if locally unfolded states are not already marginally probable in the wild type, stabilizing such states by around 1.0 kcal mol$^{-1}$ should have no effect. Thus, this strategy is a direct probe of functionally important locally unfolded states in both the LID and the AMPbd domains.

The conformational stability and dynamics of each Gly mutant was investigated using differential scanning calorimetry (DSC) (Fig. 2a, Extended Data Fig. 4). LID and AMPbd mutations affected adenylate kinase differently, with a substantial diminution in the area of the main unfolding transition and the appearance of a low temperature peak in the LID mutations (Fig. 2a). Fitting of the data (Extended Data Fig. 5 and Extended Data Table 1) indicates that the origin of the effect is an intermediate (that is, I-state), which is 5% populated at physiological temperature for wild type (37°C) and is increased to roughly 40% in the LID mutants (Fig. 2b), consistent with previous circular dichroism and isothermal titration calorimetry (ITC) results. By contrast, mutations in the AMPbd show no effect on the I-state at 37°C (Fig. 2c).

Importantly, because the LID comprises part of the binding site, the LID-unfolded I-state should display decreased affinity relative to the fully folded N-state. If so, mutations that destabilize the LID and increase the probability of the I-state should decrease the affinity of adenylate kinase for ligand. Consistent with this prediction, ITC experiments that monitored the binding of the wild-type and the mutant adenylate kinases to the bi-substrate analogue Ap5A (Extended Data Fig. 6) revealed that although the affinity of the LID mutants is decreased relative to wild type, as shown previously, the AMPbd mutations have no effect on affinity. Furthermore, inspection of binding enthalpies (that is, ∆Hₜₐₜ) from ITC (Fig. 2d) corroborates this model and reconciles the thermodynamics of binding with the thermodynamics of the conformational changes observed from DSC analysis (Fig. 2a–c). Thus, the ITC and DSC results provide a self-consistent picture of the adenylate kinase ensemble. Namely, at physiological temperature, the LID domain is unfolded 5% of the time, effectively modulating substrate affinity, and the AMPbd has no role in modulating affinity.

The relationship between the thermodynamics and conformational dynamics was further investigated using nuclear magnetic resonance (NMR). Relaxation studies of the ligand-bound (that is, holo) form of adenylate kinase implicate LID conformational fluctuations to be rate limiting for enzyme turnover. To determine the nature of conformational fluctuations that affect affinity, backbone dynamics were measured for the ligand-free (that is, apo) forms using 15N Carr–Purcell–Meiboom–Gill (CPMG) NMR relaxation dispersion experiments, which provide direct access to processes occurring on the milli- to microsecond timescale. Previously, we showed that at 19°C the single LID mutation Val142Gly has a similar population of the LID unfolded
I-state to the wild-type protein at 37 °C. The lower temperature thus permits direct comparison of LID fluctuations between the wild type and all mutants.

As expected, the chemical shifts for LID residues showed conformational exchange in LID mutants, whereas the fluctuations of the remaining positions in the protein (with the exception of CORE residues 6 and 10, proximal to the LID) showed no exchange, resembling wild type (Fig. 3a). Importantly, the population of the LID-unfolded state determined from the fit of the CPMG data (either individually or globally), I-state population (P_I) = 3–9% agrees quantitatively with the population of the LID-unfolded state determined calorimetrically (P_I ≈ 5%). The fact that three independent techniques (that is, ITC, DSC and NMR), each sensitive to different properties of the protein, yielded similar results supports a model in which unfolding of the LID (instead of a rigid-body opening of an always-folded LID) is the minor conformational state of the apo protein that regulates substrate affinity. By contrast, analysis of the 15N NMR relaxation dispersion data for the AMPbd mutants showed no such effect (Fig. 3a), consistent with the fact that these mutants do not affect the substrate binding of adenylate kinase.

To determine the relationship between conformational dynamics and activity, turnover was measured as a function of temperature for each mutant (Fig. 3b, Methods and Supplementary Table 3). As expected, the activity of each mutant increased until reaching a rollover temperature, after which the activity decreased owing to denaturation (Fig. 3b). Paradoxically, although the single LID mutants each destabilized the LID (Fig. 2a, b) and increased its dynamics (Fig. 3a), such changes had no effect on the activity (k_cat) of adenylate kinase (Fig. 3b). By contrast, single AMPbd mutants, which did not affect the stability (Fig. 2a, c) or the dynamics (Fig. 3a), significantly increased k_cat at every temperature (Fig. 3b). In other words, the dynamics and stability of the LID domain, and therefore the affinity, are uncoupled from the activity of the enzyme.

