

The Enzymatic Activities of the *Escherichia coli* Basic Aliphatic Amino Acid Decarboxylases Exhibit a pH Zone of Inhibition

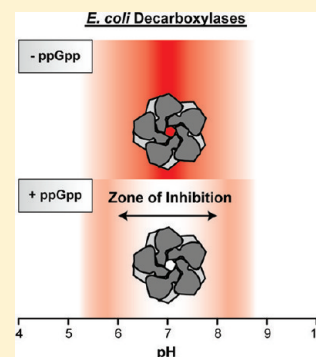
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S Supporting Information

ABSTRACT: The stringent response regulator ppGpp has recently been shown by our group to inhibit the *Escherichia coli* inducible lysine decarboxylase, LdcI. As a follow-up to this observation, we examined the mechanisms that regulate the activities of the other four *E. coli* enzymes paralogous to LdcI: the constitutive lysine decarboxylase LdcC, the inducible arginine decarboxylase AdiA, the inducible ornithine decarboxylase SpeF, and the constitutive ornithine decarboxylase SpeC. LdcC and SpeC are involved in cellular polyamine biosynthesis, while LdcI, AdiA, and SpeF are involved in the acid stress response. Multiple mechanisms of regulation were found for these enzymes. In addition to LdcI, LdcC and SpeC were found to be inhibited by ppGpp; AdiA activity was found to be regulated by changes in oligomerization, while SpeF and SpeC activities were regulated by GTP. These findings indicate the presence of multiple mechanisms regulating the activity of this important family of decarboxylases. When the enzyme inhibition profiles are analyzed in parallel, a “zone of inhibition” between pH 6 and pH 8 is observed. Hence, the data suggest that *E. coli* utilizes multiple mechanisms to ensure that these decarboxylases remain inactive around neutral pH possibly to reduce the consumption of amino acids at this pH.



The decarboxylation reactions of the basic aliphatic amino acids L-lysine, L-arginine, and L-ornithine are important for the production of polyamines¹ and for cell survival during the acid stress response.^{2,3} In *Escherichia coli*, there are six pyridoxal-5'-phosphate (PLP)-dependent inducible and constitutive enzymes that catalyze such decarboxylation reactions: LdcI (inducible) and LdcC (constitutive) convert L-lysine to cadaverine,^{4,5} AdiA (inducible) and SpeA (constitutive) convert L-arginine to agmatine,^{6,7} and SpeF (inducible) and SpeC (constitutive) convert L-ornithine to putrescine.^{8,9} Putrescine can also be produced in a two-step reaction initiated by the decarboxylation of L-arginine to agmatine by SpeA followed by the conversion of agmatine to putrescine and urea via the activity of the agmatine ureohydrolase SpeB.¹

The polyamines produced by the constitutive decarboxylases (LdcC, SpeA, and SpeC) are important cellular components and are involved in a variety of processes including DNA replication, transcription, and protein synthesis,¹ membrane permeability,^{10,11} and biofilm formation.¹² The inducible decarboxylases, on the other hand, are mainly involved in the cellular acid stress response.

E. coli possesses a very robust acid stress response system that allows survival over a wide range of acidic conditions.^{2,3} To operate, an amino acid decarboxylase consumes an intracellular proton during the reaction with the substrate, following which the reaction products are exchanged for fresh substrate from the external media via specific inner-membrane antiporters. Two systems function under extreme acid stress conditions: the arginine decarboxylase AdiA coupled with the arginine/

agmatine antiporter AdiC and the homologous PLP-dependent GadA/GadB glutamate decarboxylases and glutamic acid/ γ -amino butyric acid antiporter GadC.¹³ The inducible lysine decarboxylase LdcI and the lysine/cadaverine antiporter CadB provide limited protection against extreme acid stress¹⁴ but are very effective under milder acid stress conditions (\sim pH 5.0).^{15,16} The inducible ornithine decarboxylase SpeF and the ornithine/putrescine antiporter PotE are induced by anaerobic growth at low pH and may play a role in the acid stress response.¹⁷

Oligomerization is an important aspect for the function and regulation of the various decarboxylases. LdcI and its paralogous enzymes LdcC, AdiA, SpeF, and SpeC share the same structural fold,^{16,18} and these enzymes exist at minimum as homodimers but are often observed as higher order oligomers. Some of the enzymes form decamers composed of five homodimers as in the case of LdcI,^{4,15,16} LdcC,¹⁵ and AdiA,^{6,19} or they form homodimers as in the case of SpeF⁸ and SpeC.²⁰ The SpeF homologue from *Lactobacillus* 30A (OdcI) has been crystallized as a dodecamer.²¹ The other decarboxylases involved in polyamine biosynthesis or acid stress response are from different structural fold families, but they all form higher-order oligomers: SpeA forms a homotetramer,⁷ while the glutamate decarboxylases GadA²² and GadB^{23,24} exist as homo-hexamers.

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We have recently determined the X-ray crystal structure of LdcI (PDB ID: 3N75)^{16,25} where we discovered that this protein binds to the effector of the stringent response, guanosine 3',5'-bis(diphosphate) (ppGpp)—also known as the alarmone. The stringent response is induced when cells experience unfavorable growth conditions, such as nutrient deprivation and stress conditions, and results in significant changes to cell physiology including the induction of stress response genes and amino acid biosynthesis pathways.^{26,27} The stringent response effector ppGpp is produced by either the ribosomal bound RelA or the cytoplasmic SpoT proteins, and its main target is RNA polymerase. Unexpectedly, we found ppGpp copurified with LdcI overexpressed in *E. coli* and then cocrystallized with the enzyme.¹⁶ Ten ppGpp molecules were observed to bind to LdcI at specific interaction sites between neighboring monomers in the LdcI decamer. While there was no significant effect on LdcI oligomerization, ppGpp binding resulted in a substantial inhibition of LdcI activity over a wide range of pH values. As a follow-up to our findings on LdcI, we examined the ability of ppGpp and other nucleotides to regulate the activity of the LdcI paralogs LdcC, AdiA, SpeF, and SpeC. Furthermore, the effect of the oligomeric state on the catalytic activity of these enzymes was also determined. It was found that ppGpp inhibited LdcC and SpeC, while GTP regulated the activities of SpeF and SpeC. AdiA activity was found to be regulated by changes in oligomerization. Enzyme inhibition profiles display a “zone of inhibition” between pH 6 and pH 8. These findings shed important insights into the multilevel regulation of these enzymes in the cell.

