Molecular Adaptation and Allostery in Plant Pantothenate Synthetases*\[^{a, b}\]

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Pantothenate synthetase catalyzes the ATP-dependent condensation of pantoate and \(\beta\)-alanine to yield pantothenate, the essential precursor to coenzyme A. Bacterial and plant pantothenate synthetases are dimeric enzymes that share significant sequence identity. Here we show that the two-step reaction mechanism of pantothenate synthetase is conserved between the enzymes from *Arabidopsis thaliana* and *Escherichia coli*. Strikingly, though, the *Arabidopsis* enzyme exhibits large allosteric effects, whereas the *Escherichia coli* enzyme displays essentially non-allosteric behavior. Our data suggest that specific subunit contacts were selected and maintained in the plant lineage of the pantothenate synthetase protein family and that the resulting allosteric interactions are balanced for efficient catalysis at low pantoate levels. This is supported by mutations in the putative subunit interface of *Arabidopsis* pantothenate synthetase, which strongly attenuated or otherwise modified its allosteric properties but did not affect the dimeric state of the enzyme. At the molecular level, plant pantothenate synthetases exemplify functional adaptation through allosteracy and without alterations to the active site architecture. We propose that the allosteric behavior confers a selective advantage in the context of the subcellular compartmentation of pantothenate biosynthesis in plants.

Pantothenate (vitamin B\(_5\)) is the essential precursor of coenzyme A (CoA) and acyl carrier protein, which are central to cellular metabolism. In bacteria, pantothenate is synthesized in four steps from ketoisovalerate and aspartate (1). Key enzymes of this pathway are conserved in plants and fungi but not in animals (2), and the latter must obtain pantothenate from their diet. The pantothenate biosynthetic pathway was shown to be compartmentalized in *Arabidopsis thaliana*, where synthesis of the pantothenate precursor ketopantoate takes place in the mitochondria, whereas pantothenate synthetase (PS)\(^{2}\) is found in the cytosol (3). PS converts pantoate, \(\beta\)-alanine, and ATP into pantothenate, AMP, and pyrophosphate (PP\(_i\)). The reaction was suggested to proceed in two steps via an enzyme-bound pantoaryl adenylate intermediate as shown in Scheme 1. Pantoyl adenylate is formed in the first half reaction from ATP and pantoate and reacts with \(\beta\)-alanine to give pantothenate and AMP in the second half reaction (4). Pantoyl adenylate is subject to rapid hydrolysis in solution, but stable analogs could be demonstrated as kinetically competent intermediates of PS from *Escherichia coli* (5). The kinetic mechanism of PS from *E. coli* (6) and *Mycobacterium tuberculosis* (7) was also consistent with Scheme 1, with binding of ATP preceding binding of pantoate and release of pantothenate preceding release of AMP. The crystal structure of *E. coli* PS (8) showed that the enzyme is a homodimer. Each subunit consists of two well-defined domains that are connected by a flexible hinge region. The active site is found in the N-terminal domain, which forms a Rossmann fold, and structural elements involved in dimerization form an insertion in this domain. *M. tuberculosis* PS was crystallized in the presence of various substrates, and the resulting structures confirmed pantoyl adenylate as the reaction intermediate that is protected from hydrolysis by tight binding within the active site cavity (9, 10). We previously identified PS from *Lotus japonicus*, which was, in agreement with bacterial PS, a dimeric enzyme (11). However, the *L. japonicus* enzyme showed strong substrate inhibition by pantoate (Pt; Scheme 1), whereas PS from *E. coli* and *M. tuberculosis* followed hyperbolic kinetics throughout (6, 7).

PS is conserved across bacterial and eukaryotic organisms, and *E. coli* PS shows greater than 50 and 40% sequence identity with plant and fungal homologues, respectively (2). PS belongs to the HIGH superfamily of nucleotidyltransferases and is, thus, related to class I aminocyl-tRNA synthetases (12). Aminocyl-tRNA synthetases attach amino acids to their cognate tRNAs and in a two-step process by ATP activation of the amino acid and subsequent transfer to the tRNA (13). The first half-reactions of PS and aminocyl-tRNA synthetase are analogous in that both enzymes utilize ATP to form an acyl adenylate intermediate and release PP\(_i\).