Further analysis of the activity using a previously described method provides mechanistic insight into the role of unfolding in adenylate kinase activity (Fig. 4). The activation enthalpy (ΔH^‡), which is proportional to the slope of rate constant versus inverse temperature, is identical between the wild type and all four single mutants (ΔH^‡ = 10.5 kcal mol⁻¹ K⁻¹). Indeed, the major difference between mutants is the average activation entropy (ΔS^‡; that is, the intercepts of the lines in Fig. 4), which for the AMPbd mutants is lower than the wild type and LID mutants, increasing the catalytic rate at every
temperature. The similarity in activation enthalpy suggests that the degree of structural change associated with the transition state is identical for the wild type and all four mutations, but that the free energy difference is less for the two AMPbd mutations, with the difference being almost entirely entropic. Remarkably, the free energy difference between the AMPbd mutations and the wild type and LID mutants is ∆ΔS = 2.5 cal mol⁻¹ K⁻¹ (Fig. 4, inset), corresponding to a change in activation energy (∆ΔG‡) at 25°C of approximately 770 cal mol⁻¹, which is the expected result if the rate-limiting transition state for product release is in fact a locally unfolded state of the AMPbd (see Fig. 1b).

The quantitative agreement with theoretical expectations for two different surface-exposed positions in the AMPbd supports an interpretation that conformational change in the AMPbd, but not the LID, is the rate-limiting step in product release for adenylate kinase. Importantly, the nature of the AMPbd conformational change is also local unfolding, but because the unfolding in AMPbd is extremely rare, the millisecond timescale dynamics are unaffected. There is no evidence that the chemistry of the catalytic event is affected by local unfolding⁶ (nor is it likely to be based on the nature of the surface mutations), but merely the rate-limiting product release step. Interestingly, these results also provide insight into a previous thermo-adaptation study⁷, which reports that the increased curvature in Eyring plots is a signature of increased transition-state heat capacity in ancestral warm-adapted adenylate kinase variants. The origin of this effect could involve local unfolding, although a definitive claim awaits further study.

For more than a century, biochemists have used the Arrhenius equation, k = Aexp(−Ea/RT), to characterize temperature effects on enzyme reaction rates. This expression reveals that the reaction rate k increases exponentially with temperature (T, in K), with the increase determined by the activation energy Ea (in which R is the gas constant and A is a reaction-specific pre-exponential term). For typical activation energies, the rate increases approximately twofold for every 10°C environmental temperature rise. If such a model sufficiently describes activity, an enzyme from a psychrophilic organism (which thrives at approximately 0°C) should have activity more than 20 times lower than an orthologous enzyme from a mesophilic organism (which thrives at around 40°C). This, however, is not the case. In several well-documented examples, it has been shown that although the activity of each orthologous enzyme obeys an Arrhenius relationship, increasing with temperature, the enzymes evolved such that the respective kcat and Km values are similar at the physiological temperature of each organism⁴⁻⁵. Clues to the origin of this effect can be gleaned from sequence analysis. Indeed, in studies of lactate dehydrogenase from a series of notothenioid fish adapted to different environmental temperatures⁶, no changes to active site residues were found, but instead Gly was shown to be prevalent at surface sites in cold-adapted species, a result in agreement with the observations reported here for adenylate kinase.

In fact, the mechanism of this type of adaptive tuning can now be directly dissected. Our results indicate that unfolding in both the LID and AMPbd domains contribute to function, although the specific roles are segregated in adenylate kinase (Fig. 5). LID unfolding modulates the affinity of the enzyme for substrate (Fig. 5b) and is thus tuned to a level of approximately 5% for the wild type. As local unfolding is favoured entropically⁶⁻⁷, mutation from Ala to Gly (Fig. 1b) increases the stability of the locally unfolded state of AMPbd, lowering the activation barrier for product release, and increasing the apparent rate (Fig. 5c, step 1). Because unfolding is high enthalpy, and disfavoured at lower temperatures⁶⁻⁷, a decrease in temperature reduces the LID-unfolded equilibrium to the original level (Fig. 5b, step 2). Conversely, unfolding in AMPbd modulates the rate-limiting process for turnover, using the same principles at play in the LID. Mutations from Ala to Gly (Fig. 1b) increase the stability of the locally unfolded state of AMPbd, lowering the activation barrier for product release, and increasing the apparent rate (Fig. 5c, step 1). As local unfolding in AMPbd is the rate-limiting step, mutation to Gly lowers the activation barrier for product release. Lowering temperature again dampens fluctuations, resetting the transition state equilibrium, and lowering the activity to the original level (Fig. 5c, step 2).

