# Modification of Residue 42 of the Active Site Loop with a Lysine-Mimetic Side Chain Rescues Isochorismate-Pyruvate Lyase Activity in *Pseudomonas aeruginosa* PchB

José Olucha, Kathleen M. Meneely, and Audrey L. Lamb*

Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045, United States
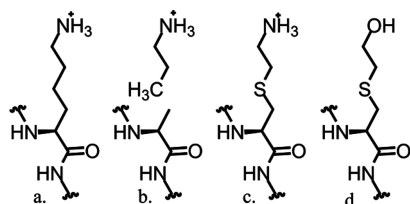
S *Supporting Information*

ABSTRACT: PchB is an isochorismate-pyruvate lyase from *Pseudomonas aeruginosa*. A positively charged lysine residue is located in a flexible loop that behaves as a lid to the active site, and the lysine residue is required for efficient production of salicylate. A variant of PchB that lacks the lysine at residue 42 has a reduced catalytic free energy of activation of up to 4.4 kcal/mol. Construction of a lysine isosteric residue bearing a positive charge at the appropriate position leads to the recovery of 2.5−2.7 kcal/mol (about 60%) of the 4.4 kcal/mol by chemical rescue. Exogenous addition of ethylamine to the K42A variant leads to a neglible recovery of activity (0.180 kcal/mol, roughly 7% rescue), whereas addition of propylamine caused an additional modest loss in catalytic power (0.056 kcal/mol, or 2% loss). This is consistent with the view that (a) the lysine-42 residue is required in a specific conformation to stabilize the transition state and (b) the correct conformation is achieved for a lysine-mimetic side chain at site 42 in the course of loop closure, as expected for transition-state stabilization by the side chain ammonio function. That the positive charge is the main effector of transition state stabilization is shown by the construction of a lysine-isosteric residue capable of exerting steric effects and hydrogen bonding but not electrostatic effects, leading to a modest increase of catalytic power (0.267−0.505 kcal/mol of catalytic free energy, or roughly 6−11% rescue).
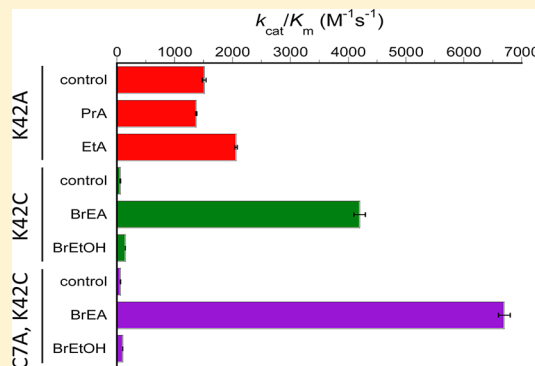
Chemical rescue experiments have been widely used in enzymology studies to obtain insight into the function of important active site amino acid side chains.[1] Noncovalent rescue experiments usually include mutation of the residue of interest to an alanine and the addition of excess compound mimicking the lost side chain (e.g., propylamine to mimic a lysine as depicted in Scheme 1A,B). The first chemical rescue, by Toney and Kirsch, restored activity in a lysine-to-alanine variant of aspartate aminotransferase using aliphatic amines as the rescue agents.[2,3] Since then, a variety of amino acid variants that suffered loss of activity have had restored activity in the presence of exogenous compounds, including but not limited to lysine to alanine or cysteine variants rescued with aliphatic amines,[4−7] arginine to alanine variants rescued with guanidinium or imidazole,[8−13] histidine to glycine or alanine variants rescued with imidazole,[14−17] and an aspartate to serine variant rescued with acetate.[18] These experiments have sometimes been called "cavity mutants" because the exogenous rescue agent is thought to bind in the cavity generated by the mutational variation.[19] Binding of the rescue agent in the vacated site has been confirmed in crystallographic studies of myoglobin[14] and trypsin.[18] However, the exogenous rescue agent does not always bind in the cavity, as in the case of carbonic anhydrase, where the rescue agent reestablished a hydrogen bonding network by binding in a site remote to the mutation.[15] Alternatively, mutation of the residue of interest to cysteine allows for covalent chemical modification by alkylating agents (e.g., bromoethylamine to generate γ-thialysine, Scheme 1C). Covalent modification may allow for better control over the position and orientation of the rescue mimetic, with the caveat that any other reactive cysteines may be modified as well. Covalent modification has been shown to be effective in a variety of systems, including

Scheme 1. Structures of the Lysine at Residue 42 of WT PchB (a), a Lysine Mimetic Assembly with Propylamine (b), a Lysine Mimetic Residue Resulting from Covalent Modification of the Cysteine Variant with Bromoethylamine (c), and a Lysine-Isosteric Residue Lacking Electrostatic Charge Resulting from Covalent Modification of the Cysteine Variant with Bromoethanol (d)

acetoacetate decarboxylase,[20] ketopantoate reductase,[7] fructose 1,6-bisphosphate aldoloase,[21] glutamine synthase,[22] arginase I,[23] and aspartate aminotransferase.[24,25]