Biosynthesis of CoA occurs in nearly all organisms. There is evidence that the synthesis of CoA from pantothenate is mainly controlled by pantothenate kinase, *i.e.* at the step after PS in the CoA biosynthetic pathway. Pantothenate kinase is feedback-inhibited by CoA or CoA thioesters in bacteria, fungi, plants, and animals, and this property is thought to constitute a universal mechanism for the control of intracellular CoA levels (14). *E. coli* produces pantothenate in large excess over the amount incorporated into CoA, and excess pantothenate is excreted into the medium, suggesting that pantothenate biosynthesis proceeds largely unregulated (15). The regulation of

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\(^{b}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 and Table 1.

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2 The abbreviations used are: PS, pantothenate synthetase; ATP, A. thaliana PS; MES, 4-morpholinooethanesulfonic acid; AMP, 2-amino-2-methyl-1-propanol; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; ORF, open reading frame; Pt, pantoate.
Expression constructs for wildtype and mutagenized AtPS were yield expression constructs for AtPS-E132A and AtPS-Mut1 (extension PCR (19) using appropriate mutagenesis primers to GAGACA-3 derived from a previously described cDNA (3) that correspond to the single Arabidopsis gene encoding PS (At5g48840) in the ecotype Landsberg erecta. The AtPS open reading frame (ORF) was amplified from the cDNA using Pfu polymerase (Stratagene) and the primers 5'-CGGCGCATATGAGAAGTAATCA-3' and 5'-CGCGGTCGACTTAGAGAGACATT-3'. The AtPS ORF was mutagenized by overlap extension PCR (19) using appropriate mutagenesis primers to yield expression constructs for AtPS-E132A and AtPS-Mut1 (cf. Fig. 1B). E. coli PS is encoded by the panC gene, and the panC ORF was amplified from genomic DNA of E. coli strain XL1Blue (Stratagene) using the primers 5'-CGGCGGCTCAGGATTTCCG-GAGACA-3' and 5'-CGGCGCGCTCGAGTTACGC-3'. To remove an internal NdeI restriction site, a silent A→G mutation was introduced at position 112 of the panC ORF by overlap extension PCR. The amplified ORFs for AtPS, AtPS mutants, and E. coli PS were subcloned via NdeI and Sall (AtPS) or NdeI and Xhol (E. coli PS) restriction sites into the pET28-a vector (Novagen). The resulting plasmids contain the respective ORFs in translational fusion with the vector-encoded N-terminal His tag, leading to the expression of, for example, NH2-MGSSHHHHHH-HSLGLVPRGSH-AtPS. After subcloning into pET28-a, the complete nucleotide sequences of the respective wild type and mutagenized ORFs were verified by automated DNA sequencing. DNA sequencing also revealed a G at position 432 of the Landsberg erecta AtPS ORF, whereas the AtPS ORF in the Columbia ecotype has a T at this position (gi:30695550 or gi:51968617). This non-silent polymorphism causes the Columbia and Landsberg erecta alleles of the gene for AtPS to encode Phe and Leu at amino acid 144, respectively. Inspection of the Monsanto Landsberg erecta sequence collection confirmed this polymorphism.

Expression and Purification of PS Enzymes—AtPS and E. coli PS were expressed in E. coli BL21(DE3) cells (Stratagene) and purified on nickel-nitrilotriacetic acid-agarose (Qiagen) using standard procedures. After affinity chromatography, PS proteins were dialyzed exhaustively against 50 mM Tris-HCl (pH 8.0), 0.1 mM dithiotheriol, frozen in liquid N2, and stored in aliquots at −70 °C, where no loss of activity occurred within 6 months. Subunit and native molecular weights of affinity-purified PS proteins were estimated by SDS-PAGE and gel filtration chromatography, respectively. Briefly, a HiLoad 16/60 Superdex 200 column was equilibrated in 50 mM Tris-HCl (pH 8.0), 100 mM KCl. PS proteins and standards for calibration (Sigma) were eluted in the same buffer at 0.5 ml/min. Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin as standard.

Purified AtPS was digested with thrombin (4 NIH units/mg of AtPS) or E. coli BL21(DE3) cells containing the CarA plasmid (Stratagene) and purified on nickel-nitrilotriacetic acid-agarose (Qiagen) using standard procedures. After affinity chromatography, PS proteins were dialyzed exhaustively against 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2.5 mM CaCl2 for 1 h at 25 °C. The sample was dialyzed against 50 mM Tris-HCl (pH 8.8), 1 mM phenylmethylsulfonyl fluoride and then loaded onto a Mono Q anion exchange column equilibrated in 50 mM Tris-HCl (pH 8.8). Proteins were eluted in a linear 0–0.5 M KCl gradient. Fractions containing AtPS were pooled, dialyzed against 50 mM Tris-HCl (pH 8.0), 0.1 mM dithiotheriol, and stored at −70 °C. Removal of the N-terminal His tag was confirmed by SDS-PAGE.