Taken together, the results reveal that affinity and turnover in adenylate kinase can be independently regulated through these similar, but spatially distinct, dynamic allosteric mechanisms. Illustrating this point, relative affinity and activity (Fig. 5b and c, respectively) are displayed for the wild-type enzyme and each of the mutants. Figure 5d shows that the independent regulation is physiologically significant, as a single point mutation can substantially change one function (>30%) while leaving the second function comparatively unaltered (<10%), resulting in as much as a 10°C downward shift in adaptive temperature. To our knowledge, such spatially segregated and dynamic (that is, entropic) regulation of several functions within the same enzyme has yet to be documented.

The results described here for adenylate kinase also suggest a general strategy for adaptation. By using unfolded states, adaptation can...
be facilitated by introducing as few as one conformational entropy-enhancing mutation anywhere on the surface of the protein structure that becomes unfolded. Such changes would energetically oppose the increased stability of the unfolded state of any domain that results at lower temperatures. Considering that the enrichment of flexibility-promoting residues in psychrophilic enzymes has long been identified from sequence analysis, it is possible that the local unfolding mechanism identified here is driving cold adaptation in numerous systems, even when structural data exists supporting alternative hypotheses, as shown here for adenylate kinase (Extended Data Figs. 1, 2). Indeed, the Protein Data Bank contains numerous ligand-bound (holo) structures in which the active site occludes the ligand, and the structural transitions associated with the substrate gaining access to and from the active site are, as yet, unknown (Supplementary Tables 6, 7, Extended Data Fig. 7), leaving open the possibility of local unfolding.

Finally, using local unfolding as a mechanism for regulating key enzymatic parameters such as $K_m$ and $k_{cat}$ confers an additional potential advantage to organisms. Because the chaperone machinery has evolved to recognize unfolded states of proteins, utilization of local unfolding as part of the functional energy landscape provides an opportunity to tie the overall regulation of enzymes to the proteostatic state of the cell, and thus, indirectly, to any process that is co-regulated with chaperone activity. The validity of this hypothesis is currently under investigation.

Online content

Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586-018-0183-2.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

Identification of entropy-enhancing mutation sites. Mutation sites were objectively identified based on a strict set of geometric criteria, to isolate positions distal to the active site that also had no side-chain enthalpic interaction in the folded structure. These criteria were as follows: 1) the side chain of a mutated position should be highly surface exposed and devoid of intramolecular contacts; 2) the mutated side chain should not have polar or charged groups that could be involved in long-range interactions; 3) the mutated side chain should be distant (that is, >8 Å) from the active site; and 4) the side chain should not make contact with the ligand. All these criteria were satisfied for all mutated positions in both the open (PDB code 4AKE) and closed (PDB code 1AKE) states of wild-type adenylate kinase. Exactly four positions in the LID domain (Val127, Val135, Val142 and Val148), and two positions in the AMPbd domain (Ala37 and Ala53) were identified. Thus, the reported results pertain to the majority of all possible entropy enhancing mutations for this enzyme.

Protein mutagenesis and purification. The wild-type adenylate kinase amino acid sequence contained no affinity tag and was identical to that described previously. The gene corresponding to this sequence was codon-optimized, synthesized, and inserted into an IPTG-inducible pJ414 bacterial expression vector conferring ampicillin resistance (DNA 2.0). This DNA template was used to make the single Gly variants using PCR site-directed mutagenesis. The adenylate kinase-containing plasmid was used to transform Rosetta2(DE3)pLysS Singles, with the transformed cells plated on LB plates and grown overnight. The next day, a single colony was picked from the Amp plate and a new culture was started in the presence of 60 µg/ml ampicillin. Protein was purified from sonicated E. coli lysate under native conditions using fast protein liquid chromatography (FPLC). Adenylate kinase for all variants was at least 95% pure as assayed by 12% SDS–PAGE. Full details of expression, purification, and handling are described in the Supplementary Information.

