# Investigating the Evolution of Negative Cooperativity in Phosphagen Kinases

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## Abstract

Complex biochemical traits arise through protein evolution. Mutation of a single residue can alter the structure of a protein, changing the position or potential interactions of other residues within the protein. Cooperativity, when substrate binding of one subunit changes substrate affinity of the other, is an example of a complex biochemical trait that may evolve. Phosphagen kinases (PKs) are a large, diverse protein family that can be used as a model system to investigate the evolution of cooperativity. An internal hydrogen bond network connecting the active site to the dimer interface has been observed in cooperative PKs. To investigate the functional and evolutionary significance of this hydrogen bond network, this study substituted S194, a central residue in the network, of *Stichopus japonicus* arginine kinase (SjAK), a known cooperative PK. The variants were characterized via size exclusion chromatography, UV-Vis kinetic assays, and ITC binding assays to determine the quaternary structure, kinetic parameters, and degree of cooperativity, respectively. Kinetic assays revealed that both SjAK S194A and S194T have reduced catalysis, but only S194T has reduced substrate affinity. Cooperativity assays were inconclusive, but structural analysis suggests that SjAK S194T does not properly convert to the 'closed' conformation upon ATP binding, as seen in SjAK WT and S194A. These findings show how residues located outside of the active site or dimer interface can impact protein structure and affect enzymatic activity and highlight the evolutionary significance of S194.

## Protein evolution allows for the development of complex new traits

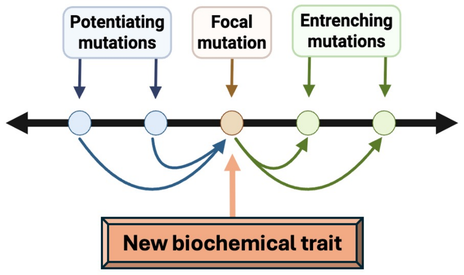



**Fig. 1. Evolution of a focal mutation.** The acquisition of a focal mutation depends on the presence of prior potentiating mutations, which can form direct or interact interactions that affect the possible interactions that the focal mutation may partake in. Subsequent mutations may then entrench the focal mutation, making it deleterious to lose the focal mutation. A double-headed arrow is used to indicate that many mutations may need to occur before the acquisition and entrenchment of a focal mutation. Figure adapted from Shah et al. (2015).

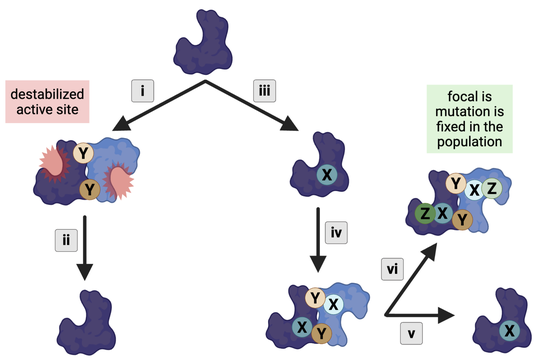



**Fig. 2. An example of how potentiating and entrenching mutations could support the evolution of dimerization.** A focal mutation (Y) may allow a protein to develop a new biochemical trait. When the independent evolution of a focal mutation causes deleterious effects, it may be contingent upon prior potentiating mutations (X) and further stabilized by entrenching mutations (Z). The following describes possible evolutionary pathways of a protein as it acquires dimerization as a new biochemical trait. (i) A hypothetical protein acquires focal mutation Y, allowing for dimerization but is deleterious due to destabilization of the active site. (ii) Purifying selection removes this deleterious mutation. (iii) Neutral mutation X is acquired and fixed due to chance. (iv) Focal mutation Y is acquired, allowing for dimerization. X alters the protein structure, changing the positionality of Y so that the active site is not affected. (v) Focal mutation Y is not fixed and is lost from the population due to chance. (vi) Entrenching mutation Z interacts with Y, making the loss of Y deleterious. Y then becomes fixed in the population, and the dimerization of this protein is evolutionarily protected.

