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Halophilic enzyme activation induced by salts

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Halophilic archea (halobacteriae) thrive in hypersaline environments, avoiding osmotic shock by increasing the ion concentration of their cytoplasm by up to 3–6 M. To remain folded and active, their constitutive proteins have evolved towards a biased amino acid composition. High salt concentration affects catalytic activity in an enzyme-dependent way and a unified molecular mechanism remains elusive. Here, we have investigated a DNA ligase from *Haloferax volcanii* (*Hv* LigN) to show that K⁺ triggers catalytic activity by preferentially stabilising a specific conformation in the reaction coordinate. Sodium ions, in turn, do not populate such isoform and the enzyme remains inactive in the presence of this co-solute. Our results show that the halophilic amino acid signature enhances the enzyme's thermodynamic stability, with an indirect effect on its catalytic activity. This model has been successfully applied to reengineer *Hv* LigN into an enzyme that is catalytically active in the presence of NaCl.

S alines are harsh environments where the high salt concentration precludes the survival of almost any living form but the halophilic archaea. To avoid osmotic shock, these extremophiles equilibrate their ionic strength with the environment, and K⁺ (or Na⁺) ions can increase the cytoplasm's osmolarity up to 4 Osm/L [1]. To remain folded and active at this high intracellular ionic concentration, proteins from halophilic organisms have evolved towards a biased amino acid composition: glutamate and aspartate are found at higher frequencies compared to their mesophilic counterparts [2–4]. This increase in acidic residues is accompanied by a low content of bulky hydrophobic side chains and a drastic reduction in the number of lysines. Such amino acid composition places halophilic proteins amongst the most acidic proteins naturally found [5]. Genomic analyses of taxonomically unrelated species reflect similar signatures in the amino acid composition, indicating that this is a universal evolutionary trait [2].

Biophysical characterization of a plethora of examples has demonstrated that salt (NaCl and/or KCl) always increases the stability of halophilic proteins [6,7]. More detailed studies in which proper thermodynamic descriptors were obtained also support this conclusion [8,9]. The monotonic stabilization of halophilic proteins by salt contrasts with the complex effect that NaCl or KCl has on the functionality of halophilic enzymes [10,11]: some of them decrease their catalytic activity at increasing salt concentrations, showing suboptimal activity at physiological ionic strength [12,13]. On the other hand, proteins like β-galactosidase from *Haloferax alicantei* are activated by salt [14] and, perhaps more strikingly, the two activities for the catalase peroxidase from *Halobacterium salinarium* show contrasting dependence on NaCl concentration [15]. Some reports indicate that the activity of halophilic enzymes can also be modulated by anions other than chloride [7,16], but a unified molecular mechanism to explain these differences remains elusive.

Ligase N from *Haloferax volcanii* (*Hv* LigN) is a NAD⁺-dependent enzyme that joins the end of DNA molecules during replication and repair [17]. Full length *Hv* LigN has the characteristic amino acid composition found in halophilic proteins (Figure 1A) [2,8]. Such features allow the protein to remain folded in hypersaline environments and *Hv* LigN requires KCl to be active and shows increased activity upon addition of this salt. Surprisingly, the enzyme remains inactive in the presence of NaCl [17]. The full length LigN has 699 amino acids and shares its domain structure with all the bacterial NAD⁺-dependent ligases including a DNA binding domain, an adenylation domain (divided into the sub-domains 1A and 1B) and an OB fold domain [17] (Figure 1B). During catalysis, an AMP molecule is transferred from NAD⁺ to the protein (adenylation of ligase) resulting in the closure of subdomains 1A and 1B [18]. Here, we have investigated the putative coupling between changes in stability induced by salt and its effect on enzyme activity. Our results show that, unlike Na⁺, potassium ion has the ability to stabilize the enzyme as well as to modulate *Hv* LigN ligase activity. The two mechanisms are coupled, and the differential effect of salt on two different enzyme conformations, both part of the reaction pathway, is the key element to understanding the activation of the enzyme by KCl, the lack of activation by NaCl and the effect induced by other salts.