The enzyme under study here is the isochorismate-pyruvate lyase from *Pseudomonas aeruginosa* (PchB). PchB is a structural homologue of the *Escherichia coli* chorismate mutase[26] and indeed has adventitious chorismate mutase activity, although the efficiency of the reaction is lower.[27,28] The chorismate mutase reaction is pericyclic with concerted but asynchronous bond breaking and bond forming and a cyclic transition state.[29] The isochorismate-pyruvate lyase activity has been shown by NMR to occur via a quantitative hydrogen transfer from C2 to C9, which led to the hypothesis that this reaction is also pericyclic with the hydrogen atom as one of the atoms of the cyclic transition state (Scheme 2).[30] The hypothesis was supported computationally.[31]

Scheme 2. Isochorismate-Pyruvate Lyase Activity Catalyzed by PchB[a]
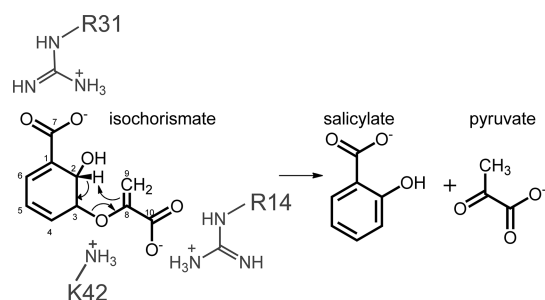
[a]The arginines that arrange the substrate carboxylates (R14 and R31) are shown in grey, as is the lysine (K42) of interest in this study, which is hypothesized to stabilize the developing negative charge of the transition state.

In our investigations of the pericyclic reactions catalyzed by the isochorismate-pyruvate lyase from *Pseudomonas aeruginosa* (PchB), we have provided evidence that catalytic power is derived from the organization of the substrate in the active site and from a positive charge at the 42 site of the mobile active site loop. This hypothesis has a basis in three previous experiments: (1) mutational analyses showed that variants at the K42 site did not alter the active site architecture, but a change in the chemical nature of the side chain from positively charged to neutral or negatively charged reduced or abolished activity;[28] (2) pH titrations of the K42H variant of the enzyme showed that with a protonated histidine at the 42 site the enzyme had nearly full activity whereas a deprotonated histidine at the same site was an inefficient catalyst;[32] and (3) thermodynamic data suggested that organization of the substrate in the active site does not account for the observed entropic change thereby implicating loop organization in catalysis.[33] With these data in mind, we performed several chemical rescue experiments. It is important to note that since the reaction is pericyclic, the lysine of interest is not involved in a proton transfer (general acid–base chemistry), does not participate in a covalent intermediate, and is not a metal ligand as frequently evaluated by chemical rescue. Instead, K42 is the residue of debate in the controversy over electrostatic transition state stabilization versus reactive substrate destabilization in the structurally homologous *E. coli* chorismate mutase. This lysine residue (or the comparable arginine in the *Bacillus subtilis* chorismate mutase) has been hypothesized either to be important in stabilizing the developing negative charge of the

ether oxygen at bond breaking[34−41] or to be unimportant in catalysis.[42−46]

The first chemical rescue experiment described was to determine whether exogenous rescue agents in a K42A variant were sufficient for restoration of activity, which might suggest that the aliphatic amine could bind in a preformed cavity vacated by the variant side chain. This hypothesis seemed unlikely because the variant side chain is found in the mobile active site loop that undergoes a disorder to order transition upon binding of ligands in the active site, as evidenced by crystallography (Figure 1).[26] In the second series of experiments, the K42C and
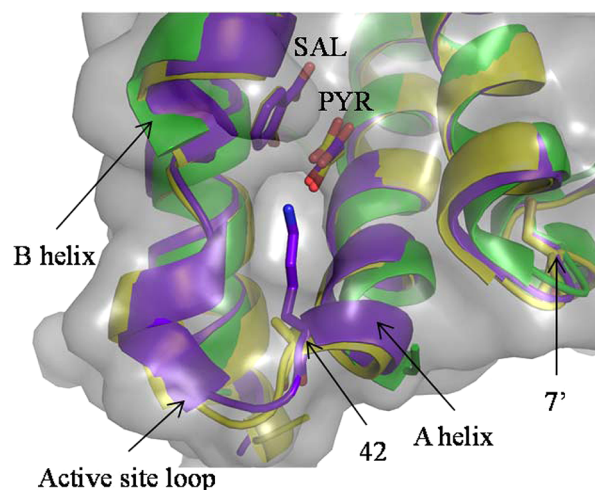

Figure 1. Active site of PchB in the open and closed wild-type and closed K42A-PchB structures. Overlay of the wild-type closed (PDB 3REM, purple), wild-type open (PDB 2H9C, green), and K42A-PchB (PDB 3HGX, yellow) structures. The open structure, solved in the absence of products, has no electron density for the active site loop, which connects the A and B helices and contains a single turn of helix when ordered in the closed structures. The amino acid at the 42 position, which is the first residue of the active site loop, is shown as sticks, as is the amino acid at the 7 position from the opposing monomer (7′). The salicylate and pyruvate from the closed structures are also shown as sticks, all colored like the cartoon. The solvent accessible surface (gray) for the K42A structure shows a hole leading to the active site, highlighting the location that a chemical rescue agent must bind and be organized. Figure was generated with PyMOL.[52]