DSC and ITC. For DSC, 20 µM protein was measured in calorimetric buffer (60 mM PIPES, 1 mM EDTA, pH 7.2). A MicroCal VP-DSC (Malvern) was set at: 10 °C start, 80 °C final, 60 °C per h scan rate, 15 min pre-scan thermostat, 0 min post-scan, 20 s filtering period, no feedback mode/gain. For ITC, 40 µM protein was measured in calorimetric buffer. Initial Ap5A inhibitor (Sigma–Aldrich) was 400 µM in calorimetric buffer. A MicroCal VP-ITC was set at: 55 total injections, 1 each hexokinase and G6PDH diluted in 50% (w/v) glycerol, pH 7.0. Protein was concentrated to 1 mM in NMR sample buffer (50 mM HEPES, 20 mM MgCl2, 5 mM TCEP, pH 7.0). Most assignments for all variants were conservatively transferred from previously reported spectra. Relaxation–dispersion experiments used a pulse sequence with 50 ms constant time at 800 MHz field, with data collected at relaxation delays of 9.55, 0.25, and 0.0 (t) ms. TROSY-HSQC spectra were collected in an interleaved manner with 16 transients, 90 ms T1, and 2.5 s delay between transients for each relaxation delay at 19 °C. R2 values were calculated from differences of peak intensities. Full details of NMR data acquisition, processing, analysis, and interpretation are given in the Supplementary Information.

Ensemble model and estimation of conformational population. The ensemble allosteric model used experimentally determined stabilities and domain interaction energies (Extended Data Table 1) to compute Boltzmann-weighted temperature-dependent adenylate kinase ensemble populations (Fig. 2b, c). Three thermodynamic states were permitted: fully folded (P1), binding incompetent locally unfolded LID (P2), and fully unfolded (P3). Full details are provided in the Supplementary Information.

Statistical information. In all figures, error bars indicate the standard deviation around the mean value for at least three independent data points. There is one exception, Fig. 5d, in which the bars are derived from propagation of errors between differences of mutant and wild-type activity and affinity measurements (which are explicitly reported in the Supplementary Information).

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

Data availability. Most data generated or analysed during the current study are included in the published manuscript (and its Supplementary Information). All other data are available from the corresponding author upon reasonable request.