Results

Effect of K⁺ on the enzymatic activity and the thermodynamic stability of Hv LigN. The ligase activity of *Hv* LigN is fully dependent on the KCl concentration: the enzyme cannot ligate DNA in the absence of this salt, it increases its activity upon KCl addition and it reaches a measurable maximum at 3.2 M KCl [19]. *Hv* LigN selectively joins fragments I (8454 bp) and IV (5686 bp) of *Bst*EII-digested λ DNA to produce a new oligomer that runs in an agarose gel at the molecular weight of the sum of the two fragments [19]. This reaction was used to show that *Hv* LigN activity is dependent on KCl concentration up to 3.2 M (Figures 1C and S1).

It has been shown in the literature that the NAD⁺-dependent ligase N from *Enterococcus faecalis* undergoes a conformational rearrangement upon adenylation, resulting in the closure of subdomains 1A and 1B [20]. Using homology programs [21,22] and the available high resolution structures [18], we modelled the deadenylated (*Hv* dea-LigN, open) and adenylated (*Hv* ade-LigN, closed) conformations of the adenylation domain in *Hv* LigN (Figure 1B). The modelled structures retained the fold of the mesophilic counterparts, consistent with the fact that halophilic proteins concentrate the changes in the surface [23,24]. Interestingly, the adenylated protein has less exposed area, burying 331 Å² more than *Hv* dea-LigN to the solvent. Adenylation of LigN is a required step for DNA ligation to occur, and the two conformations correspond to two instances in the enzymatic reaction coordinate.

In contrast to thermal denaturation, *Hv* LigN unfolds reversibly in response to chemical agents. We adapted a previously reported protocol[25] to obtain fully deadenylated (*Hv* dea-LigN) and fully adenylated (*Hv* ade-LigN) ligase and we explored the stability of the two enzyme isoforms in the presence of KCl. Figure 1D shows the guanidinium chloride melting curves for *Hv* dea-LigN and *Hv* ade-LigN enzyme isoforms. Inspection of the melting curves clearly shows that unfolding does not obey a two-state model. This is not surprising given that *Hv* LigN is a multidomain protein of high molecular weigth. Previous thermodynamic characterization of the NAD⁺-dependent ligase N from *Thermus scotoductus* also showed a non-two-state behavior [26]. Instead, the experimental data fit well to a three-state model (fitting the experimental data to this model was validated according to the F-test analysis) and the solid lines in Figure 1D represent the best fits to this model. We employed the overall free energy upon unfolding ($\Delta G^0_{U-N}(0) = \Delta G^0_{U-I}(0) + \Delta G^0_{I-N}(0)$) as a reporter for protein stability.

Free energies of *Hv* dea-LigN and *Hv* ade-LigN were determined at increasing concentrations of KCl (Figure 2A). In the absence of K⁺, *Hv* dea-LigN was more stable than *Hv* ade-LigN, providing an explanation for the lack of activity. This is in contrast to non-halophilic ligases, where the protein becomes stabilised upon adenylation so the reaction can proceed.[26] KCl, in turn, stabilised both isoforms, showing a linear dependence with the molar concentration of salt (Figure 2A), with slopes (m_{KCl}) shown in Table S1. Interestingly, m_{KCl} (*Hv* ade-LigN) was higher than m_{KCl}(*Hv* dea-LigN) and, at a concentration around 1M, *Hv* ade-LigN became more stable than the deadenylated form. The apparent populations of the adenylated and deadenylated species as a function of KCl concentration (obtained from the free energy values) were highly consistent with the experimental enzyme activity values (Figure 2B): at KCl concentrations below 1 M there was no enzyme activity, in agreement with *Hv* dea-LigN as the only populated molecule. At concentrations higher than this value, *Hv* ade-LigN became thermodynamically more stable and the enzyme started showing ligase activity.