Enzyme Assays—Initial rate data for AtPS and AtPS mutants were obtained using a spectrophotometric, coupled assay procedure (11). The standard assay contained 100 mM Tris-HCl (pH 8.0), 10 mM MgSO4, 10 mM (NH4)2SO4 (from coupling enzymes), 10 mM ATP, 0.5 mM pantoate, 5 mM β-alanine, 1 mM potassium phosphoenolpyruvate, 0.3 mM NADH, myokinase (8 units), pyruvate kinase (12 units), lactate dehydrogenase (12 units) (Sigma), and AtPS (20 μg) in a final volume of 1 ml. The reaction was initiated by the addition of the variable substrate after preincubation at 25 °C for 15 min. Initial velocities were determined for 5–10 min at 25 °C using a Beckman DU 640 spectrophotometer with a circulating water bath. In this coupled assay, two mole-
cules of NADH ($e_{340} = 6220 \text{ m}^{-1} \text{ cm}^{-1}$) were oxidized per molecule of pantothenic acid formed. The purified PS enzymes used in this study gave no background activity when one of the three substrates was absent. Potassium D-pantoate was prepared by hydrolysis of D-pantoyl lactone (Aldrich) using KOH (King et al. 20)). The coupled assay was linear with the amount of AtPS, and the addition of up to 100 mM KCl or 5 mM pantoyl lactone to the standard assay had essentially no effect on AtPS activity, indicating that pantoyl lactone or high concentrations of K$^+$ are not inhibitory.

Alternatively, PS was assayed by monitoring the incorporation of β-[3-14C]alanine into [14C]pantothenate. This isotopic assay contained 100 mM buffer (MES-NaOH (pH 5.5), 10 mM AMP, 10 mM pantoyl lactate, 0.32 mM β-alanine, 0.18 mM β-[3-14C]alanine (55 mCi/mmol), and 0.05 μg/μl ATP or E. coli PS. The assay was initiated by the addition of pantoyl lactate and 5-μl aliquots were removed from the reaction at 0.5-, 1-, 2-, 3-, 4-, 5-, and 6-min time points, combined with 2 volumes of acetone, and immediately frozen in liquid N$_2$. Precipitated protein was removed by centrifugation, and 17-nCi aliquots of the reaction products were spotted onto silica gel 60 F$_{254}$ thin layer chromatography (TLC) plates. Pantoyl lactate and β-alanine were separated using dioxane, 25% NH$_3$, H$_2$O (9:1:4) as a mobile phase. The amounts of 14C label associated with β-alanine and pantoyl lactate were quantitated using a Storm 860 PhosphorImager (GE Healthcare). Quantitation of the β-[3-14C]alanine standard was linear between 0.1 and 20 nCi. Initial rates were calculated from progress curves of pantoyl lactate formation corrected for non-enzymatic activity by using the "exact" numerical method described in Cornish-Bowden (21).

The standard assay of the pantoyl lactate:β-alanine isotope exchange was carried out at 25 °C and contained 100 mM MES-NaOH (pH 5.5), 10 mM AMP, 10 mM pantoyl lactate, 0.32 mM β-alanine, 0.18 mM β-[3-14C]alanine (55 mCi/mmol), and 0.05 μg/μl ATP or E. coli PS. The assay was initiated by the addition of pantoyl lactate, and 5-μl aliquots were removed from the reaction at 1-, 3-, 6-, 9-, 12-, and 24-h time points. 14C label associated with pantoyl lactate and β-alanine in 17-nCi aliquots was determined as described above and corrected for non-enzymatic activity. The pH dependence of the exchange reaction catalyzed by AtPS or E. coli PS was studied by replacing MES-NaOH (pH 5.5) by an equimolar amount of sodium acetate (pH 4.5–5.0), MES-NaOH (pH 5.0–6.5), or Tris-HCl (pH 6.5–9.0).