C7A/K42C variants were generated and tested for restoration of activity after treatment with bromoethylamine, which generates a lysine mimetic, or treatment with bromoethanol, which generates an isosteric alcohol (Scheme 1D). We hypothesized that restoration of activity would be more efficient in covalent rescue experiments. The comparison of the amine versus alcohol derivatives was to consider the importance of a positive charge or hydrogen bond donors/acceptors at the 42 site. We report our findings here.

■ EXPERIMENTAL PROCEDURES

Protein Preparation. Wild-type and K42A-PchB were prepared as previously described.[26] Site-directed mutants of PchB were prepared with the plasmid used for wild-type PchB overexpression as the template and Quickchange II (Agilent) mutagenesis kit, using the protocol as per manufacturer's instructions. The K42C mutation was generated using a primer of sequence 5′-GGCGTCGCGCTTCTGCGCCAGC-GAGGCGG-3′ (mutated codon is underlined) and its reverse complement. The C7A mutation was generated using a primer of

sequence 5′-AACTCCGAAGACGCCACCGGCCTGGCG-3′ (mutated codon is underlined) and its reverse complement. In order to prevent disulfide bond formation and aggregation of the mutants, which generated a new cysteine, 20 mM β-mercaptoethanol was added to the resuspension buffer. All buffers used in the purification process, including 30Q and gel filtration buffers, contained 1 mM tris(2-carboxyethyl)phosphine (TCEP, Sigma).

Preparation of Isochorismate. Isochorismate was prepared as described previously.[28]

Enzymatic Assays. All assays were performed using a TgK Scientific Stopped Flow device operated at 20 °C, with a xenon lamp with the monochromator set at 310 nm and a 360 nm cutoff filter. Equal 50 μL volumes of enzyme and substrate were injected into a 22 μL cell, and initial velocity of salicylate production was measured as an increase in fluorescence at 430 nm for 30 s during which the reaction was linear. Preinjection enzyme and substrate concentrations were twice the final enzyme/substrate concentration in the cell (postmixing). Unless otherwise specified, all concentrations reported are final postmixing concentrations. Both the substrate isochorismate and enzyme were prepared in the same buffer system to prevent buffer dilution effects postmixing (50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM TCEP, and 500 mM rescue agent [where applicable]). Isochorismate was prepared to a desired premix final concentration of 0−800 μM. Enzyme concentrations used were 100 nM for wild-type and C7A-PchB experiments and samples treated with bromoethylamine, 2.5 μM for K42A-PchB, and 5 μM for PchB-K42C and C7A/K42C and samples treated with bromoethanol. Michaelis−Menten kinetic data were fit using Kaleidagraph (Synergy Software) using nonlinear regression.

Covalent Chemical Rescue of K42C- and C7A/K42C-PchB Variants with Bromoethylamine and Bromoethanol. PchB variants and controls were incubated for 22−24 h at 18 °C in 2.5 mL of 1 M Tris pH 8.8 with and without 1 mM bromoacylating reagent added. Enzyme was concentrated using Microcon centrifuge concentrators, and unreacted alkylating agent was removed by buffer exchange into 50 mM Tris HCl, pH 8.0, 150 mM NaCl, 10% glycerol, and 1 mM TCEP using a PD-10 desalting column (GE Healthcare) as per manufacturer's specifications. Protein concentration was determined by Bradford assay. Enzyme was added to 10 mL of reaction buffer without bromoacylating agent to final concentration of 200 nM to 10 μM (twice the postmix enzyme concentration). Substrate was prepared with the same buffer components.

Circular Dichroism Spectroscopy. CD spectra of wild-type and variant PchB enzymes (~10 μM protein in 20 mM potassium phosphate, pH 7.0) with and without modification with bromoethylamine were measured with a Jasco J-815 spectrometer (Easton, MD) at 25 °C with a path length of 1 cm. Spectra were recorded three times for each sample and averaged. The step size and bandwidth were 1 nm, and the averaging time at each wavelength was 1 s.