EXPERIMENTAL PROCEDURES

Bioinformatics. A molecular phylogeny of the PLP-dependent decarboxylases belonging to the Clusters of Orthologous Groups²⁸ COG1982 was performed based on the method described in Snider and Houry.¹⁵ The amino acid sequence of *E. coli* LdcI (SwissProt # P0A9H3) was searched against the National Center for Biotechnology Information (NCBI) completed microbial genomes database using BLAST²⁹ with an expect value of 1 and all other parameters set to default. The list of retrieved sequences was pruned so that only one representative from each phylogenetic family was included. In addition, short fragments (<100 amino acids) and long fragments (>1000 amino acids) were discarded. Multiple sequence alignment on the 199 remaining sequences was performed using MUSCLE.³⁰ The aligned sequences were used to generate a phylogenetic tree using PHYLIP.³¹ The first 500 bootstrapped permutations of the data set were produced using SEQBOOT following which PROTDIST was used to generate protein distance matrices using the Henikoff/Tillier Probability Matrix from Blocks model.³² Finally, a consensus phylogenetic tree was generated using NEIGHBOR via the neighbor joining method. Analysis of the multiple sequence alignment was carried out using JALVIEW,³³ and the phylogenetic tree was visualized using HYPERTREE.³⁴

Cloning. All restriction enzymes were purchased from New England Biolabs (NEB), and all cloning procedures involved DH5 α cells (Invitrogen). The *adiA*, *speF*, and *speC* genes were PCR amplified from MG1655 K12 genomic DNA using *Pfx* polymerase (Invitrogen). The PCR product was purified and digested with *NdeI* (NEB) and *XhoI* (NEB) and ligated with T4 DNA ligase (Fermentas) into a pET-22b (Novagen) vector cut with the same restriction enzymes and treated with calf intestinal alkaline phosphatase (CIAP) (Fermentas) to generate the C-terminal His₆-tagged constructs: pET-22b-AdiA-H₆,

pET-22b-SpeF-H₆, and pET-22b-SpeC-H₆. The untagged clone of LdcC in pET-16b was the same as that used in ref 15.

Protein Purification. Purification of both C-terminally hexahistidine tagged and untagged LdcC was attempted from a ppGpp⁰ strain CF1693³⁵ but because of protein stability problems was unsuccessful. Instead, LdcC was purified from BL21 Gold (DE3) pLysS cells (Stratagene) as described previously.¹⁵ AdiA-H₆, SpeF-H₆, and SpeC-H₆ were expressed from a ppGpp⁰ strain CF1693³⁵ bearing a T7 polymerase,¹⁶ and the proteins were then purified using a standard Ni-NTA purification procedure (Qiagen). After elution from the Ni-NTA column, the protein sample was concentrated using an Amicon Ultra 15 centrifugal concentrator at 4 °C. The concentrated protein was diluted 10-fold with buffer A [10 mM sodium 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid (HEPES), pH 7.5, 10% (w/v) glycerol, and 1 mM dithiothreitol (DTT)] and loaded onto a MonoQ 5/50 GL (GE) anion exchange column at 4 °C using an ÄKTA FPLC (GE) chromatography system. Protein was eluted using a linear gradient from 0% buffer A to 100% buffer B (same as buffer A but with 500 mM KCl) over 50 mL. Peak fractions were identified via absorbance at 280 nm and sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. The appropriate protein fractions were pooled, concentrated, and then applied to a Superdex 200 10/300 GL (GE) size exclusion chromatography column equilibrated in buffer C (10 mM sodium HEPES, pH 7.5, 300 mM NaCl, 0.1 mM PLP, and 1 mM DTT). Purified protein fractions were pooled, concentrated, and quantified using the Bradford assay³⁶ with reagent purchased from Bio-Rad and finally flash frozen with liquid nitrogen and stored at –80 °C until use.

(p)ppGpp Purification. Synthesis and purification of ppGpp were carried out as described.¹⁶ All other nucleotides were purchased from SigmaAldrich.

Biochemical Assays. The 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay was used to measure enzyme activity as described previously.¹⁶ The following standard solution was used in the assay (0.1 mM PLP and 1 mM β -ME) and the different pH values were obtained using a 100 mM concentration of the following buffers: pH 3.0–4.5, sodium citrate; pH 5.0–5.5, sodium acetate; pH 6.0–6.5, sodium 2-(*N*-morpholino)-ethanesulphonic acid (MES); pH 7.0–8.0, sodium HEPES; pH 8.5–9.0, sodium [(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-amino]-1-propanesulfonic acid (TAPS); pH 9.5, sodium 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO); and pH 10.0, sodium 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS). The concentrations of substrates were as follows: 5.0 mM L-lysine, 5.0 mM L-arginine, 5.0 mM L-ornithine (SpeF), and 7.5 mM L-ornithine (SpeC). Furthermore, for the LdcC assay, the buffer was supplemented with 300 mM NaCl. The protein concentration for each assay was as follows: LdcC (25 nM), AdiA (15 nM), SpeF (400 nM), and SpeC (50 nM).

An isothermal titration calorimetry (ITC) based assay was used to determine the inhibition kinetics for LdcC as described¹⁶ using a VP-ITC instrument, ORIGIN 7.0 for data analysis, and SIGMAPLOT 11.0 for enzyme kinetics fits. ITC-based binding experiments for SpeC with ppGpp and GTP were performed as described.¹⁶

Size exclusion chromatography experiments were performed on a Superose 6 HR 10/30 column (GE) using an ÄKTA FPLC system (GE). A total of 20 μ g of each of the proteins was loaded onto the columns using the following standard buffer: 100 mM buffer (selected as for the enzyme assays based on pH), 300 mM NaCl, 0.1 mM PLP, and 1 mM DTT.

Electron Microscopy. Prior to observation, the protein solution was diluted in the buffer of the desired pH to the same final concentration as used for the enzyme assays described above. Substrate, NaCl, ppGpp, and GTP were added, where necessary, at the same concentrations as in the enzyme assays described above. After an incubation period of 1 min, established based on the time frame of the enzyme assays, protein solution was applied to the clear side of carbon on a carbon–mica interface and negatively stained with 2% (w/v) sodium silicotungstate, the pH of which was previously adjusted to correspond to each of the analyzed buffer conditions, from pH 3.5 to pH 9.5. Images were recorded under low-dose conditions with a JEOL 1200 EX II microscope at 100 kV at a nominal magnification of 40000. Selected negatives were digitized on a Zeiss scanner (Photoscan TD) at a step size of 14 μm giving a pixel size of 3.5 Å at the specimen level.

RESULTS

Bioinformatic Analysis of LdcI Paralogs. LdcI, LdcC, AdiA, SpeF, and SpeC are paralogous enzymes with sequence and structural homology and belong to the prokaryotic ornithine decarboxylase (pODC) subclass of Fold Type I PLP-dependent decarboxylases.¹⁸ These proteins possess three domains: an N-terminal Wing domain, a Core domain composed of a short linker region followed by a PLP-binding subdomain (PLP-SD) and subdomain 4 (SD4), and a C-terminal domain (CTD)¹⁶ (Figure 1A). Comparison of the pairwise sequence identity between the *E. coli* decarboxylases shows that two pairs of proteins have very high residue conservation (sequence identity >60%): LdcI and LdcC, as well as, SpeF and SpeC (Figure 1B). The constitutive arginine decarboxylase SpeA and the inducible glutamate decarboxylases GadAB belong to different fold families of PLP-dependent decarboxylases^{12,37} and are, therefore, not included in the following examination. Furthermore, SpeA is found in the periplasm and not the cytoplasm.³⁸

A total of 199 sequences homologous to *E. coli* LdcI were obtained by BLAST analysis against the NCBI database of completed prokaryotic sequences, aligned by multiple sequence alignment, followed by generation of a phylogenetic tree (Figure 1C, see also the Supporting Information, Table 1). The sequences can be divided broadly into two major groups: Group 1 has longer sequences that contain an N-terminal Wing domain, and group 2 has shorter sequences that lack a Wing domain (Figure 1C, inset). There are also a few sequences that possess an extended N terminus that is not a Wing domain. The Wing domain belongs to the CheY-like response regulator receiver domain family of proteins^{12,16} and has been shown to be required for the formation of higher-order oligomers (decamers and dodecamers) (see below). Group 1 proteins are found almost exclusively in the Gram-negative Proteobacteria phylum, while group 2 is found in the mostly Gram-positive Firmicutes, Cyanobacteria, and Actinobacteria phyla. In addition to the lack of an N-terminal Wing domain, group 2 sequences also have three short deletions, two in SD4 and one in the CTD. On the basis of the sequence and structure of *E. coli* LdcI, these deletions are between D460 and G475 (loop region before and after strand β 17 in SD4), between S505 and S512 (sequence between β 19 and α 17 in SD4), and between E579 and V612 (part of α 21 and all of α 22 in CTD) (refer to Figure 1 of ref 16). These deletions are on the outer face of the protein and reduce the size of the SD4 and CTD.

The *E. coli* lysine, arginine, and ornithine decarboxylases fall into three separate branches of the phylogenetic tree within

group 1. An attempt was made to try to distinguish sequence features that might play a role in substrate selection between these enzymes; however, no obvious trends were observed. On the basis of the overall multiple sequence alignment, highly conserved residues (>70% identity) were found to be either components of the PLP binding site, located in structural elements that are part of the stabilizing interfaces between different subdomains of the protein, or involved in dimerization.

The ppGpp binding site of LdcI is found between neighboring dimers in the LdcI decamer¹⁶ and involves residues from all of the domains of the protein (Figure 2A). A portion of the alignment showing the residues of the ppGpp binding site in LdcI and the corresponding aligned residues in LdcC, AdiA, SpeF, SpeC, and *Lactobacillus* 30A OdCI is given in Figure 2A. On the basis of this alignment, we predicted that LdcC might be regulated by ppGpp in a manner similar to that of LdcI.

SpeF and SpeC are known to be regulated by GTP,²⁰ and a cocrystal structure of the *Lactobacillus* 30A OdCI bound to GTP has been determined (PDB ID: 1C4K).³⁹ There is a single GTP-binding site per monomer, found on the outer face of the PLP-SD (Figure 2B). On the basis of the full alignment of 199 sequences, residues involved in GTP binding were found predominantly in the group 1 Proteobacterial sequences and become more degenerate in the sequences of enzymes from other organisms. A portion of the alignment showing the GTP binding site residues from OdCI and the corresponding aligned residues in LdcI, LdcC, AdiA, SpeF, and SpeC is given in Figure 2B. On the basis of the alignment, it is difficult to predict what other decarboxylases will be regulated by GTP.

Effect of Nucleotides on the Activity of the Decarboxylases. To explicitly determine which decarboxylases are affected by the different nucleotides, the initial rate of activity of the various decarboxylases was measured over a range of pH values in the absence and presence of 100 μM GTP, GDP, ppGpp, or guanosine 3'-diphosphate, 5'-triphosphate (pppGpp) (Figure 3). The data for LdcI are reproduced from Kanjee et al.¹⁶ and are shown for completion. LdcI has a peak activity between pH 5.5 and pH 6.0, and the enzyme is unaffected by GTP or GDP. The presence of ppGpp results in robust inhibition from pH 5.5 to 8.5, while pppGpp inhibits over a narrower range from pH 6.5 to pH 8.5. LdcC alone has a peak activity at pH 7.5 and very little activity at pH values lower than 5.0. The LdcC activity is unaffected by the addition of GTP or GDP; however, there is strong inhibition observed for the protein from pH 5.0 to pH 9.5 in the presence of (p)ppGpp. Michaelis–Menten enzyme kinetics data were collected for LdcC over a range of lysine and ppGpp concentrations, and the resulting curves were fit to standard competition models (Supporting Information, Figure 1) giving a K_i value in the 100–500 nM range. The K_i values obtained are consistent with the K_i (and K_d) values measured for LdcI.¹⁶

In the case of AdiA, the enzyme alone had an activity maximum at pH 5.0; this activity drops off very dramatically at pH values higher than 6.0 and lower than 4.0 (Figure 3). Within the error of the measurements, AdiA is not inhibited by the addition of any of the nucleotides.

SpeF, with a pH optimum of 7.0, has a very low intrinsic decarboxylation activity, close to 100-fold lower than the activity of the other decarboxylases (Figure 3). The addition of GTP, GDP, or pppGpp results in a 10–12-fold activation of SpeF activity, while the addition of ppGpp results in only a 3-fold activation of the enzyme. Hence, the alarmone activates rather than inhibits SpeF.

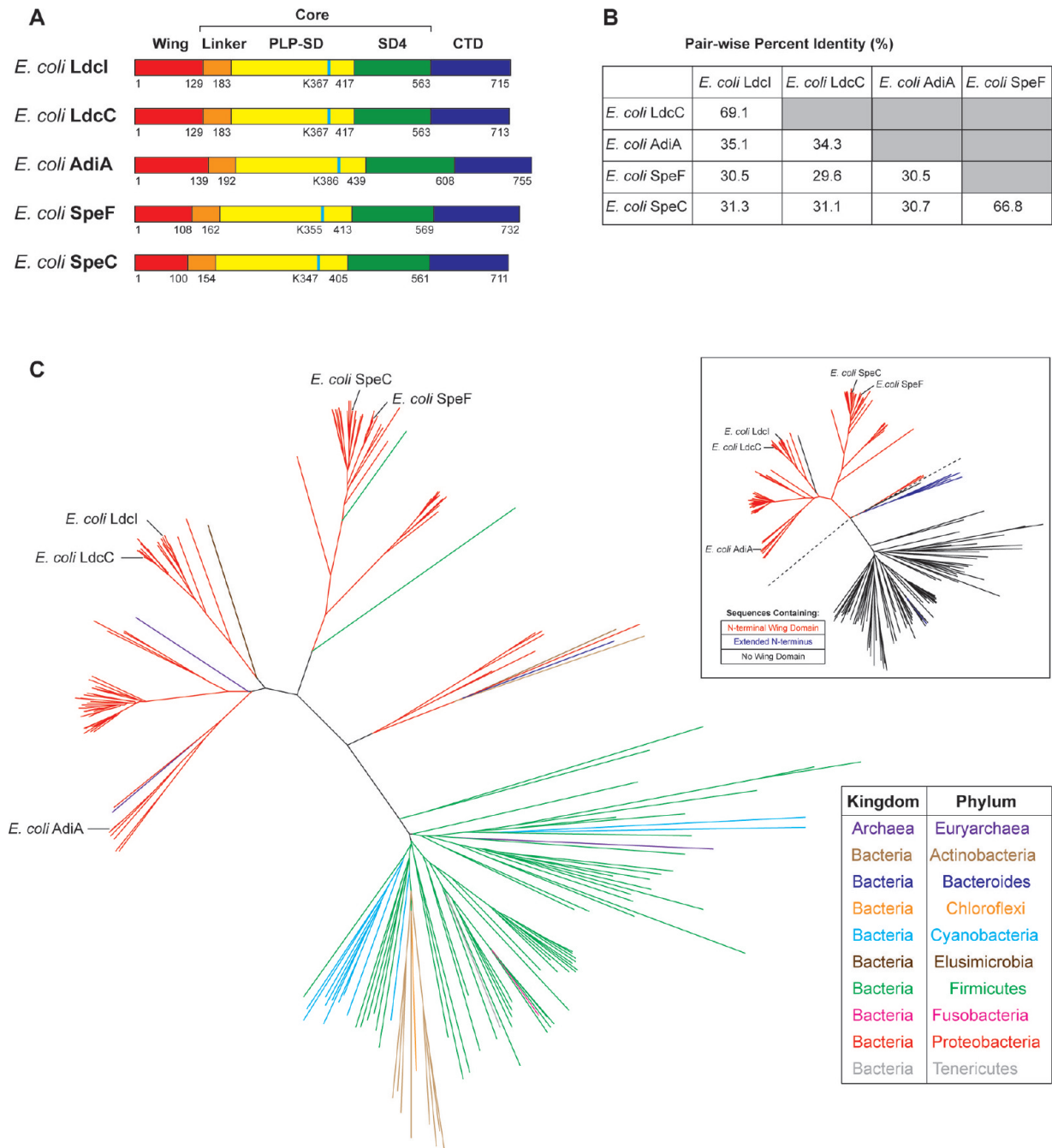


Figure 1. Comparison and phylogeny of the bacterial decarboxylases. (A) The protein domains for the *E. coli* LdcI, LdcC, AdiA, SpeF, and SpeC decarboxylases are indicated as follows: Wing domain, red; Core domain consisting of linker region, orange; PLP-SD, yellow; SD4, green; and the CTD, blue. The conserved lysine that forms the aldimine bond with PLP is indicated in cyan. (B) The percent identity of pairwise alignments between the decarboxylases is shown calculated using JALVIEW.³³ (C) A phylogenetic tree based on the amino acid sequences of 199 sequences homologous to *E. coli* LdcI. The tree has been colored according to the phylum of each species as indicated in the key, and the locations of the *E. coli* decarboxylases are shown directly on the tree. The inset shows the phylogenetic tree colored based on the presence (red, group 1) or absence (black, group 2) of the N-terminal Wing domain. Sequences colored blue have an extended N terminus that is not a Wing domain. The list of sequence accession numbers is included in the Supporting Information, Table 1.

The activity of SpeC alone peaks between pH 8.0 and 8.5 but drops off sharply at pH values higher than 9.5. Both GTP and GDP are capable of stimulating enzyme activity by approximately 50% at pH 8.0, as has been reported previously.²⁰ pppGpp does not have an observable effect on enzyme activity, while ppGpp is capable of inhibiting SpeC by 40% at pH 8.0. This inhibition is weaker than that observed for LdcI¹⁶ or LdcC under similar conditions.

To test whether ppGpp was able to regulate the activity of SpeF and SpeC in the presence of the activator GTP, the activities of the enzymes were measured over a range of ppGpp and GTP concentrations (Figure 4). For SpeF (at 400 nM), we observed a 2–4-fold increase in activity in the presence of increasing amounts of ppGpp from 50 to 1000 μ M (Figure 4A). In the presence of 50–1000 μ M GTP, the activity seems to be maximally saturated and did not increase beyond 6–8-fold. When the

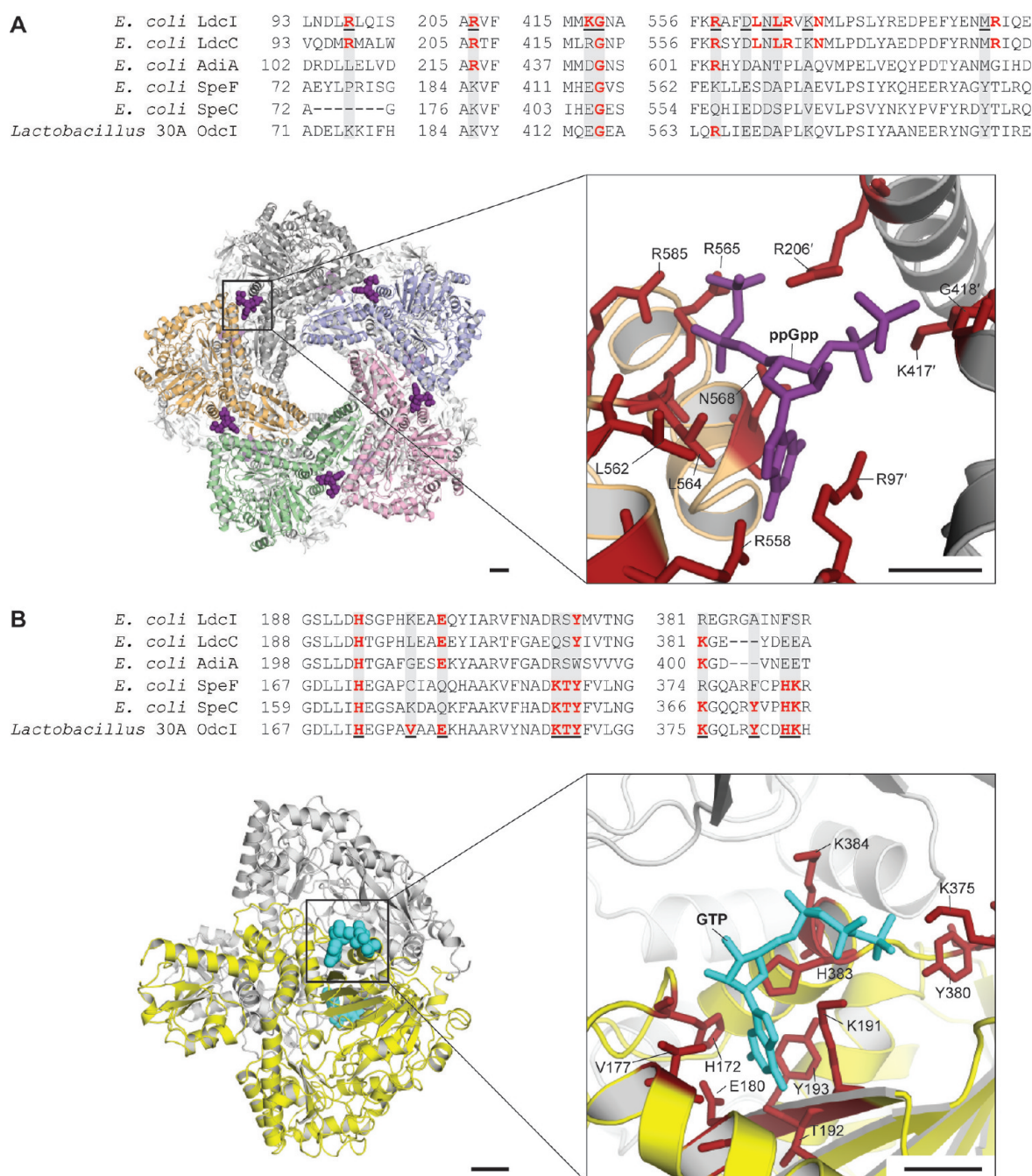


Figure 2. Binding of ppGpp and GTP to the decarboxylases. (A) Residues involved in binding ppGpp in *E. coli* LdcI are shown underlined, aligned to equivalent residues in *E. coli* LdcC, AdiA, SpeF, SpeC, and *Lactobacillus* 30A Odci. Conserved binding site residues are shown in red text. The top view of the *E. coli* LdcI decamer (PDB ID: 3N75) is shown with each of the top ring monomers shaded with a different color and the ppGpp molecules shown in purple. The inset shows the ppGpp binding pocket in LdcI between two protomers. The two subunits are colored tan and gray, and the ppGpp is colored purple; the residues in the binding site are colored red. (B) Residues involved in binding GTP in *Lactobacillus* 30A Odci are shown underlined, aligned to equivalent residues in *E. coli* LdcI, LdcC, AdiA, SpeF, and SpeC. Conserved binding site residues are shown in red text. A side view of the *Lactobacillus* 30A Odci dimer (PDB ID: 1C4K) with the bottom monomer colored yellow and the top monomer colored gray. The residues in the GTP-binding pocket from the structure (PDB ID: 1C4K) are shown in the inset. The PLP-SD is colored yellow, GTP is shown in cyan, and the residues in the binding site are colored red. The scale bars for the LdcI decamer and Odci dimer are 10 Å, and the scale bars for the ppGpp binding site and the GTP binding site are 5 Å.

concentration of GTP was fixed at 100 μ M and the concentration of ppGpp was varied from 50 to 1000 μ M, no significant inhibition or activation of the enzyme was observed, suggesting that ppGpp is ineffective at inhibiting (or activating) SpeF in the presence of GTP.

For SpeC (at 50 nM), adding increasing concentrations of ppGpp resulted in a 50–60% inhibition of activity, while the

addition of GTP resulted in a \sim 2-fold stimulation of activity (Figure 4B). Importantly, in the presence of 100 μ M GTP, the addition of 50–1000 μ M ppGpp resulted in inhibition of the enzyme to levels close to that observed for ppGpp alone. This indicates that ppGpp is capable of inhibiting SpeC even in the presence of GTP.

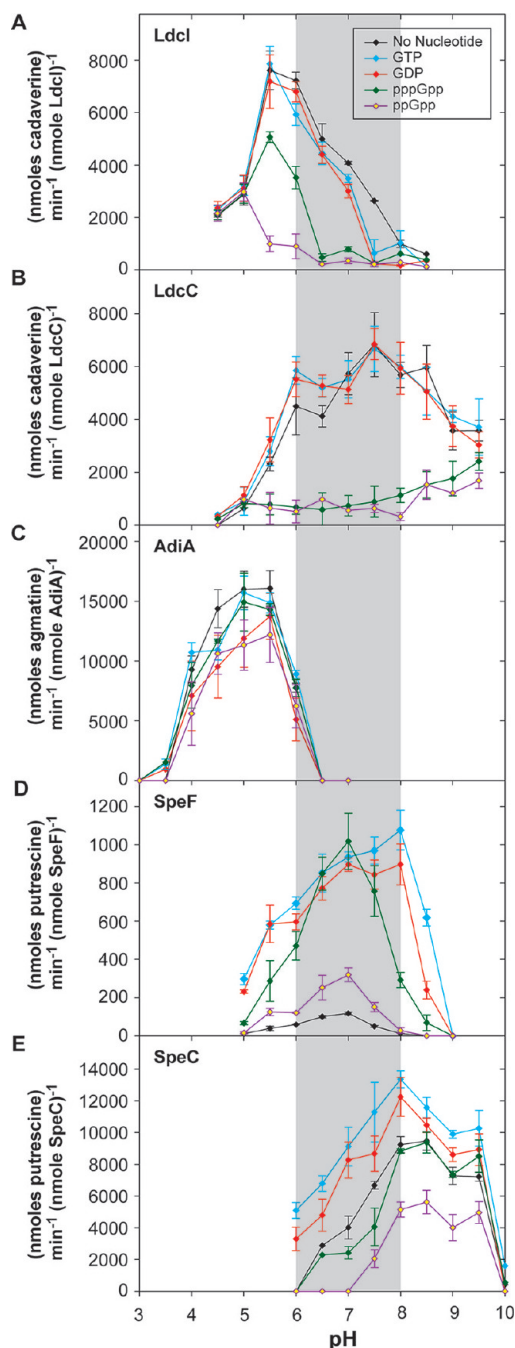


Figure 3. Effect of nucleotides on the enzymatic activity of the decarboxylases. The activities of 25 nM LdcI (A), 25 nM LdcC (B), 15 nM AdiA (C), 400 nM SpeF (D), and 50 nM SpeC (E) were determined over a range of pH values in the absence of nucleotide (black) and in the presence of 100 μ M each of GTP (cyan), GDP (red), pppGpp (green), and ppGpp (purple). The key in panel A applies to panels B–E. The error bars represent the standard error from a minimum of three repeats. The values for LdcI are from ref 16 and are shown for completion. The gray shading denotes the proposed “zone of inhibition” found between pH 6 and pH 8 (see the text).

To further investigate the binding of nucleotides to the ornithine decarboxylases, ITC binding experiments were performed with ppGpp and GTP. Under the conditions tested (15 μ M SpeF, 160 μ M GTP, pH 7.0 buffer), we did not observe measurable heat of binding between SpeF and ppGpp or GTP. By performing a Scatchard analysis on the SpeF

activation data from Figure 4A, we determined that the K_d for binding ppGpp is ~ 12.2 μ M per SpeF monomer. On the other hand, we were able to observe robust binding of ppGpp (Figure 4C) and GTP (Figure 4D) to SpeC. The binding stoichiometry for ppGpp indicates one binding site per dimer with a K_d close to 600 nM, while for GTP, the binding is more complex with at least two binding sites: one is of high affinity, K_d of 20.5 nM, and the other of lower affinity, K_d of 403.2 nM. For comparison, LdcI decamer binds ppGpp with similar affinity—There are five binding sites of high affinity, K_d of 12.8 nM, and five binding sites of lower affinity, K_d of 684.9 nM, per LdcI decamer.¹⁶ Hence, the different nucleotides have varying effects on the decarboxylases with ppGpp significantly inhibiting LdcI, LdcC, and SpeC and GTP/GDP activating SpeF and SpeC (Table 1).

Table 1. Summary of the Decarboxylase Interactions with ppGpp^a

protein	ppGpp interaction	GTP interaction	evidence
<i>E. coli</i> LdcI	+++	—	enzyme assays ^b , ITC ^b , X-ray crystallography ^b
<i>E. coli</i> LdcC	+++	—	enzyme assays ^b
<i>E. coli</i> AdiA	—	—	enzyme assays ^b
<i>E. coli</i> SpeF	+	+	enzyme assays ^c , Scatchard analysis ^c
<i>E. coli</i> SpeC	+++	+++	enzyme assays ^c , ITC ^c , X-ray crystallography ^d

^aThe ability of the proteins to interact with ppGpp is scored as strong (+++), weak (+), or no interaction (—), and the experimental evidence for these conclusions is indicated. ^bData are from ref 16. ^cThis work. ^dRef 39.

Effect of Oligomerization on the Enzymatic Activity of the Decarboxylases. The pODC subclass of Fold Type I PLP-dependent decarboxylases dimerize via their Core domains, and the dimerization is necessary to form a complete active site in each monomer.¹⁸ The Wing domain is important for mediating interaction between protein dimers and leads to decamer formation for LdcI,^{4,15,16} LdcC,¹⁵ and AdiA^{6,19} and dodecamer formation for the *Lactobacillus* 30A OdcI.²¹ On the other hand, *E. coli* SpeF and SpeC have generally been found as dimers.²⁰

The oligomerization of LdcI has been extensively characterized.^{4,15,16} LdcI is predominantly decameric at neutral pH and dissociates into dimers at high pH and low enzyme concentrations.^{15,16} At pH 5.0, the LdcI decamers are seen to associate into high molecular weight stacks of decamers, and this process is sensitive to the addition of ppGpp. The dimeric form of LdcI has lower specific activity than the decameric enzyme.¹⁶

Oligomerization of the LdcI paralogs was investigated over a range of pH values by size exclusion chromatography (SEC) at 1 μ M protein concentration and by negative stain electron microscopy (EM) at ~ 0.4 μ M protein concentration (Figure 5). The effect of the addition of 100 μ M ppGpp on enzyme oligomerization was investigated by EM. LdcC was found to be predominantly decameric by SEC (Figure 5A), with a greater proportion of dimers visible at pH 9.0 as compared to pH 6.0. By EM, LdcC was also predominantly decameric with very few dimers visible at any concentration (Figure 5B).

AdiA eluted as a decamer at pH 5.0 and 6.5 by SEC (Figure 5C), while at pH 3.5, no protein was eluted, possibly due to nonspecific interactions with the SEC bed matrix or aggregation of the protein at

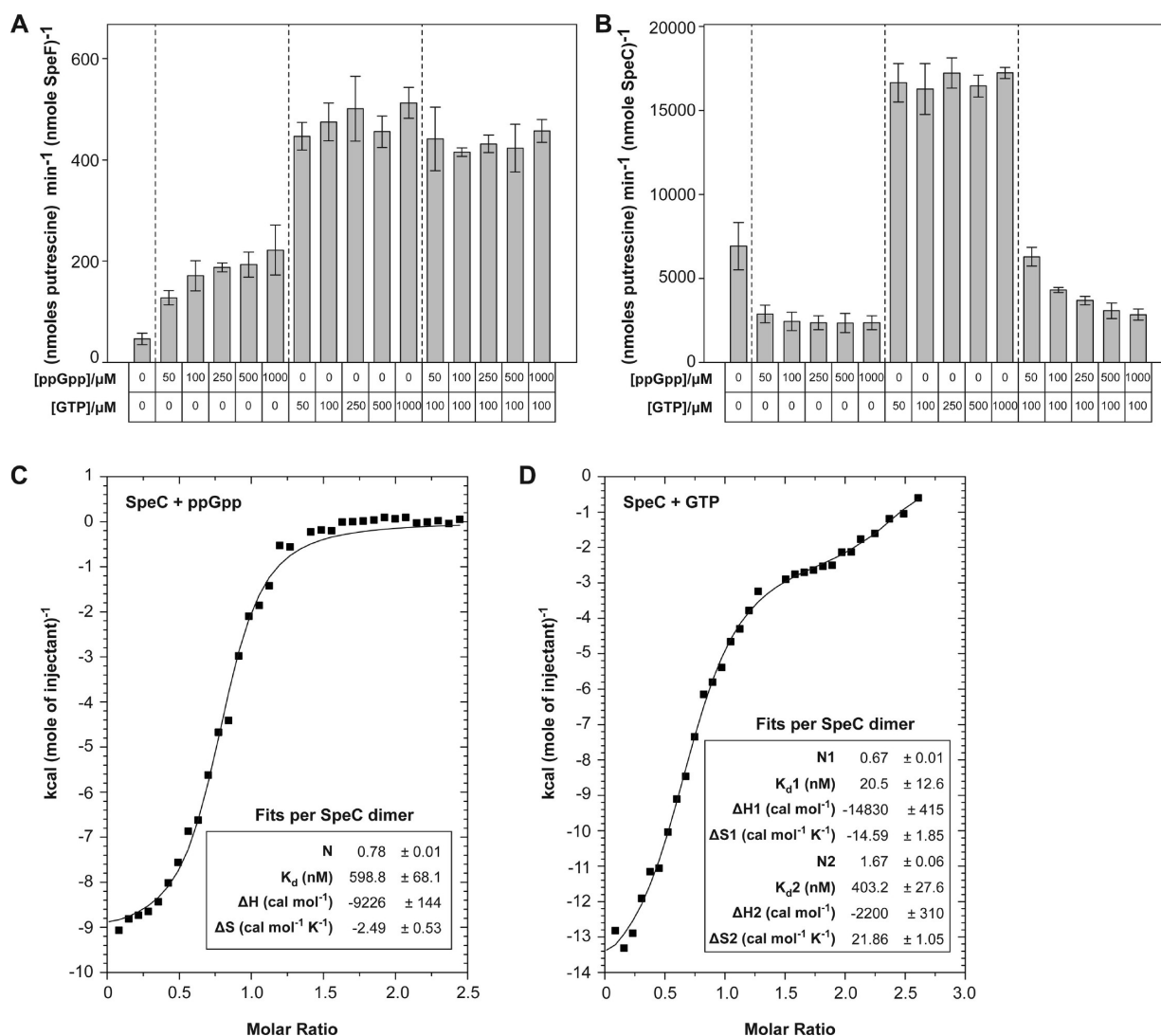


Figure 4. Modulation of SpeF and SpeC activity by ppGpp and GTP. The effect of ppGpp on ornithine decarboxylase activity in the presence of the activator GTP was tested at pH 7.0 for SpeF (A) and SpeC (B). The error bars represent the standard deviation from the measurement of at least six repeats. The binding of ppGpp (C) and GTP (D) to SpeC at pH 7.0 was measured using ITC. The insets show the derived number of binding sites (N), K_d , ΔH , and ΔS for each type of binding site.

this pH. Under EM conditions (Figure 5D), AdiA decamers were observed predominantly at pH 3.5 and 5.0, while at pH 6.5 a pronounced dissociation of AdiA decamers into dimers was visible.

SpeF at pH 7.0 and 8.5 (Figure 5E) and SpeC at pH 6.5, 8.0, and 9.0 (Figure 5G) eluted as dimers by SEC, while at pH 5.5, SpeF did not elute from the column, again possibly due to nonspecific interactions or due to aggregation. For both SpeF and SpeC, only dimers were observed under all conditions by EM (Figure 5F,H).

Finally, the addition of 100 μ M ppGpp did not affect the oligomerization of any of the decarboxylases under the EM conditions tested. Similarly, the addition of 100 μ M GTP to SpeF and SpeC did not change the oligomerization of these enzymes (data not shown).

DISCUSSION

The division of the bacterial pODC Fold Type I decarboxylases into a long-form group 1 class of enzymes containing a Wing domain and a short-form group 2 class of enzymes lacking a Wing domain very closely mirrors the phylogenetic division of

bacteria into Gram-positive (group 1 sequences) and Gram-negative (group 2 sequences) bacteria (Figure 1). The group 2 enzymes, which function as biosynthetic arginine decarboxylases,¹² are likely the ancestral form that through evolution acquired a CheY-like N-terminal Wing domain that is involved in higher-order oligomer formation and enzyme regulation.¹⁶

We recently determined the X-ray crystal structure of the *E. coli* inducible lysine decarboxylase LdcI, and we discovered that this protein was cocrystallized with the stringent response regulator ppGpp.¹⁶ The ppGpp binding site was found at the interface between neighboring monomers in the LdcI decamer, and binding of ppGpp leads to inhibition of LdcI activity in vitro and in vivo. By analyzing the ability of ppGpp to inhibit the paralogous *E. coli* decarboxylases, we intended to better understand the regulation of these enzymes during stringent response and acid stress response conditions. Table 1 shows a summary of the interactions between the ppGpp/GTP/GDP and the *E. coli* pODC decarboxylases.

On the basis of the sequence alignment of the pODC decarboxylases in *E. coli*, we expected to observe regulation of LdcC by ppGpp as this enzyme has the highest sequence identity

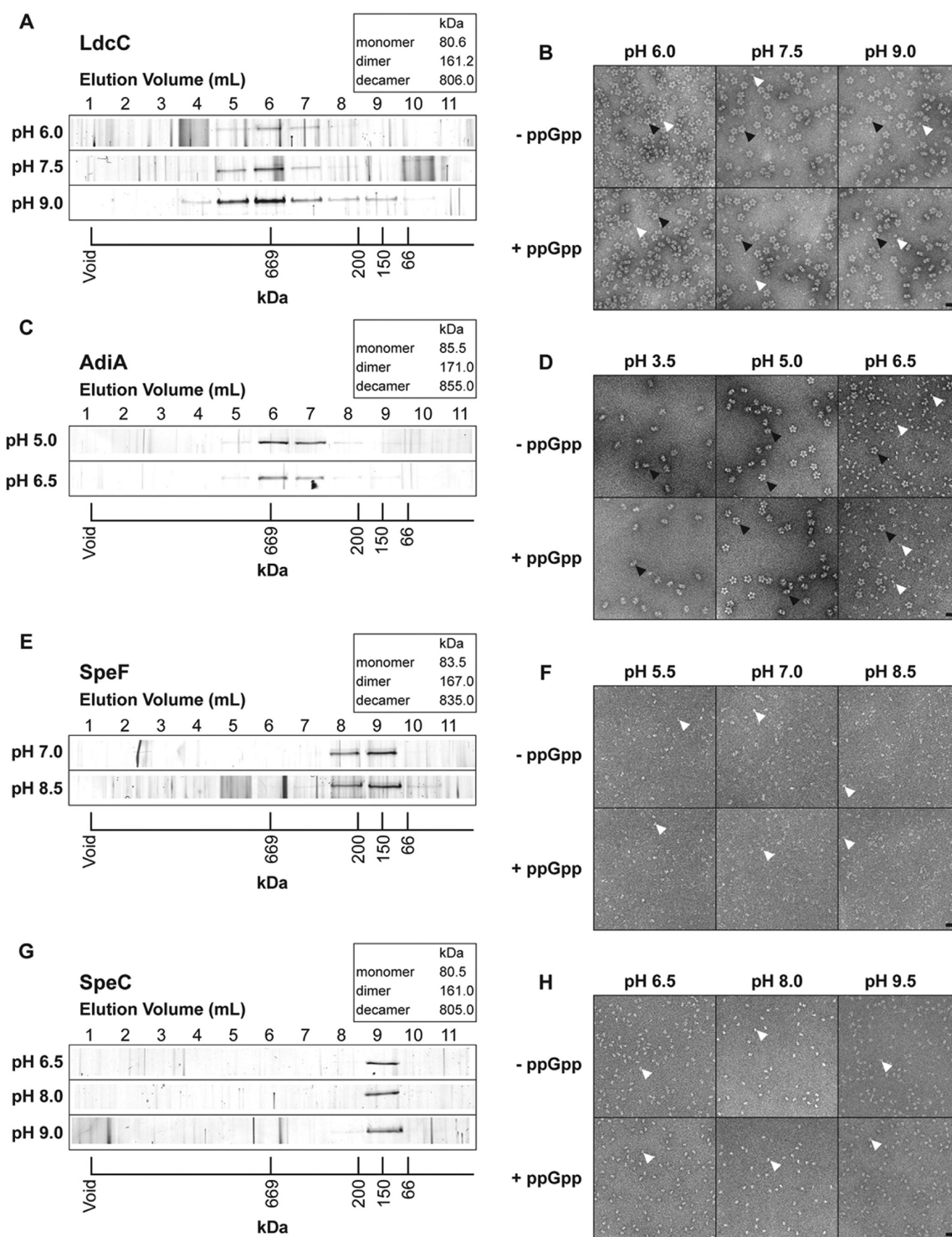


Figure 5. Effect of pH and ppGpp on the oligomerization of the decarboxylases. The oligomerization of the decarboxylases was investigated by SEC and negative stain electron microscopy. For SEC, 1 μ M LdcC (A), AdiA (C), SpeF (E), and SpeC (G) were separated at the indicated pH values on a Superose 6 HR 10/30 column. The top row indicates 1 mL elution fractions, and the positions of SEC molecular weight standards are indicated schematically along the bottom. Each panel represents a slice from a silver-stained polyacrylamide gel. The boxed table indicates the expected molecular mass in kDa for the monomer, dimer, and decamer of each protein. For negative strain EM, 400 nM LdcC (B), AdiA (D), SpeF (F), and SpeC (H) were prepared at the indicated pH values in the absence and presence of ppGpp. Black arrow heads point to decamers, and white arrow heads point to dimers. The scale bar is 20 nm.

to LdcI (69.1%, Figure 1B), has an almost complete conservation of ppGpp binding residues identified for LdcI (Figure 2A), and is found predominantly as a decamer (Figure 5A,B). As such, we observed strong inhibition of this enzyme over a wide range of pH

values with both ppGpp and pppGpp (Figure 3B). The next most likely enzyme to show inhibition by ppGpp was AdiA as there is reasonable conservation of ppGpp binding residues (Figure 2A) and the protein forms decamers below pH 5.0 (Figure 5C,D).