Steady-state kinetic Analysis—Initial rate data were obtained using the coupled assay at variable pantoyl lactate (0.01–20 mM) or β-alanine (0.02–10 mM) concentrations and at different fixed ATP levels (1–10 mM). Initial velocity data were fitted to four rate equations by least-squares non-linear regression. The rate equations used were the Michaelis-Menten equation (Equation 1), the Hill equation (Equation 2), the rate equation for substrate inhibition resulting from an enzyme-substrate dead-end complex ( Equation 3) (22), and a generalized rate equation in the form of a 2:2 rational polynomial (Equation 4) (23),

$$v_0 = \frac{V_{\text{max}}[S]}{K_m + [S]} \quad \text{(Eq. 1)}$$

The true maximum velocity ($v_{\text{max}}$) is the velocity defined by Equation 4 when $[S] = [S]_{0}$, opt. The non-inhibitory and, if applicable, inhibitory substrate concentrations at which $v_0 = 0.5 v_{\text{max}}$ are defined by Equation 6,

$$[S]_{0} = \frac{1}{\alpha_1 - \beta_1 \pm \sqrt{(\beta_1 / 2)^2 - \alpha_1}}$$

where $v_0 = 0.5 v_{\text{max}}$ and rearranging. The extra-sum-of-squares F test ($\alpha = 0.05$) was used to determine which rate law among Equations 1–4 was most likely to describe the observed velocity data.

Exchange velocities for the transfer of β-[3-14C]alanine into pantoyl lactone were calculated using Equation 7 (22),

$$v^* = \left( \frac{[A][P]}{[A] + [P]} \right) \left( \frac{1}{t} \right) \ln(1 - F) \quad \text{(Eq. 7)}$$

where $v^*$ is the initial exchange velocity, [A] and [P] are the concentrations of β-alanine and pantoyl lactone, respectively, and $F$ is the fraction of isotopic equilibrium attained at time $t$. Because there is no label in pantoyl lactone at time 0, $F$ is given by the ratio $a/a_\infty$, where $a$ and $a_\infty$ are the fractions of 14C label in pantoyl lactone at time $t$ and at isotopic equilibrium, respectively. Plots of $\ln(1 - F)$ versus $t$ were linear, and the slope was used to obtain $v^*$.Alignment of PS Protein Sequences—GenBank$^\text{TM}$ was searched for plant ESTs encoding full-length or partial homologues to AtPS (gi:15239721) using the tblast program (E-value cutoff 10$^{-50}$) (24). EST-encoded amino acid sequences with homology to AtPS that were supported by at least two independent EST sequences were aligned with annotated PS
sequences from plants, algae, fungi, and bacteria by using the ClustalX program (Version 1.82) (25) with the Gonnet weight matrix and penalties for gap opening and extension of 10.0 and 0.2, respectively.

RESULTS

Conservation of Active Site Residues and Dimerization Contacts in the PS Protein Family—Crystal structural analyses of PS from *E. coli* and *M. tuberculosis* have previously afforded a detailed picture of the active site and the dimerization in these enzymes. Fifteen active site residues with various functions in catalysis were identified in the liganded structures of *M. tuberculosis* PS (9, 10), and six of these active site residues were confirmed by kinetic analysis of the respective Ala mutants (26). The structure of *E. coli* PS (8) revealed 11 residues specifically involved in forming dimerization contacts, and the key structural elements of dimerization are conserved between *E. coli* and *M. tuberculosis* PS. We mapped these functionally important residues onto an amino acid sequence alignment containing PS homologues from bacteria, fungi, and plants (Fig. 1A). In this alignment, all active site residues of *M. tuberculosis* PS are strictly conserved, indicating that bacterial and eukaryotic pantothenate synthetases share an identical reaction mechanism. Although none of the residues involved in the dimerization of *E. coli* PS is fully conserved, the majority of these residues are functionally conserved between plants, fungi, and *E. coli* (Trp-134, Ile-135, Arg-154, Asn-165, Asp-191, Leu-192, and Phe-194 in AtPS). This suggests that the overall design of the dimer interface is largely conserved in the PS protein family. Among the remaining unconserved residues at the dimer interface, two positions in AtPS (Glu-132 and Arg-136) are conserved in plants but distinct from the corresponding residues in fungi or bacteria, and this pattern can be taken as an indication that these residues are involved in specialized subunit interactions occurring only in plant pantothenate synthetases. The alignment in Fig. 1A also shows that PS sequences from plants and euascomycete fungi contain specific insertions relative to *E. coli* PS, respectively.