## PKs can be used as a model system to investigate the evolution of cooperativity

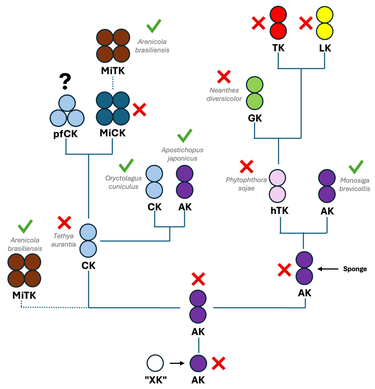



**Fig. 3. Kerwood's model of PK evolution suggests that cooperativity evolved independently in different species**. A proposed model of PK evolution is shown. A green check mark indicates cooperativity, a red X indicates noncooperativity, and a black question mark indicates that the degree of cooperativity is not known. Figure is adapted from Kerwood (2015).

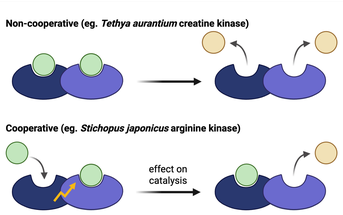



**Fig. 4. Cooperativity provides a biochemical advantage.** The subunits of non-cooperative oligomeric enzymes act independently. The subunits of cooperative enzymes, however, engage in subunit crosstalk to alter the stability and therefore substrate affinity of the other subunit, often impacting catalysis or regulation.

## Hypothesis: An internal hydrogen bond network may serve as a series of potentiating mutations for the evolution of PK cooperativity

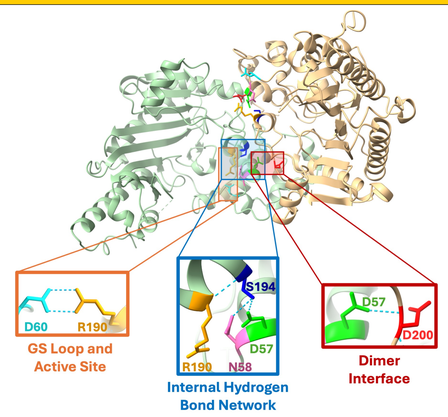
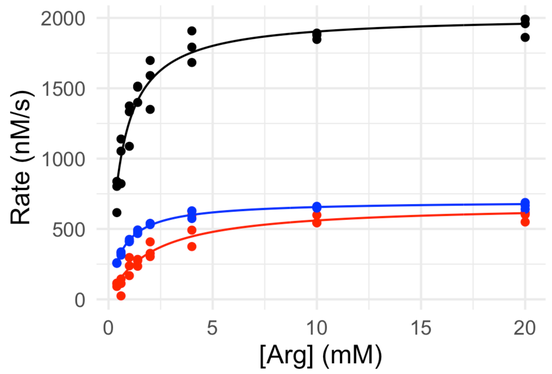



**Fig. 5. Predicted hydrogen bond network connects dimer interface to the guanidine specificity (GS) loop.** ChimeraX was used to display the crystal structure of SjAK. The two identical subunits (green and tan) are shown. D200 (red), D57 (lime), S194 (dark blue), R190 (orange), and D60 (cyan) are indicated. A cascade of hydrogen bond interactions, starting with D200, extends from the subunit interface to the GS loop. D200 interacts with D57 across the dimer interface through hydrogen bonding interactions. D57 also interacts with S194, which can rotate to interact with N58 or R190, a residue found within the GS loop. Structure obtained from protein data bank: 3ju5.

## Substituting internal residue S194 affects kinetic parameters; S194T has an increased $K_M$ suggesting reduced affinity for arginine




| Enzyme | Quaternary Structure | $K_{MArg}$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_{MArg}$ ($mM^{-1}s^{-1}$) |
|---|---|---|---|---|
| SjAK WT | Dimer | 0.6 ± 0.1 | 202 ± 2 | 337 ± 77 |
| SjAK S194A | Dimer | 0.66 ± 0.02 | 70 ± 0.6 | 106 ± 3 |
| SjAK S194T | Dimer | 2.1 ± 0.6 | 68 ± 4 | 33 ± 10 |

**Fig. 6 Michaelis Menten kinetics of SjAK WT, S194A, and S194T.** UV-Vis linked kinetic assays were performed on SjAK WT, S194A, and S194T. [MgATP] = 5 mM; [enzyme] = 10 nM. The kinetic data were fit to a Michaelis Menten plot to calculate kinetic parameters.