Ligase enzyme activity tuning in the presence of Na⁺. NaCl had a negligible effect on *Hv* LigN catalytic activity, even at very high
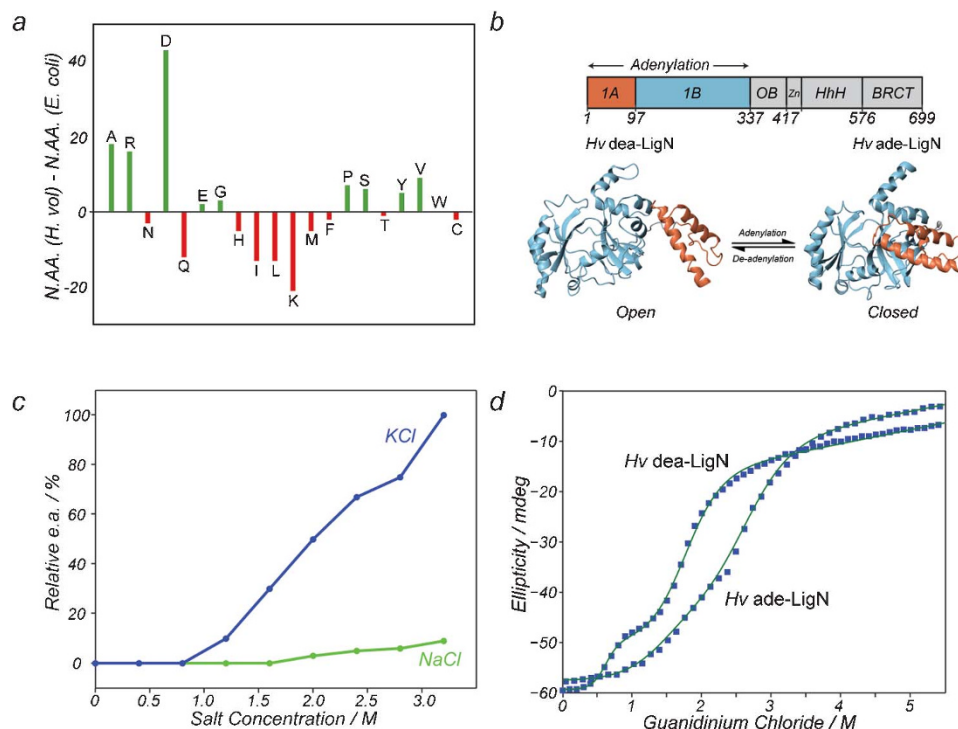

Figure 1 | (a) Amino acid composition difference between the primary sequences of *H. volcanii* and *E. coli* Lig N. Green (red) bars indicate an increase (decrease) of the given amino acid. (b) *Top.* Domain structure for the *Hv* LigN enzyme. The adenylation domain is divided into two subdomains: 1A (orange) and 1B (light blue). *Bottom.* Modelled structures of the de-adenylated (open, left) and adenylated (closed, right) conformations. The model was obtained using the homology modelling program Phyre.[21] (c) Relative enzyme activity as a function of the salt concentration and referenced to the catalytic activity in the presence of 3.2 M KCl. (d) Representative examples of the chemical denaturation profiles monitored by CD, for *Hv* dea-LigN and *Hv* ade-LigN. Solid lines correspond to the best fit using a three-state model.
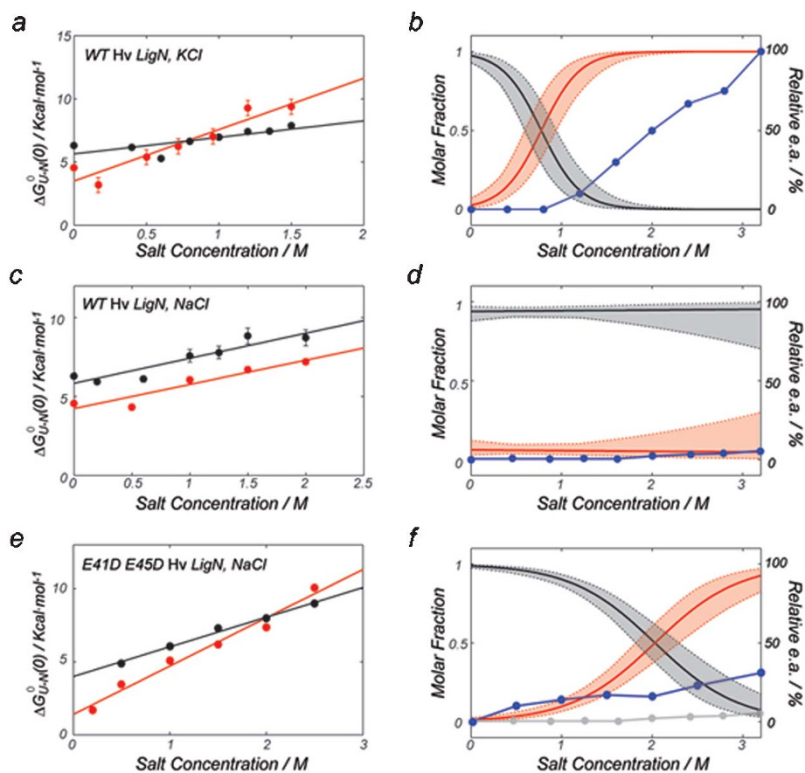
Figure 2 | **Plots of the free energy upon unfolding (ΔG_{U-F}) versus the molar concentration of salt are shown in panels (a), (c) and (e).** The salt and the protein used are indicated in the panels. *Hv* dea-LigN and *Hv* ade-LigN are represented by black and red circles, respectively. A linear dependence between stability and the molar concentration of salt was found and the solid lines correspond to the linear regressions. The apparent populations of *Hv* dea-LigN and *Hv* ade-LigN are plotted in panels (b), (d) and (f) using the same colour code (left ordinate axes). The shaded areas in red (black) correspond to the estimated uncertainties for the *Hv* ade-LigN (*Hv* dea-LigN) populations. The blue circles in the same panels represent the enzyme activity (normalised with respect to 3.2 M KCl, right ordinate axes). In panel (f), WT *Hv* LigN activity as a function of NaCl concentration is also shown in gray.