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Extended Data Fig. 1 | Kinases of known structure that may exhibit similar open/close architecture as *E. coli* adenylate kinase. a–d, In each panel, the left cartoon represents a putative 'lid open' apo-structure, and the right panel represents a putative 'lid closed' holo-structure. Protein chains are rainbow-coloured from blue (N terminus) to red (C terminus). Note that in b and c there is crystallographic evidence of disorder (magenta) in the conformationally changing 'lid' domain, a situation very similar to that seen for the low-population locally unfolded state in *E. coli* adenylate kinase, given in a for comparison. a, *E. coli* adenylate kinase; PDB accessions 4AKE (left) and 1AKE (right). b, *Mycobacterium tuberculosis* adenylyl sulfate kinase; PDB accessions 4RFV (left) and 4BZX (right). c, *Helicobacter pylori* shikimate kinase; PDB accessions 1ZUH (left) and 1ZUI (right). d, *Sulfolobus tokodaii* hexokinase; PDB accessions 2E2N (left) and 2E2O (right).
Extended Data Fig. 2 | Other enzymes of known structure that may exhibit similar open/close architecture as E. coli adenylate kinase. a–d, In each panel, the left cartoon represents a putative 'lid open' apo-structure, and the right panel represents a putative 'lid closed' holo-structure. Protein chains are rainbow-coloured from blue (N terminus) to red (C terminus). Note that in a–c there is crystallographic evidence of disorder (magenta) in the conformationally changing 'lid' domain, a situation very similar to that seen for the low-population locally unfolded state in E. coli adenylate kinase. a, E. coli 2-glycinamide ribonucleotide transformylase: PDB accessions 1CDD (left) and 1CDE (right). b, l,d-carboxypeptidase: PDB accessions 4JID (left) and 4OX5 (right). c, Thermus thermophilus ribosomal protein L11 methyltransferase PrmA: PDB accessions 2NXC (left) and 2NXE (right). d, Lactobacillus casei dihydrofolate reductase: PDB accessions 1l7o (left) and 2HQP (right).
Extended Data Fig. 3 | Comparison of wild-type adenylate kinase HSQC spectrum with mutants A55G and V142G. a, b, Overlay HSQC spectra at 19 °C for A55G (blue, a) and V142G (red, b); in both panels an identical wild-type spectrum is shown in black. Peak dispersion in all spectra is consistent with folded protein and also is not inconsistent with a similar ground state structure shared among all three proteins. Individual resonances for both mutants exhibited minimal shifts from the wild type, and thus generally permitted transference of assignments from the wild type.
Extended Data Fig. 4 | DSC control experiments. a, Test of the two-state model using wild-type adenylate kinase. Wild-type thermal denaturation is not consistent with a two-state process, as data simulated under the two-state assumption do not agree with experiment, and calorimetric to van’t Hoff enthalpy ratio is substantially greater than 1. Results represent \( n = 1 \) independent experiments. b, Reversibility test. Wild-type adenylate kinase exhibited approximately 80% of the original calorimetric area upon re-heating. Results represent \( n = 1 \) independent experiments. c, High temperature test. Wild-type adenylate kinase demonstrates complete reversibility when extreme high temperature is avoided. Results represent \( n = 1 \) independent experiments. d, Calorimetric heat capacity (\( \Delta C_p \)) of adenylate kinase LID variants. Dependence of enthalpy on melting temperature for wild type, V135G and V142G results in \( \Delta C_{p,LID} \) of \( 0.7 \pm 0.1 \) kcal mol\(^{-1}\) K\(^{-1}\) (mean \( \pm \) s.d.). This value is reasonably consistent with energetics determined from accessible surface areas. Results represent mean \( \pm \) s.d. of \( n = 3 \) independent experiments.
Extended Data Fig. 5 | Modelled domain stabilities and ensemble probabilities of adenylate kinase variants. The DSC data for wild type, LID and AMPbd mutants were each fit to three-state transitions. The fitted parameters correspond to population profiles that differ dramatically between the LID and AMPbd mutants (Fig. 2b and c, respectively). As determined previously from circular dichroism and ITC\(^{25}\), the locally unfolded intermediate (LU), which is 5% populated at physiological temperature for wild-type adenylate kinase (37 °C), is increased to approximately 40% in the LID mutants (Fig. 2b). In contrast to the LID, mutations to the AMPbd do not stabilize the intermediate. Instead, the unfolded (U) state is stabilized, accounting for the decrease in the apparent \(T_{\text{m}}\) of the main peak, with no change in the temperature of onset of the intermediate (Fig. 2c).

\(\Delta G_{\text{total}} = \Delta G_{\text{CA}} + \Delta G_{\text{LID}}\) in which ‘CA’ denotes ‘CORE-AMPbd’. Mutations other than those in the LID domain have a small effect on the stability of adenylate kinase. a, Wild type. b, A37G AMPbd mutant. c, A55G AMPbd mutant. d, V135G LID mutant. e, V142G LID mutant. f–j, Ensemble probability calculations were based on values in Extended Data Table 1. LID mutations V135G and V142G clearly modulate the ensemble by reducing the population of fully folded state and increasing population of unfolded LID domain. f, Wild type. g, A37G AMPbd mutant. h, A55G AMPbd mutant. i, V135G LID mutant. j, V142G LID mutant.
Extended Data Fig. 6 | Representative ITC data. All measurements were obtained at 37 °C, fitting parameters are indicated in each panel. Results represent $n = 1$ independent experiments. a, Wild type. b, A55G AMPbd mutant. c, V135G LID mutant.
Extended Data Fig 7 | Examples of holo-enzymes’ degree of ligand burial. a–c, In each panel, atoms are shown as van der Waals’ spheres. Dark grey indicates protein atoms and yellow indicates ligand. The left side of each panel shows the protein and ligand together, and the right side shows ligand alone. a, ‘Little’ ligand surface area is buried in the complex of deoxyhypusine synthase and nicotinamide adenine-dinucleotide inhibitor, PDB accession 1RLZ. b, ‘Partial’ ligand surface area is buried in the complex of glyceraldehyde-3-phosphate dehydrogenase, nicotinamide adenine-dinucleotide cofactor, and glyceraldehyde-3-phosphate substrate, PDB accession 1NQA. c, ‘Mostly’ ligand surface area is buried in the complex of chorismate-pyruvate lyase and p-hydroxybenzoic acid product, PDB accession 1TT8.
## Extended Data Table 1 | Summary of thermodynamic parameters obtained from DSC