To reveal the structure of the plant-specific insertion, which was previously noted to precede the dimerization domain of *E. coli* PS (8), we re-aligned the residues in the dimerization region that are situated between the β3 and the α3 structural elements of *E. coli* PS (8). With a view to broaden the basis of this analysis, EST-encoded, partial PS sequences from a range of angio- and gymnosperm plants were included in the alignment. Additionally, we identified a cDNA clone for PS in an EST library of the bryophyte Physcomitrella patens (27) and obtained the full-length sequence (gi:84871839). Finally, the alignment includes gene models for PS from the chlorophyte Chlamydomonas reinhardtii and the rhodophyte Cyanidioschyzon merolae (28). The resulting alignment (Fig. 1B) shows that the insertion in plant PS sequences is framed by the structural elements αD and βD. The insertion varies relative to *E. coli* PS between 15 amino acids in *Allium cepa* and 32 amino acids in Saruma henryi and is absent from all known bacterial or fungal PS homologues. The inserted residues are poorly conserved except for the 131HETWIRVER139 motif. The alignment also reveals the conserved motif 131HETWIRVER139, which is present in PS sequences from plants and algae but not outside the Viridiplantae kingdom. This motif coaligns with the βD strand, which forms an intersubunit antiparallel β sheet and is central to the dimerization of bacterial PS (8, 9). It is possible, therefore, that the 131HETWIRVER139 motif is equally important for the dimerization of plant pantothenate synthetases. Furthermore, it is noteworthy, that the 131HETWIRVER139 motif contains basic residues (His-131 and Arg-139) and acidic residues (Glu-132 and Glu-138) in a symmetrical fashion because repulsion between like-charged residues flanking the predicted βD strand may have a role in intersubunit communication. We did not detect analogous symmetrical arrangements of like-charged residues in non-plant PS sequences.

To evaluate the functional role of the divergent dimerization insertion in plant pantothenate synthetases, we constructed two AtPS mutants and compared their catalytic properties with those of wild type AtPS (see below). In one of the mutants (AtPS-Mut1), the bulk of the insertion occurring between the strictly conserved Pro-97 and the putative βD strand (Trp-134—Arg-136) was deleted and replaced with the corresponding residues from *E. coli* PS (Fig. 1B), creating a mutant enzyme that is shorter than wild type AtPS by 22 residues. The second mutant (AtPS-E132A) carries a single amino acid exchange (Glu-132 replaced by Ala) that eliminates the symmetry of negative charges in the 131HETWIRVER139 motif. Also, the amino acid sequence alignment in Fig. 1A implied that Glu-132 may be important for divergent subunit interactions in AtPS and other plant pantothenate synthetases (see above).

Evidence for a Conserved Reaction Mechanism between A. thaliana PS and *E. coli* PS—AtPS, AtPS mutants, and *E. coli* PS were overproduced in *E. coli* as N-terminal His-tagged proteins and had subunit molecular masses in good agreement with their predicted sizes (34–36 kDa by SDS-PAGE). The native molecular weights of these enzymes were estimated by gel filtration and lay between 68,000 and 73,000, indicating that the enzymes were dimers in solution. This result also shows that the mutations in the dimerization region of AtPS did not affect the dimeric subunit structure. AtPS was digested with thrombin and had very similar activity with or without the His tag, indicating that the His tag does not interfere with catalysis. This is supported by the structure of *M. tuberculosis* PS, which showed that the N and C termini are away from the active site cavity and unlikely to affect pantothenate synthesis (9). Interestingly, however, we were not able to obtain *E. coli* PS in an active form when the enzyme was fused to a C-terminal His tag. Consequently, all steady-state kinetic experiments reported below were carried out using N-terminal His-tagged PS enzymes.

Based on the conservation of catalytic residues, AtPS is expected to catalyze the formation of pantothenate by the same reaction mechanism that was previously established for bacterial PS (Scheme 1). To obtain experimental evidence supporting this prediction, we compared AtPS and *E. coli* PS with respect to the pH dependence of two reactions, the formation of pantothenate and the isotope exchange between pantothenate and β-alanine. The activity of AtPS and *E. coli* PS in the forward direction was assayed between pH 5.5 and 11.0 by monitoring the incorporation of β-[1^4^C]alanine into pantothenate. For this reaction AtPS and *E. coli* PS had the same optimum pH of 9.0 in...
Tris buffer (Fig. 2, A and B), which is close to the previously reported optimum for E. coli PS (pH 10, Miyatake et al. (6)). At optimum pH AtPS and E. coli PS had turnover rates of 0.5 and 2.0 s\(^{-1}\) in this assay, respectively. More importantly, the enzymes had little or no activity at neutral or acidic pH, and the largest change of activity occurred between pH 7.5 and 8.0. Thus, the forward rates of both AtPS and E. coli PS depend on the basic form of an anionizable group of pKa between 7.5 and 8.