concentrations of this salt. We reproduced the thermodynamic characterization of the adenylated and deadenylated forms of *Hv* LigN at increasing concentrations of NaCl (up to 2.0 M) and the results are shown in Figures 2C and 2D. Interestingly, *Hv* dea-LigN was more stable than *Hv* ade-LigN at all tested concentrations of NaCl (Figure 2C), and the equilibrium was never displaced towards the adenylated form. In other words, the red and black lines in Figures 2C–D do not cross (m_{NaCl}(*Hv* ade-LigN) $\approx$ m_{NaCl} (*Hv* dea-LigN)). To our knowledge, this is the first report for differential stabilisation of a protein induced by KCl when compared to NaCl, even though computer simulations had predicted a minor stabilising effect for the Na$^+$ due to the higher affinity of this cation for the carbonyl groups of the protein.[27–29]

We have recently shown that a change in the hydrophobic content of the protein exposed to the solvent can modulate the salt effect on its stability.[8] To test whether this strategy is useful in restoring enzyme activity, we created two very conservative mutations (E41D and E45D) in the 1A domain of *Hv* LigN that slightly alter the hydrophobic character of the protein by removing two methylene groups without affecting the overall charge. The circular dichroism spectrum for the mutant was identical to wild type *Hv* LigN (Figure S2), indicating that no structural perturbation occurred upon mutation. Enzyme activity for the mutant was tested as a function of NaCl and KCl concentration. In the presence of NaCl, there was a clear recovery of catalytic activity upon mutation (Figures 3 and S1). A similar increase in the activity of the mutated enzyme was also observed when KCl was added instead, and the enzyme starts being catalytically active at a much lower concentration of this salt. Free energies of the *Hv* ade-LigN and *Hv* dea-LigN isoforms for the engineered protein showed that the two species were stabilised by NaCl with different slopes (Figure 2E–F),

progressively favouring the adenylated form, the key requisite to trigger the reaction. Thus, very subtle modifications in the protein's surface can modulate salt-induced enzyme activity, a property that could be exploited for the biotechnological use of these enzymes in algaculture.
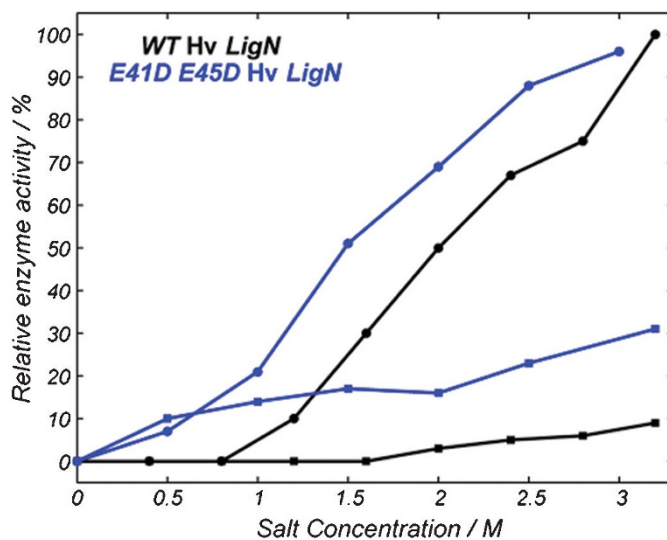

Figure 3 | **Dependence of the enzyme activity with the molar salt concentration (referenced to the wild type catalytic activity in the presence of 3.2 M KCl).** Squares (circles) represent the enzyme activity of Hv LigN in the presence of NaCl (KCl). Wild type and mutant proteins are shown in black and blue, respectively, as indicated in the figure.
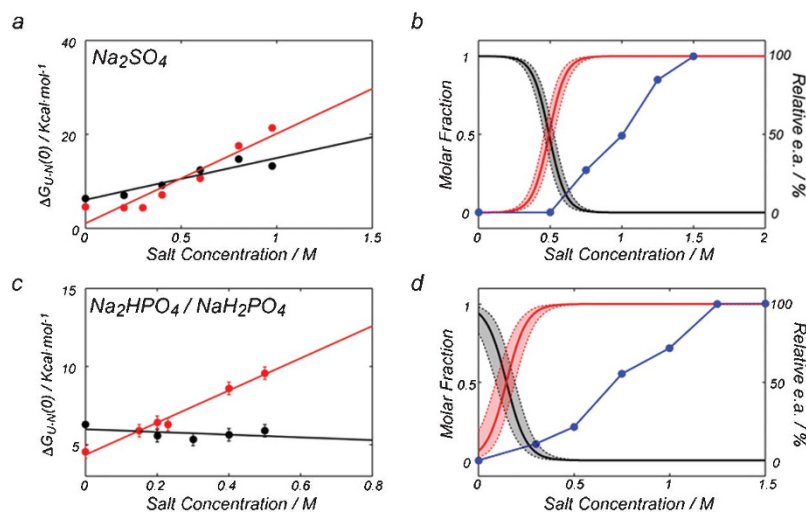
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Figure 4 | Plots of the free energy upon unfolding (ΔG^0_{U-F}) for WT *Hv* LigN versus the molar concentration of salt are shown in panels (a) and (c) for the salts indicated in the panels. *Hv* dea-LigN and *Hv* ade-LigN are represented by black and red circles, respectively. A linear dependence between stability and the molar concentration of salt was found and the solid lines correspond to the linear regressions. The populations of *Hv* dea-LigN and *Hv* ade-LigN are plotted in panels (b) and (d) using the same colour code (left ordinate axes). The shaded areas in red (black) correspond to the estimated uncertainties for the *Hv* ade-LigN (*Hv* dea-LigN) populations. The blue circles in the same panels represent the enzyme activity (normalised with respect to 3.2 M KCl, right ordinate axes).

Effect of the anion on Hv LigN catalytic activity. To account for the effect of the anion, we also explored the stability of wild type *Hv* ade-LigN and *Hv* dea-LigN in the presence of other salts (sodium sulphate and sodium phosphate). Sodium is chosen as counterion instead of potassium due to the higher solubility of guanidinium chloride in the presence of sodium salts. In both cases, the co-solute preferentially stabilised *Hv* ade-LigN and there was also a good agreement between the relative population of the two species and the catalytic activity of the enzyme (Figure 4). Consistent with data reported in other systems, [30,31] protein stabilisation by salt is the sum of the contributions from the anion and the cation.