| AK Variant | $T_{m,\text{LID}}$ $^a$ ($^\circ$C) | $\Delta H_{m,\text{LID}}$ (kcal/mol) | $\Delta C_p,\text{LID}$ (kcal/mol K) | $T_{m,\text{CA}}$ $^a$ ($^\circ$C) | $\Delta H_{m,\text{CA}}$ (kcal/mol) | $\Delta C_p,\text{CA}$ (kcal/mol K) | $\Delta G_{\text{total},298^b}$ (kcal/mol) |
|------------|---------------------------------|----------------------------------|----------------------------------|---------------------------------|----------------------------------|----------------------------------|----------------------------------|
| WT         | 50.7 ± 0.1 $^c$                 | 46.0 ± 0.3                       | 0.7 ± 0.1                        | 55.8 ± 0.1                      | 132.3 ± 1.5                     | 2.7 ± 0.4                        | 7.8 ± 0.2                       |
| A37G       | 50.0 ± 0.1                      | 44.5 ± 0.1                       | 0.7 ± 0.1                        | 53.5 ± 0.1                      | 125.7 ± 0.6                     | 2.7 ± 0.4                        | **6.8 ± 0.2**                   |
| A55G       | 50.2 ± 0.1                      | 44.5 ± 0.1                       | 0.7 ± 0.1                        | 54.8 ± 0.1                      | 126.0 ± 0.1                     | 2.7 ± 0.4                        | **7.1 ± 0.2**                   |
| V135G      | **38.1 ± 0.1**                   | **37.4 ± 0.6**                    | 0.7 ± 0.1                        | 56.3 ± 0.1                      | **111.7 ± 0.1**                 | 2.7 ± 0.4                        | **6.1 ± 0.3**                   |
| V142G      | 41.8 ± 0.1                      | 40.6 ± 1.5                       | 0.7 ± 0.1                        | 54.5 ± 0.1                      | **110.3 ± 0.1**                 | 2.7 ± 0.4                        | **6.2 ± 0.2**                   |

$^a$ CORE–AMPbd domains unfolding transition; $^b$ intermediate, binding-incompetent LID domain unfolding transition; $^c$ van’t Hoff. $\Delta G_{\text{total}} = \Delta G_{m,\text{LID}} + \Delta G_{m,\text{CA}}$. Data are mean ± s.d. Values in bold differ substantially from the wild type.
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Reporting Summary

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- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

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Software and code

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Data collection

No custom software was used for data collection. Commercial software used for data collection was as follows: for DSC data collection, Origin version 7.0 with VP-DSC module was used; for ITC data collection, Origin version 7.0 with VP-ITC module was used; for UV-Vis and kinetic data collection, Shimadzu UV-Probe version 2.33 was used.

Data analysis

No custom software was used for data analysis. Commercial software used for data analysis was as follows: Origin 7.0 for DSC and ITC analysis; Microsoft Excel for linear analysis and for generating plots. Public open-source software R was used for generating plots.

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Life sciences study design

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| Sample size | Since the interpretations and conclusions of the manuscript do not rely on p-values, sample size was primarily determined by the effort and resources required to complete all experiments for all mutants. Most kinetic measurements were performed three times (sample size n = 3) to obtain a standard deviation error estimate. The robust temperature trends exhibited by DSC and ITC were obtained with a sample size n = 1 for each enzyme variant. These sample sizes are sufficient because the conclusions rely on trends over the entire data set rather than on strict confidence in the precision of any one data point. |
| Data exclusions | No data were excluded. |
| Replication | Most kinetic measurements were performed three times (sample size n = 3) to obtain a standard deviation error estimate; we consider those experiments to be reproducible. All other experiments were measured one (sample size n = 1), but we have no reason to doubt their reproducibility. |
| Randomization | Samples were not allocated into groups, as the experiments were always interpreted relative to the wild-type enzyme, as a control. |
| Blinding | The investigators were not blinded during data collection and analysis. Blinding was not relevant to this study because the co-authors performing experiments were not solely responsible for interpretation and analysis. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|------|-----------------------|
| ☒ | Unique biological materials |
| ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☒ | Palaeontology |
| ☒ | Animals and other organisms |
| ☒ | Human research participants |

Methods

| n/a | Involved in the study |
|------|-----------------------|
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |