Part 1: EVB Computational Protocol for LmrR_pAF Friedel–Crafts Alkylation with Alanine Scanning

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1. Define the EVB Reaction Coordinate

- Key catalytic step: pAF (at V15) forms an iminium ion intermediate with enal (1a); indole (2a) attacks the β-carbon forming the C–C bond.
- For one-step EVB, model the whole reaction as:

Enal+Indole+pAF→Iminium C–C adduct (pre-reduction product)Enal+Indole+pAF→Iminium C–C adduct (pre-reduction product)

- Reactive bond changes to describe:
    - Breaking: enal C=C π bond weakening
    - Forming: new C–C bond between indole C3 and enal β-carbon
- Proton/electron transfers handled implicitly; focus on bond reorganization.

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2. Define EVB States

- State I (Reactants): Iminium ion formed between pAF and enal, indole unbound.
- State II (Products): Covalent Friedel–Crafts C–C adduct (indole-enal), still attached as iminium.

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3. Build the Model System

- Use LmrR_pAF crystal structure (PDB: 6I8N).
- Model noncanonical amino acid pAF at position 15.
- Place substrates (enal and indole) in the hydrophobic pocket guided by paper's docking data.
- Select key active site residues around pAF (all from paper) for alanine scanning mutagenesis in silico.
- Setup individual systems where each residue is mutated to alanine one at a time (alanine scanning) and also include the wild-type for comparison.

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4. Calibration of EVB Diagonal Terms

- Extract reaction free energies and barriers (ΔG0, ΔG‡Δ) for calibration.
- Adjust EVB diagonal Hamiltonians to reproduce aqueous reference barrier.
- Use this calibrated model in enzyme environment to focus on protein electrostatic and steric effects.

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5. Simulation Procedure

- Perform EVB free energy simulations for each system.

- Calculate activation free energies ΔG‡Δ*G*‡ for all variants.

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7. Validation

- Compare EVB-predicted changes in activation barrier upon alanine mutagenesis with experimental alanine scanning data reported in the paper.
- Focus on qualitative trends and mechanistic insights rather than exact numerical match.

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8. Expected Outcome (Report Writing)

- EVB simulations will capture the catalytic role of the pAF residue through iminium ion stabilization and transition state lowering.
- Alanine mutations disrupting critical noncovalent or steric interactions will increase activation free energies, reducing catalysis efficiency.
- The LmrR hydrophobic pocket and electrostatic environment will be shown to significantly contribute to catalysis beyond pAF alone.
- Computational alanine scanning results will align well with the experimental alanine scanning data from the paper, confirming the mechanistic importance of key residues.

Part 2: Experimental Validation

A. Kinetic Measurements

- Perform enzyme kinetics assays with LmrR_pAF and alanine mutants using trans-2-hexenal and 2-methylindole substrates.
- Determine Michaelis-Menten parameters kcat and KM for wild-type and mutants under conditions from Leveson-Gower et al.
- Measure product yield and enantioselective excess through HPLC.

B. Correlation with EVB Predictions

- Compare computed EVB activation energies (ΔG‡) with experimental catalytic efficiencies kcat/KM
- Correlate predicted effects of alanine mutations on activation barriers with observed changes in yield and enantioselectivity.
- Use correlation to validate EVB reaction coordinate, supporting it as a model for the key transition state of iminium ion formation and C–C bond formation in catalysis.

Combined Relevance and Integration of Computational and Experimental Alanine Scanning

- The experimental alanine scanning identifies residues in LmrR_pAF that, when mutated, reduce catalytic efficiency or stereoselectivity, highlighting their essential roles.
- The EVB computational alanine scanning quantifies the impact of these residues on the activation free energy barrier of the critical reaction step, directly linking structure to catalysis.
- Agreement between experimental and computational trends provides robust mechanistic insight that local protein environment and specific side chains cooperate with pAF in catalysis.
- This combined approach strengthens confidence in the EVB model and guides future enzyme engineering by pinpointing hot-spot residues.