Ellman's Reagent Competition Assay. In order to determine whether the cysteine at position 7 was solvent-accessible and thus alkylatable, 1 mL of 1 mM protein sample (wild-type, K42C-, C7A- or C7A/K42C-PchB) was incubated for 24 h at 18 °C in the same buffer used in the alkylation process as described above but without alkylating reagent. A volume of 100 μL of each of the samples was reacted with 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) in 300 mM Tris, pH 8.0, 25 mM sodium acetate buffer for 5 min at room temperature. Absorbance was measured at 412 nm and

concentration of NTB^{-2} was determined (ε = 13 600 M^{-1} cm^{-1}).[47] For these experiments, protein concentration was determined by measuring the absorbance at 280 nm and using the extinction coefficient calculated by ProtParam (ε = 20 970 M^{-1} cm^{-1}).[48] This concentration corresponds to a 1:1 ratio with the solvent-accessible cysteines in solution. Wild-type (one cysteine at position 7) and C7A-PchB variant (no cysteines in sequence) showed no change in absorbance, indicating no cysteines are solvent-accessible in these enzymes. K42C- (two cysteines at positions 7 and 42) and C7A/K42C-PchB (one cysteine at position 42) both showed a similar absorbance change corresponding to one cysteine per protein molecule being solvent-accessible. Presumably, this is the cysteine at position 42, since the cysteine at position 7 is not accessible in the wild-type and only one cysteine is accessible in the K42C-variant; however, it is possible that both cysteines are modified at 50% efficiency in the K42C-variant. Samples of each enzyme were then incubated as described above for the controls but with excess alkylating agent (10 mM). A DTNB competition assay was performed to determine the percent alkylation for bromoethylamine and bromoethanol using the equation

$$\%\text{alkylation} = 1 - \frac{[\text{sample with alkylated agent}]_{\text{DTNB}}}{[\text{sample without alkylated agent}]_{\text{DTNB}}}$$

(1)

Wild-type and the C7A-variant showed no alkylation (0%). K42C-PchB showed 82% alkylation for two cysteines. C7A/K42C-PchB showed 98% alkylation for one cysteine with derivatization with bromoethylamine and 100% alkylation for one cysteine with derivatization with bromoethanol. The values (% alkylation) were used to adjust the kinetic parameters found in Tables 1 and 2.

$\Delta\Delta G^{\ddagger}$ Calculations. To quantify chemical rescue, variant effects were calculated in terms of changes in energies of activation:

$$\Delta\Delta G^{\ddagger} = -RT \ln\left[\frac{(k_{\text{cat}}/K_{\text{m}})_{\text{var}}}{(k_{\text{cat}}/K_{\text{m}})_{\text{ctrl}}}\right]$$

(2)

where R represents the gas constant (1.98 × 10^{-3} kcal K^{-1} M^{-1}) and T is the experimental temperature, 293 K (20 °C). Propagation of the error of $k_{\text{cat}}/K_{\text{m}}$ to the $\Delta\Delta G^{\ddagger}$ was calculated as described previously [49] using eq 3.

$$\text{error} = \left(-RT \ln\left[\frac{(k_{\text{cat}}/K_{\text{m}})_{\text{var}}}{(k_{\text{cat}}/K_{\text{m}})_{\text{ctrl}}}\right]\right)\sqrt{\left[\frac{\left(\frac{\partial k_{\text{cat}}}{K_{\text{m}}}\right)_{\text{var}}}{\left(\frac{k_{\text{cat}}}{K_{\text{m}}}\right)_{\text{var}}}\right]^2 + \left[\frac{\left(\frac{\partial k_{\text{cat}}}{K_{\text{m}}}\right)_{\text{ctrl}}}{\left(\frac{k_{\text{cat}}}{K_{\text{m}}}\right)_{\text{ctrl}}}\right]^2}$$

(3)

RESULTS

Kinetic Measurements of Isochorismate-Pyruvate Lyase Activity. The experiments presented were conducted in a new buffer system from previously reported work, and the experiments were performed using a stopped-flow spectrometer. While the values are different by 3-fold from those previously reported,[28] the K42A-PchB variant was shown previously to have 100-fold reduction in $k_{\text{cat}}/K_{\text{m}}$ relative to wild-type, which is consistent with the values presented in Table 2.

Attempted Chemical Rescue of K42A-PchB with Exogenous Alkylamines. The K42A-PchB variant was employed to further gauge the catalytic contribution of the
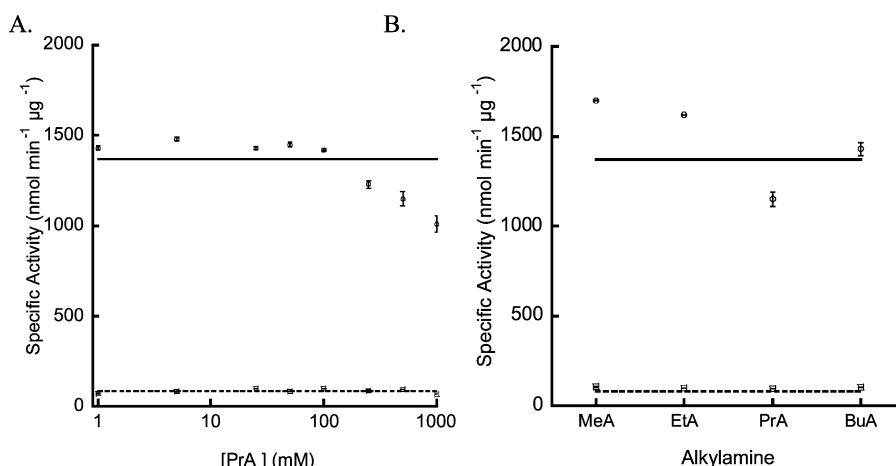
Figure 2. No chemical rescue of isochorismate-pyruvate lyase activity by exogenous amines. (A) There was no recovery of K42A-PchB activity (□) with the addition of propylamine. The value without the addition of exogenous amine is shown as a dashed line (86.2 nmol/(min·μg)). Propylamine similarly had no effect on the activity of wild-type enzyme (○). The value without the addition of exogenous amine is shown as a solid line (1370 nmol/(min·μg)). (B) The amine molecular volume was unimportant in the lack of rescue, because methylamine (MeA), ethylamine (EtA), propylamine (PrA), and butylamine (BuA) were all equally ineffective as chemical rescue agents at 0.5 M. Wild-type activity without exogenous amine is shown as the upper solid lined (1370 nmol/(min·μg)) and with the addition of rescue agents shown as circles (○). The activity of the K42A-PchB variant without exogenous amine is shown as the lower dashed line (94.4 nmol/(min·ng)) and with the addition of rescue agents shown as squares (□).