## Structural analysis suggests that S194T does not undergo the proper conformational changes upon ATP binding

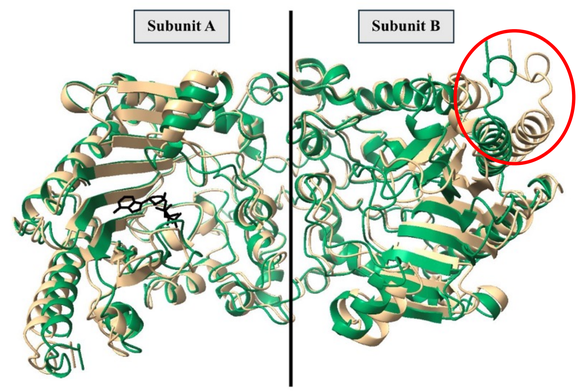



**Fig. 7. Conformational changes occur in SjAK WT upon ATP binding.** Bound SjAK WT (green) is depicted, where Subunit A is bound to ATP (black) while Subunit B remains unbound. SjAK WT with neither subunit bound (tan) is also shown. Significant conformational changes due to ATP binding are observed in the unstructured loops and supporting alpha helices (circled in red). Structures were generated with AlphaFold2 and analyzed on ChimeraX.

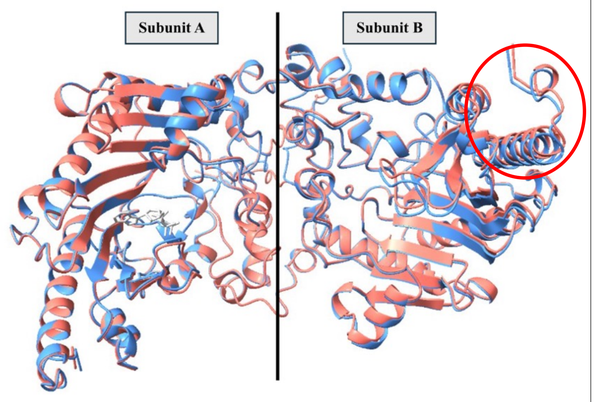



**Fig. 8. ATP binding induces fewer conformational changes in SjAK S194T than in WT.** Bound SjAK S194T (blue) is depicted, where ATP (silver) is docked in Subunit A while Subunit B remains unbound. SjAK S194T with neither subunit bound (pink) is also shown. Structures were generated with AlphaFold2 and analyzed on ChimeraX.

## Conclusions

- Substitution of S194 affects the enzymatic activity and conformations of SjAK
- Size and steric hinderance of S194, rather than conservation of the hydrogen bond, has a greater impact on enzymatic activity
- S194 may serve as a potentiating mutation that helps stabilize the ATP bound form of SjAK
- Internal residues can play a key role in protein structure and function

## Future directions

- Determine whether S194A and S194T are cooperative
- Investigate and mutate other residues within the internal hydrogen bond network
- Use a directed evolution as a less biased approach to investigate PK evolution (Fig. 9)

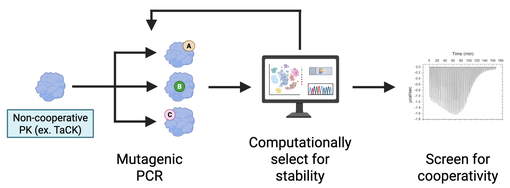



**Fig. 9 Using directed evolution to explore the evolution of cooperativity in PKs.** Directed evolution is a technique in which mutagenic PCR is used to generate a library of protein variants, which are then screened for stability and tested for cooperativity. Few PK studies investigate the importance of internal residues, making this a unique and unbiased approach to study the evolutionary significance of seemingly neutral residues and determine whether they play a role in enzyme structure and function.

## Acknowledgements

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