FIGURE 1. Multiple sequence alignment of PS proteins from plants, algae, fungi, and bacteria. A, mapping of residues with established functional roles in PS from E. coli or M. tuberculosis. Active site residues ( ), with various functions in the catalytic cycle, residues involved in dimerization ( ), the HIGH and KMSKS sequence motifs that characterize the HIGH superfamily of nucleotidyltransferases, and the start of the C-terminal domain ( ) were previously identified by crystal structural analysis of E. coli PS (8) and M. tuberculosis PS (9, 10). The secondary structural elements of E. coli and M. tuberculosis PS are highlighted in black and numbered according to von Delft et al. (8). Asterisks indicate strictly conserved positions. Colors and periods indicate full conservation of strong and weak groups, respectively. B, re-alignment of the residues in the dimerization region. The SCVE\(_1\) and HETWIRVER\(_2\) motifs from PS1, which are conserved in plant PS sequences, are highlighted in gray. The two AtPS mutants analyzed in this study, AtPS-E132A and AtPS-Mut1, are indicated. The alignments in A and B contain annotated PS sequences from A. thaliana var. Columbia (gi:15239721), L. japonicus (gi:2292921), Oryza sativa (gi:50920129), M. tuberculosis (gi:15610738), and E. coli (gi:16128126). Also included are conceptual translations of full-length cDNAs for PS from Triticum aestivum (gi:32128941) and P. patens (gi:84871839). Algal PS sequences correspond to gene models from C. reinhardtii (e.g., gw01715313) and available at www.jgi.doe.gov and C. merolae (CMC138C (28)). The remaining plant PS sequences were derived from public EST sequences as described under “Materials and Methods.”
The second reaction used to probe the reaction mechanism of AtPS was the isotope exchange between pantothenate and $\beta$-[14C]alanine. According to the established kinetic mechanism of bacterial PS, this exchange reaction should be dependent on AMP as was previously demonstrated for PS from *M. tuberculosis* (7). We found that both AtPS and *E. coli* PS catalyze the exchange between $\beta$-[14C]alanine and pantothenate in the presence of AMP with a pH optimum of 5.5 (Fig. 2, C and D). Again, the pH profile was very similar in both enzymes and indicated that the pantothenate-$\beta$-alanine exchange rate depends on the acidic form of an ionizable group of $pK_a$ between 6 and 7. At the pH optimum, similar isotope exchange rates ($5 \to 6 \times 10^{-2} \text{ min}^{-1}$) were observed for AtPS and *E. coli* PS, respectively. A 10-fold lower exchange rate was reported for *M. tuberculosis* PS (7), presumably because of the suboptimal pH of 7.8 employed there.

When AMP was removed from the exchange reaction or replaced with an equimolar amount of cAMP, the exchange rates of both AtPS and *E. coli* PS were reduced by $\sim\text{50- or 20-fold}$, respectively (Fig. 3). Thus, hydrolysis and re-synthesis of the amide bond in pantothenate depend on AMP. We used cAMP as a non-reactive analog of AMP, and the inability of cAMP to promote the pantothenate-$\beta$-alanine exchange supports the direct involvement of AMP in the catalytic process. In summary, the behavior of AtPS and *E. coli* PS supports the view that these enzymes share an identical two-step reaction mechanism with pantoyl adenylate as the key reaction intermediate as shown in Scheme 1. Furthermore, the great similarity between the pH profiles of AtPS and *E. coli* PS is fully consistent with conserved catalytic residues involved in the rate-limiting steps of the forward and reverse exchange reactions, respectively.

**Allosteric Properties of AtPS**—The Bi Uni Uni Bi kinetic mechanism of *E. coli* PS requires that the enzyme obeys Michaelis-Menten behavior with each of its substrates, i.e. ATP, pantoate, and $\beta$-alanine (Scheme 1). However, despite the above evidence for a conserved reaction mechanism, initial rate kinetic analysis revealed that AtPS deviates from Michaelis-Menten kinetics on several accounts.

Initial rates of AtPS were obtained using the coupled enzyme assay and either pantoate or $\beta$-alanine as the variable substrate at different fixed ATP concentrations. In the absence of a rate equation that can globally explain allosteric effects in a terreactant enzyme such as AtPS, each substrate-velocity curve was analyzed separately. Individual sets of rate data were fitted to the Michaelis-Menten equation, the Hill equation, an equation for substrate inhibition resulting from dead-end complexes, and a generalized rate equation in the form of a 2:2 rational function (Equations 1–4). The rate law most likely to explain the rate data was selected for each substrate-velocity curve (Fig. 2).