side chain at this position by determining the rescue efficiency in the presence of exogenous amines. Figure 2 shows the effect of exogenous amines on specific activity of wild-type and K42A-PchB. Since the molecular void volume created by the formation of the K → A variant is the same as that of propylamine,[4,7] this compound was used for an initial analysis. Panel A shows the effect of increasing propylamine concentration on lyase activity for wild-type (circles) and K42A-PchB (squares). If there were rescue, then we would expect a hyperbolic curve. Instead, a line with a slope of 0 is shown at the value of activity without exogenous amine added. Methylamine, ethylamine, and butylamine were also tested at a single concentration of 0.5 M with no rescue seen for these alkylamines of smaller or larger molecular volume (Figure 2B). The effective binding constants for these compounds must be higher than 2-fold the highest concentration tested, giving a minimum K_d of 2 M. All of the alkylamines showed a specific activity that was within 1.3-fold the specific activity of the K42A-PchB variant without exogenous amine.

Attempted Chemical Rescue of K42A-PchB with Exogenous Ethylamine or Propylamine. Michaelis−Menten kinetic analyses of K42A-PchB with 0.5 M ethylamine or 0.5 M propylamine were carried out. A comparison of the kinetic parameters for wild-type and the K42A-PchB variant is found in Table 1. The K42A-PchB variant has altered values for both k_{cat} and K_m relative to wild-type. The k_{cat} value is reduced by 2.9-fold, and the K_m is increased by 27-fold, suggesting that the lysine at the 42 site is important in substrate binding. The rate of enhancement upon the addition of ethylamine or propylamine is compared relative to the variant. K42A-PchB in the presence of either ethylamine or propylamine demonstrates a 2-fold increase in k_{cat} relative to K42A-PchB without exogenous amine. There was a comparable change in K_m. The change in the K_m may suggest that the lysine has a role in binding substrate, but the addition of the exogenous amine does not rescue substrate binding. A comparison of the k_{cat}/K_m values (Table 2) shows the lack of rescue more clearly. The k_{cat}/K_m values for K42A-PchA with and without exogenous amine are all approximately 100-fold less than the wild-type value. The addition of ethylamine to K42A-PchB increased the k_{cat}/K_m by 1.4-fold relative to the

Table 1. Catalytic Constants for Wild-Type PchB and Variants at Residue 42 with or without Exogenously Added Ethylamine or Propylamine or Covalent Modification of the Side Chain with Bromoethylamine or Bromoethanol

sample[a]	k_{cat} (×10⁻³ s⁻¹)	$k_{cat}^{var}/k_{cat}^{ctrl}$	K_m (μM)	K_m^{var}/K_m^{ctrl}
WT[b]	130 ± 0		1.1 ± 0.1	
K42A	45 ± 1	1	30 ± 1	1
K42A + EtA	83 ± 1	1.8	60 ± 1	2
K42A + PrA	98 ± 1	2.2	48 ± 1	1.6
WT[c]	178 ± 1		1.39 ± 0.03	
K42C	8.28 ± 0.04	1	134 ± 1	1
K42C + BrEA	94.6 ± 0.1	11.4	23 ± 1	0.17
K42C + BrEtOH	18.4 ± 0.1	2.2	125 ± 1	0.93
C7A/K42C	8.96 ± 0.01	1	123 ± 2	1
C7A/K42C + BrEA	105 ± 1	12	15.6 ± 0.3	0.13
C7A/K42C + BrEtOH	12.8 ± 0	1.4	114 ± 2	0.93
C7A	200 ± 10		2.10 ± 0.02	

[a]EtA = exogenous ethylamine; PrA = exogenous propylamine; BrEA = treatment with bromoethylamine; BrEtOH = treatment with bromoethanol. [b]Exogenous amine conditions. [c]Covalent rescue conditions.

variant without exogenous amine, whereas propylamine reduced the value by 0.9-fold.