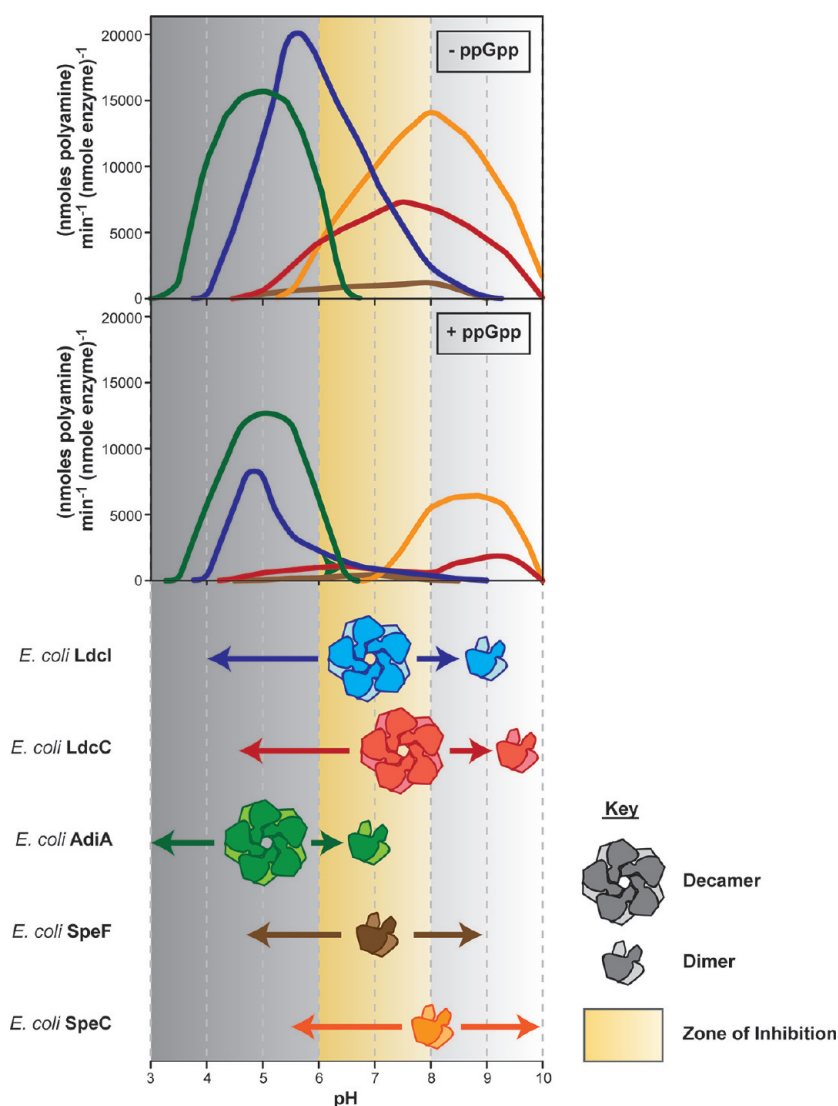


Figure 6. Model for the multiple mechanisms regulating the activity of the decarboxylases. Smoothed curves representing the specific activities of the five decarboxylases are shown for enzyme alone (LdcI, LdcC, and AdiA) and in the presence of GTP (SpeF and SpeC) (top panel) and for all of the enzymes in the presence of ppGpp (middle panel). The approximate oligomerization states for each decarboxylase are indicated as a function of pH in the bottom panel, with the decamer and dimer species indicated as shown in the key. The activity curves and oligomerization cartoons are colored based on the following scheme: LdcI, blue; LdcC, red; AdiA, green; SpeF, brown; and SpeC, orange. The yellow shaded area between pH 6 and pH 8 represents the zone of inhibition.

However, AdiA activity was found not to be regulated by ppGpp (Figure 3C) but rather seems to be regulated by oligomerization (Figure 5C,D).

The observation that SpeC is also inhibited by ppGpp (Figure 3E) was a surprise in light of the lack of conservation in the ppGpp binding residues (Figure 2A) and the exclusively dimeric state of the protein (Figure 5G,H). While inhibition of SpeC by ppGpp has been reported previously,⁴⁰ we believe that the inhibition mechanism is distinct from the one employed by LdcI and LdcC. SpeC differs from the lysine and arginine decarboxylases due to its activation by GTP (Figure 3E and Figure 4B) (also refer to ref 41), and there is a single distinct GTP binding site per SpeC monomer (two per homodimer) as seen in the X-ray crystal structure of Odcl from *Lactobacillus* 30A.³⁹ ppGpp is capable of inhibiting SpeC in the presence of GTP (both GTP and ppGpp bind with nanomolar affinity, Figure 4C,D), and we observe a single ppGpp binding site per SpeC dimer (Figure 5), suggesting that ppGpp and GTP either

have separate binding sites or that ppGpp and GTP compete for the same binding site but have opposite effects on enzyme activity.

SpeF has very low intrinsic activity and a much weaker binding to ppGpp as compared to SpeC and LdcI. SpeF shows activation by all guanosine nucleotides but less so with ppGpp.

When the enzyme inhibition profiles are analyzed in parallel, we observe a “zone of inhibition” between pH 6.0 and pH 8.0 (Figure 3 and shown schematically in Figure 6). Within this pH range, the cell uses multiple mechanisms to regulate the activities of the decarboxylases and, thus, the consumption of amino acids. In unstressed cells, the internal pH will be close to 7.0,⁴² and the constitutive decarboxylases SpeC and LdcC will be present. During amino acid starvation, ppGpp binds to and directly inhibits SpeC and LdcC, and this reduces the consumption of lysine and ornithine. However, the inhibition of SpeC is not as effective as for LdcC, possibly to ensure a continued production of the polyamine putrescine. During an

extreme acid stress response, with an external pH (pH_{ex}) between 2 and 3, the internal pH (pH_{in}) of the cell may drop to 4–5, which corresponds to the pH optimum of AdiA and the homologous GadA/B decarboxylases.⁴³ In the case of AdiA, the enzyme activity is regulated by dissociation of the active decamer into inactive dimers as the internal pH increases (Figure 5D), and by pH 6.5, AdiA is essentially inactive.⁴⁴ Under milder acid stress conditions (pH_{ex} 4–5), the internal pH of the cell remains close to neutral,⁴² but LdcI and SpeF are induced.^{16,17} The cell is able to regulate these enzymes either by ppGpp binding (LdcI) or through the change in concentration of GTP (SpeF) that occurs during the stringent response.²⁶

In conclusion, our data have shown that multiple strategies can be used to regulate the decarboxylases, either via inhibition by ppGpp (LdcI, LdcC, and SpeC), loss of the active decamer (AdiA under acid stress conditions and possibly LdcI and LdcC at high pH values), or through changes in the levels of GTP (SpeF and SpeC). While we have shown the regulation of these purified enzymes in vitro, complementary studies will further elucidate the existence of such an exquisite regulation within the cell.

■ ASSOCIATED CONTENT

● Supporting Information

Figure of enzyme kinetics of LdcC inhibition by ppGpp and table of sequences used in Figure 1C. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS USED

PLP, pyridoxal-5'-phosphate; ppGpp, guanosine 3',5'-bis(diphosphate); pppGpp, guanosine 3'-diphosphate, 5'-triphosphate; pODC, prokaryotic ornithine decarboxylase; ITC, isothermal titration calorimetry; SEC, size exclusion chromatography

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