![FIGURE 2. The effect of pH on PS from *A. thaliana* and *E. coli*. A and B, pH dependence of pantothenate synthesis (forward direction). The enzymes were assayed by monitoring the incorporation of $\beta$-[14C]alanine into pantothenate in the presence of 10 mM ATP, 0.5 mM pantoate, and 0.5 mM $\beta$-alanine. C and D, pH dependence of the isotope exchange between $\beta$-[14C]alanine and pantothenate. The exchange reaction was assayed in the presence of 0.5 mM $\beta$-alanine, 10 mM pantothenate, and 10 mM AMP. MES-NaOH (●), Tris-HCl (○), AMP-HCl (□), CAPS-NaOH (▲), or sodium acetate (▲) buffers were present at a concentration of 100 mM, and the final pH of each assay mixture was verified. AtPS, *A. thaliana* PS; EcPS, *E. coli* PS; $v_0$, initial velocity; $v^*$, initial exchange rate; [E], concentration of AtPS or *E. coli* PS subunits.](Image)
test, $\alpha = 0.05$), enabling the detection of different types of kinetic behavior and the estimation of apparent kinetic parameters (supplemental Table 1).

The most obvious non-Michaelis-Menten behavior was inhibition at elevated levels of the substrate pantoate, i.e. the rate of AtPS passed through a maximum ($v_{\text{opt}}$) as pantoate was increased (Fig. 4). The true maximum rate, which is attained at the optimal pantoate concentration, correlated positively with the levels of both cosubstrates, ATP and $\beta$-alanine, and reached 1.5 s$^{-1}$ when the latter were present at 10 and 5 mM, respectively (Table 1). Two diagnostic features show that substrate inhibition by pantoate is competitive with respect to the substrate ATP (29). First, the pantoate concentration leading to $v_{\text{opt}}$ increased continuously with increasing ATP levels but was unaffected by $\beta$-alanine levels. Second, normalizing the initial rates of each substrate-velocity curve to the appropriate value of $v_{\text{opt}}$ revealed that the relative activity of AtPS at inhibitory concentrations of pantoate correlated positively with the ATP concentration (not shown). Thus, substrate inhibition by pantoate was most pronounced at low ATP levels. At a fixed ATP concentration of 1 mM, saturating pantoate led to turnover numbers as low as 0.02 s$^{-1}$ and caused 90% inhibition relative to optimal activity. However, substrate inhibition by pantoate is unlikely to function in the regulation of AtPS activity in vivo.

Non-inhibitory pantoate concentrations in the micromolar range led to half-optimal rates of AtPS. 10- and 100-fold higher pantoate levels were required for optimal rates and for inhibition back to half-optimal rates, respectively (Table 1). Therefore, even if cellular pantoate levels were high enough to cause substrate inhibition, a 10-fold change in pantoate concentration would cause at most a 2-fold change in AtPS activity.

With $\beta$-alanine as the variable substrate, AtPS exhibited Michaelis-Menten kinetics or negative cooperativity depending on the fixed levels of ATP and pantoate in the assay. Generally, AtPS followed Michaelis-Menten kinetics with respect to $\beta$-alanine when pantoate was present at lower concentrations and exhibited negative cooperativity for $\beta$-alanine when pantoate was present at elevated concentrations. As is evident

\begin{figure}
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\includegraphics[width=\textwidth]{fig3}
\caption{Dependence of the pantothenate:{$\beta$}-alanine exchange on AMP. A. thaliana PS (A) and E. coli PS (B) were assayed for the exchange between $\beta$-[\textsuperscript{14}C]alanine and pantothenate in the presence of 0.5 mM $\beta$-alanine and 10 mM pantothenate at pH 5.5. Additionally, individual reactions contained 10 mM Na-AMP, 10 mM Na-cAMP, or 10 mM NaCl. For each reaction the transfer of \textsuperscript{14}C label from $\beta$-alanine to pantothenate that occurred over 24 h was fitted to the exponential form of Equation 7. The fraction of exchange activity remaining after removal of AMP is given in percent. The reactions catalyzed by A. thaliana and E. coli PS in the presence of 10 mM AMP correspond to exchange rates of $3.6 \times 10^{-2}$ and $6.8 \times 10^{-2}$ min$^{-1}$, respectively. Different preparations of A. thaliana and E. coli PS were used for the assays in Figs. 2 and 3, respectively. $v^*$, initial exchange rate.}
\end{figure}