Successful Chemical Rescue of K42C Variant PchB by Construction of an Isosteric γ-Thialysine at Position 42 with Bromoethylamine. A K42C-PchB variant was generated and modified by reaction with bromoethylamine to form γ-thialysine (Scheme 1C) at any solvent-accessible cysteines. The K42C-PchB variant shows an increased K_m relative to wild-type, presumably due to the loss of the hydrogen bond donor or positive charge at this site (Table 1). The K_m values for K42C-PchB with the modification to make the lysine mimetic show a decrease of nearly 6-fold. However, when the K42C-PchB is modified with the bromoethylamine, the k_{cat} shows an 11.4-fold increase relative to the unmodified variant. The change in both

values represent a significant rescue of activity by the lysine mimetic. Indeed, the k_{cat}/K_m increases 68-fold (Table 2).

A double mutant was generated to remove the naturally occurring cysteine from the N-terminus of the protein, thereby generating a C7A/K42C-PchB double variant. In the absence of rescue agent, the double variant showed kinetic parameters comparable to the single variant (Table 1). With the formation of the lysine mimetic, the C7A/K42C-variant k_{cat} value was again rescued significantly (12-fold), and in this case, the K_m value was decreased by ~8-fold. Rescue is even more evident in this variant with a k_{cat}/K_m value that is 103-fold higher than the variant without the modification to make the lysine mimetic.

Attempted Chemical Rescue of K42C-PchB and C7A/K42C-PchB by Construction of an Isosteric γ-Thia-ω-oxa-lysine at Position 42 with Bromoethanol. The K42C-PchB variant and the C7A/K42C-PchB double variant were also modified with bromoethanol to generate the isosteric alcohol residue (Scheme 1D). These modified variants showed no rescue in k_{cat}, showing values within 2.2-fold of the unmodified variant, and demonstrated K_m values within 1.1-fold of the unmodified variant (Table 1). Indeed, the k_{cat}/K_m values are ~2-fold higher than the variant without the modification (Table 2).

Secondary Structure Maintained as Detected by Circular Dichroism Spectroscopy. Circular dichroism spectroscopy was used to assess the secondary structure of the variant proteins with and without modification by bromoethylamine. All variant proteins were found to be comparable to wild-type with strong minima at 208 and 212 nm, characteristic of helical structure (Supplement Figure 1, Supporting Information). The variants that contained the cysteine to alanine change at the seven position may exhibit a slightly lower helical property than those with the native cysteine. Modification of the proteins with bromoethylamine had no impact of helicity (open versus closed symbols).

■ DISCUSSION

Recent work by Richard and colleagues on triosphosphate isomerase[50] and orotidine 5′-monophosphate decarboxylase[51] provides a framework for reporting and interpreting chemical rescue experiments. In both enzymes, a cationic side chain (lysine-12 and arginine-225, respectively) is adjacent to a flexible phosphodianion gripper loop, and the side chain forms a stabilizing ion pair with the substrate. Excision of the side chain results in a large decrease in catalytic activity (10^4–10^5-fold). A substantial fraction of the catalytic activity for the variants is rescued by addition of exogenous guanidine or alkyl ammonium cations. In the work on these two enzymes, elegant calculations are used to determine the effective molarity of the side chain and the related free energies of activation: $\Delta G^{\ddagger}_{rescue-agent}$ represents the portion that can be rescued by the activator, and $\Delta G^{\ddagger}_S$ represents the advantage of having the two pieces connected, called the "connection energy." In these previous examples,

$$(\Delta G^{\ddagger}_{variant} - \Delta G^{\ddagger}_{WT}) = \Delta G^{\ddagger}_S - \Delta G^{\ddagger}_{rescue-agent}$$

where $\Delta G^{\ddagger}_{rescue-agent}$ is negative, and with the connection energy ($\Delta G^{\ddagger}_S$) sums to give the variational effect caused by mutation at the site in question ($\Delta G^{\ddagger}_{variant} - \Delta G^{\ddagger}_{WT}$). In the best case scenario, a simple model provides an estimate of the entropy advantage caused by fusing the rescue agent to the side chain. Richard and colleagues[50] also provide enthalpic arguments, stating that the connection energy is an "empirical" transition state stabilization: there may be an enthalpic penalty due to desolvation or an

enthalpic advantage due to binding interactions. The former case would be evident as a large effective molarity, since desolvation of the side chain is already "paid" in the folding of the enzyme. An enthalpic advantage from favorable binding interactions between the rescue agent and the protein is evidenced by a small effective molarity.[50]

In PchB, the site in question is the 42 position, which is located in a flexible loop as determined by crystallography (Figure 1). No electron density is observed for residues 42−48 in the wild-type structure with an open active site.[26] The open structure further shows that there is no preformed binding site for exogenous amines in this conformation. The structure of the K42A-PchA variant shows a hole in the solvent-accessible surface of this "closed" conformation where the lysine side chain is no longer present, highlighting the site where an exogenous amine would need to bind and be organized after ordering of the loop (Figure 1). This "binding site" for exogenous amines presumably has a K_d > 2 M, since no rescue was observed at concentrations up to 1 M. Previous determination of the activation thermodynamics of the reaction suggested the importance of loop organization for PchB lyase activity.[33] Therefore, organization of the loop upon active site closure must also organize the lysine side chain and lead to the formation of the transition state.