Discussion

Given the variety of possible effects that salt has on catalytic activity, its relationship with the adaptation of enzymes to hypersaline media is apparently complex. Here, we have investigated *Hv* LigN, a halophilic enzyme that requires KCl in order to be active. Prior to catalysis, *Hv* LigN undergoes cofactor binding (adenylation). The low stability of the adenylated form in the absence of salt (lower than the apo-enzyme) reveals that this procedure is not spontaneous, providing an explanation for the lack of activity of the enzyme under these conditions. The inactivation at low ionic strength may be an exclusive feature of this enzyme (perhaps shared by other halophilic enzymes as well). In support of this idea, a closely related NAD$^+$-dependent DNA ligase from *Thermus scotoductus* becomes more stable upon adenylation in the absence of salt [26], as expected for a functional non-halophilic ligase.

Why does KCl preferentially stabilise the *Hv* ade-LigN species? The effect of salt on protein stability can be understood as the balance between two contributions:[32] i) the non-specific (weak) interactions between the co-solute and the protein backbone that favour extended conformations and ii) the solvation of the hydrophobic moiety of the biomolecule that stabilises compact folding of the protein. According to our structural model, *Hv* ade-LigN buries 331 Å^2 of its surface to the solvent, adopting a more closed conformation than the dead-enylated species. We and others have shown that a reduction in the area accessible to the solvent results in higher stabilisation induced by the salt.[33–35] Thus, KCl preferentially stabilises the more compact *Hv* ade-LigN and this in turn results in the activation of the enzyme. On the other hand, upon NaCl addition, a compensation of

both mechanisms occurs and the equilibrium cannot be displaced towards the adenylated form. This mechanism can also be extrapolated to other halophilic enzymes such as malate dehydrogenase (*Hm* MalD)[36] and lipase C (*Hm* LipC)[37] from *Haloarcula marismortui* that also require KCl to be active. It has been reported that salt favours the formation of oligomers (tetramers in the case of *Hm* MalD and dimers for *Hm* LipC), the only forms for these enzymes that are catalytically active.[37,38] This simple mechanism of stabilization paves the way for potential biotechnological applications. As an example, in Figure 3 we have shown how minimal protein surface modifications successfully modulate the stability dependence of a protein induced by the salt.

In summary, halophilic enzymes all share a very biased amino acid composition shaped by evolution and this signature appears to be a universal trait.[2] Our results show that this primary sequence fundamentally alters the populations of the different states of the protein, favouring the folded versus the unfolded state, the closed versus the open conformation and the oligomeric versus the monomeric form. In cases where the enzyme reaction coordinate implies a conformational change, differential stabilisation of the species in the reaction pathway (as in *Hv* LigN) will also result in indirect modulation of the catalytic efficiency. The fact that this is an apparent effect is consistent with the very different modulations that KCl (or NaCl) can exert on the activity of halophilic enzymes.

Methods

Equilibrium denaturation experiments. The effect of inorganic salts on the stability of *Hv* LigN was determined by measuring equilibrium denaturation at increasing concentrations of the desired co-solute. The following salts were considered: sodium and potassium to explore the effect of cations, and sulfate, phosphate and chloride for the anion series. A JASCO J-810 spectropolarimeter with a quartz cuvette of 1 cm path length was used to monitor the denaturation experiments by means of circular dichroism. CD experiments were monitored at 222 nm with a bandwidth of 4 nm and collection of data every 0.2 degrees. Guanidinium chloride denaturation experiments employed an initial volume of 1.7 mL containing the protein (0.6–1.3 μM) in 20 mM phosphate buffer at pH 8.0 and at the appropriate concentration of the inorganic salt. Protein denaturation was achieved by the addition of aliquots (performed with an automatic titrator) of a solution with the same protein concentration and buffer conditions but also containing a solution of denaturant agent. The concentration of the denaturant agent was adjusted by measuring the refractive index. The range of co-solute concentrations tested was limited by the reduced solubility of the inorganic salts in the presence of denaturant. The denaturant concentration was lowered to expand this solubility without compromising the

proper determination for the unfolded state baseline (see below), with final denaturant concentrations falling in the ranges between 4.8 M–6.9 M. Between two and four independent measurements were obtained for each experimental condition and duplicate points were used to obtain an estimation of the error.