If Richard's calculations are applied to the K42A-PchB protein and the rescue attempts with exogenous propylamine (Supplemental Figure S2, Supporting Information), the effective molarity of the side chain at lysine-42 is 610 M, suggesting that if the experiments could be conducted at this concentration of propylamine, the variant would turn over at the same rate as wild-type. The effective molarity in the PchB experiments is within the range of those presented previously,[50,51] which would suggest no evidence for an enthalpic disadvantage due to desolvation. We suggest that desolvation of the rescue agent and the mobile side chain in the flexible active site loop would be comparable. The side chain is exposed to solvent in the ground state open conformation and becomes desolvated and buried upon active site closing, which is true whether or not the side chain is covalently attached. Furthermore, as mentioned above, we estimate that the K_d is >2 M, since no rescue was observed for concentrations up to 1 M. In other words, there is not tight binding of the rescue agents to PchB, since there is not a preformed cavity for binding in the ground state. A portion of the binding interactions may be required to hold the loop in the closed conformation in the transition state. Such an interaction would not be rescued by an exogenous amine, but would require a covalent connection between the amino acid side chain and the enzyme. Indeed, the connection energy in the transition state ($\Delta G^{\ddagger}_S$) is calculated from the effective molarity to be 3.8 kcal/mol, which is *greater* than the value for the variational effect between the K42A- and WT-PchB, $\Delta G^{\ddagger}_{K42A} - \Delta G^{\ddagger}_{WT}$, determined to be 3.4 kcal/mol. In keeping with this result, the $1/K_d^{\ddagger}$ for propylamine can be determined and the $\Delta G^{\ddagger}_{PrA}$ calculated, giving a value of 0.4 kcal/mol (positive, not negative as previously seen). While these values are internally consistent, this analysis highlights the inability to rescue by an exogenous amine. A different analysis is desirable for PchB to account for the placement of the amino acid to be rescued in the flexible loop and for direct comparison with covalent rescue agents.

We propose that variational effects in chemical rescue experiments can be calculated more directly from the (apparent) k_{cat}/K_m in terms of differences in the free energies of activation ($\Delta\Delta G^{\ddagger}$, Table 2). The difference in energies can be calculated using eq 2, such that the mutational effect of replacing the wild-

Table 2. Effects of Side Chain at Residue 42 on Loss or Rescue of Electrostatic Catalysis in the Lyase Activity of PchB

| entry | sample[a] | k_{cat}/K_m (M^{-1} s^{-1}) | variational effect | | $\Delta\Delta G^{\ddagger}$ (kcal mol^{-1}) ($-RT \ln$[var. effect])[b] | % rescue |
			variant/control	value		
1a	WT[c]	124000 ± 4000				
1b	K42A	1510 ± 30	K42A/WT	0.01	2.56 ± 0.09	
2a	K42A + EtA	2060 ± 20	(K42A + EtA)/K42A	1.4	−0.180 ± 0.004	7
2b	K42A + PrA	1370 ± 10	(K42A + PrA)/K42A	0.9	0.056 ± 0.001	−2
3a	WT[d]	128000 ± 3000				
3b	K42C	62 ± 0	K42C/WT	0.0005	4.4 ± 0.1	
3c	C7A	93000 ± 5000	C7A/WT	0.7	0.17 ± 0.01	
3d	C7A/K42C	65 ± 1	(C7A/K42C)/C7A	0.0007	4.2 ± 0.2	
4a	K42C + BrEA	4200 ± 100	(K42C+BrEA)/K42C	68	−2.45 ± 0.06	55
4b	C7A/K42C + BrEA	6700 ± 100	(C7A/K42C + BrEA)/(C7A/K42C)	103	−2.69 ± 0.06	64
5a	K42C + BrEtOH	147 ± 1	(K42C+BrEtOH)/K42C	2.4	−0.505 ± 0.003	11
5b	C7A/K42C + BrEtOH	103 ± 1	(C7A/K42C + BrEtOH)/(C7A/K42C)	1.6	−0.267 ± 0.005	6

[a]EtA = exogenous ethylamine; PrA = exogenous propylamine; BrEA = treatment with bromoethylamine; BrEtOH = treatment with bromoethanol. [b]Temperature is 293 K. [c]Exogenous amine conditions. [d]Covalent rescue conditions.

type lysine at position 42 with an alanine (effectively removing steric and charge effects) increases the free energy of activation compared with the wild-type lyase activity by 2.56 kcal/mol ± 0.09 kcal/mol (Table 2, entry 1b). This number represents the maximal change in free energy of activation that any given exogenous chemical would be able to rescue to reach activity levels comparable to wild-type enzyme. Addition of excess exogenous positive charge in the form of ethylamine does not rescue catalytic activity (−0.18 kcal/mol or 7% rescue of the mutational effect; Table 2, entry 2a). The addition of propylamine causes a further reduction in activity (0.056 kcal/mol, or −2% of the mutational effect; Table 2, entry 2b). Addition of alkylamines of different carbon chain length (methyl- or butylamine) at the same concentration did not rescue catalysis either (Figure 2).

A K → C variant was generated at the 42 position to enable introduction of a positive charge with bromoalkylating compounds (Scheme 1C). As with the K → A mutation, the K42C variant displayed an increased energy of activation, in this case by 4.4 ± 0.1 kcal/mol (Table 2, entry 3b). This increase in $\Delta\Delta G^{\ddagger}$ would correspond to a theoretically perfect rescue. Indeed, the covalent modification of the K42C variant with bromoethylamine (generating a γ-thialysine at this position) rescues 2.45 ± 0.06 kcal/mol, roughly 55% of the ideal rescue (Table 2, entry 4a). However, wild-type PchB treated with bromoethylamine showed a decrease in catalytic activity with a k_{cat}/K_m 2.4-fold lower (See Supplemental Table S1, Supporting Information). This may be due to the alkylation of a natural cysteine located near the N-terminus (position 7) of the enzyme. Removal of this cysteine through the generation of a C7A variant provides better control over the effects of extraneous alkylation, and the k_{cat}/K_m value for C7A-PchB is comparable to wild-type (0.7-fold change; Table 2, entry 3c). The K42C variant over the C7A background resulted in a 4.2 kcal/mol gain in energy of activation with respect to the C7A background variant (Table 2, entry 3d). Treatment of this double variant with bromoethylamine confirmed the results of the K42C single mutant experiments: generation of the γ-thialysine results in a significant rescue. In the case of the double mutant, a 2.69 kcal/mol rescue (64%; Table 2, entry 4b) of the possible ideal rescue of 4.2 kcal/mol is observed.

Previous work on the structural homologue *E. coli* chorismate mutase had suggested that a positive charge at the site equivalent to PchB 42 is not important for catalysis: organization of the

substrate carboxylates by arginines buried deep within the active site was hypothesized to generate a near attack conformation, which was sufficient for catalysis.[42−46] In order to test this hypothesis, a variant with a lysine-isosteric residue bearing a hydroxyl group in lieu of the positively charged amino group was generated using bromoethanol instead of bromoethylamine (Scheme 1D). A modest increase in activity was observed for the K42C-PchB variant (0.505 ± 0.003 kcal/mol, 11%; Table 2, entry 5a) with less observed in the C7A/K42C double variant (0.267 ± 0.005 kcal/mol, 6%; Table 2, entry 5b). Mutagenesis[28] and pH dependence[32] experiments have provided evidence that the positive charge at position 42 is important for efficient catalysis. The ability to rescue catalytic activity of the variants modified to produce a γ-thialysine at position 42, but not through the generation of the hydroxyl derivative, gives further support to the importance of electrostatic transition state stabilization in the isochorismate-pyruvate lyase reaction. Structural[26] and thermo-dynamic[33] experiments have led to the hypothesis that loop organization is important for catalysis. Indeed, this is supported by the ineffectiveness of the ethylamine or propylamine to serve as noncovalent chemical rescue reagents; the positive charge must be organized with the loop for efficient catalysis. We propose that a positive charge at position 42 *and* correct loop organization, including organization of the lysine at position 42, lead to efficient lyase catalysis and give further support to the idea that electrostatic transition state stabilization and loop dynamics work in concert for catalysis.

These data lead to the question of why the isosteric γ-thialysine at position 42 is less able to promote catalysis than the native lysine or the previously tested histidine variant[32] at the same site. The tested variants are all apparently properly folded and stable, and for those modified by alkylation, either the chemistry of modification was complete or the kinetics were adjusted by a small correction. The answer may be simply that the active site is better able to accommodate the positively charged side chains lacking sulfur; however, the geometry of the lysine and γ-thialysine would place the positive charge in a more similar position than that for histidine, which is effectively one methylene group shorter (in fairness, we must note that the histidine variant has not been tested in the buffer system detailed in this report). Alternatively, the answer may be in the role that the lysine plays in catalysis. This amino acid is not involved in covalent catalysis or acid/base chemistry but has been proposed to stabilize the developing negative charge of the transition state

in this pericyclic reaction. In PchB, closing of the active site is integral to formation of the transition state from the reactant state,[33] and this requires organization of the active site loop and the lysine at site 42. It is tempting to propose that the motion of the residue at the 42 site may *promote* development of the negative charge on the ether oxygen of bond breaking. A vibrational motion bringing the positively charged side chain into proximity of the ether oxygen of the substrate may be more easily achieved by lysine and histidine compared with the γ-thialysine.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

A complete table of the catalytic constants of all controls and variants discussed in this work, circular dichroism spectra of the variants with and without the bromoethylamine modification, and calculations based on the work of Richard and colleagues[50,51] demonstrating a lack of activation of K42A-PchB variant by the addition of exogenous propylamine. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (785)864-5075. Fax: (785)864-5294. E-mail: lamb@ku.edu.

Notes

The authors declare no competing financial interest.

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