Data analysis was completed with in-house built scripts programmed in Matlab© (Simulink). Guanidinium chloride denaturation data monitored by CD were processed assuming the linear extrapolation method[39] adapted to three states. Specifically, the equation used for the raw data fitting was:[40]

$$\theta_{obs} = \frac{\theta_N + \left\{\theta_I + (\theta_U + q[denat] \cdot e^b) \cdot e^a\right\}}{1 + (1 + e^b) \cdot e^a} \quad (1)$$

where

$$a = \frac{-\Delta G_{I-N}^0(0) + m_{IN} \cdot [denat]}{RT} \quad \text{and} \quad b = \frac{-\Delta G_{U-I}^0(0) + m_{UI} \cdot [denat]}{RT}$$

and where $\Delta G_{I-N}^0(0)$ and $\Delta G_{U-I}^0(0)$ are the free energy differences between the intermediate (I) and the native state (N) and between I and the unfolded state (U) respectively in the absence of denaturant. θ_{obs}, θ_N, θ_I and θ_U are the ellipticities observed, for the native state, for the intermediate and for the unfolded state respectively. m_{IN} and m_{NU} are the slopes of the transitions and q is an empirical factor that accounts for the linear dependence of the CD signal in the unfolded state. The total free energy of unfolding in the absence of denaturant corresponds to:

$$\Delta G_{U-N}^0(0) = \Delta G_{U-I}^0(0) + \Delta G_{I-N}^0(0) \quad (2)$$

The uncertainties in the free energies were propagated by Montecarlo analysis (with 15000 steps) to provide an estimation of the error in the populations as a function of the concentration (Figures 2B, 2D, 2F, S2B, S2D).

DNA ligase activity assay. DNA ligase assays were performed as previously described by Poidevin and MacNeill[19] with minor modifications. Ligase reactions (20 µL) contained 1.25 µg of Hν LigN in 20 mM Tris-HCl (pH 7.6 at 25 °C), 3.2 M KCl (unless otherwise indicated), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD+, 0.01% Triton ×100, 3 µL of 500 µg/mL of BstEII-digested λ DNA (New England Biolabs) and the test concentration of co-solute. Reactions were run at 45 °C for 10 minutes and stopped by the addition of 4 µL of 50 mM EDTA. The samples were then desalted using the MinElute reaction clean-up kit (Qiagen), according to the manufacturer's instructions, eluted in 20 µL of EB buffer (10 mM Tris pH 8.0), heated to 65 °C for 5 minutes and chilled on ice for 5 minutes prior to loading onto a 1% agarose gel prepared in TAE buffer. The gel contained 1× Sybr® Safe DNA gel stain (Invitrogen) and was run at 120 V for 60 minutes. Gels were visualized in the Chemidoc™ XRS System (BioRad) and band intensities were determined using ImageJ 1.41o analysis software (Wayne Rasband, National Institutes of Health, USA). Activity was calculated by measuring the net intensity of the product band (fragment I+IV) relative to the non-substrate fragments (bands II + III, V + VI or VII). The assay was tested in the presence of sodium and potassium, sulfate, phosphate, and chloride. In the case of the phosphate anion, the buffer was incubated at 75 °C for 10 min prior to running the ligase activity test, due to the low solubility of the magnesium phosphate.

Adenylation / de-adenylation assay. Due to the lower stability of the halophilic ligase, the adenylation and de-adenylation reactions were adapted from the original protocol.[25] For de-adenylation, an 80-fold excess of nicotinamide mononucleotide (NMN) and magnesium chloride was added to the protein (concentration of approximately 30 µM). The mixture was then incubated for between 75 and 100 h at 25 °C prior to purification by gel filtration chromatography (Superdex 75, Amersham). Adenylation was achieved by adding an 80-fold excess of NAD+ to the protein and subsequent incubation of the mixture for 20 h at the same temperature.

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Author contributions

G.O., A.L., X.T. and B.L. purified the proteins and performed the enzymatic assays. G.O. and X.T. measured the enzyme free energies in the presence of salts. O. M. calculated the protein models. G. O. and A.L. analyzed the data. G.O. and D.C. performed the mutagenesis studies. O.M. and G.O. designed the experiments and wrote the paper.

Additional information

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