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\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Initial rate plots for AtPS. Initial rates were obtained by using the coupled enzyme assay with pantoate as the variable substrate (0.01–20 mM) in the presence of 0.5 mM $\beta$-alanine (A) or 5.0 mM $\beta$-alanine (B). ATP was present in the assays at fixed concentrations of 1 mM (●), 2 mM (▲), 5 mM (■), and 10 mM (○). Individual sets of rate data were fitted to Equation 4, which was the preferred rate law at all cosubstrate levels (cf. supplemental Table 1). The best fit values of the coefficients $\alpha_1, \alpha_2, \beta_1$, and $\beta_2$ were used to calculate the apparent constants in Table 1. Insets, as main charts, except that rate data were fitted to Equation 3.}
\end{figure}
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TABLE 1
Substrate inhibition of AtPS by pantoate

| Fixed cosubstrates | [Pantoate]_{i0.5} | [Pantoate]_{app} | [Pantoate]_{i0.5} | k_{cat/app} | k_{cat/app} | ε_{i} |
|-------------------|-----------------|-----------------|-----------------|-------------|-------------|-------|
|                   | mm              | mm              | mm              | s^{-1}      | s^{-1}      |       |
| 0.5 mM β-alanine  |                 |                 |                 |             |             |       |
| 1 mM ATP          | 0.012           | 0.12            | 1.3             | 0.21        | 0.018       | 0.91  |
| 2 mM ATP          | 0.016           | 0.15            | 1.5             | 0.33        | 0.027       | 0.92  |
| 5 mM ATP          | 0.029           | 0.29            | 3.2             | 0.59        | 0.07        | 0.88  |
| 10 mM ATP         | 0.044           | 0.55            | 13.3            | 0.75        | 0.24        | 0.68  |
| 5.0 mM β-alanine  |                 |                 |                 |             |             |       |
| 1 mM ATP          | 0.014           | 0.12            | 1.1             | 0.24        | 0.025       | 0.90  |
| 2 mM ATP          | 0.018           | 0.15            | 1.4             | 0.41        | 0.039       | 0.90  |
| 5 mM ATP          | 0.034           | 0.29            | 2.9             | 0.89        | 0.09        | 0.90  |
| 10 mM ATP         | 0.070           | 0.60            | 6.4             | 1.48        | 0.24        | 0.84  |

* Noninhibitory and inhibitory pantoate concentrations where v_{i0.5} = 0.5 v_{cat} were calculated using Equation 6.
  * Pantoate concentration where v_{i0.5} = v_{app} was calculated using Equation 5.
  * k_{cat} and k_{cat} are equal to v_{app}/[E] and v_{cat}/[E], respectively; [E] is the concentration of AtPS subunits.
  * Degree of inhibition achieved at saturating pantoate relative to optimal activity.

from the Hill plots in Fig. 5, 5 mM pantoate induced strong negative cooperativity for β-alanine in the presence of 2 mM ATP (h = 0.34) but not in the presence of 10 mM ATP (h = 1.04). However, in the latter case negative cooperativity for β-alanine became apparent when pantoate was raised to 50 mM (h = 0.68). In summary, steady-state kinetic analysis of AtPS revealed that the enzyme is inhibited by the substrate pantoate and that pantoate can induce negative cooperativity for β-alanine. Apparently, both effects are suppressed by the substrate ATP.

The question arises of whether the non-Michaelis-Menten kinetics of AtPS are due to allostery, i.e. to interacting active sites, or have a kinetic basis. The following arguments show that “kinetic” or “monomeric” cooperativity (30) is an unlikely explanation for the kinetic behavior of AtPS. The action of pantoate as a competitive substrate inhibitor can be accounted for in kinetic terms by an enzyme-pantoate complex that is catalytically inactive or by assuming that binding of ATP and pantoate is random and not at equilibrium. Alternatively, substrate inhibition may be the result of negatively interacting sites. The “dead-end” scenario corresponds to Equation 3, whereas the “random” and “allosteric” scenarios both lead to rate laws in the form of Equation 4 (22). With pantoate as the variable substrate, Equation 4 allowed excellent fits to the initial rate data (Fig. 4, R^2 = 0.992–0.999) and was preferred over Equation 3 at all cosubstrate concentrations tested (F test, p values ≤ 0.01). Saturating pantoate concentrations reduced the rate of AtPS to a low plateau level but not to zero (see values for k_{cat/app} in Table 1), and the inability of Equation 3 to accommodate this behavior is illustrated in the insets of Fig. 4. Thus, the response of AtPS to pantoate discriminates against a dead-end kinetic mechanism but provides no basis to distinguish between interacting catalytic sites and random substrate binding. However, negative cooperativity with respect to β-alanine cannot be explained by alternative paths of substrate binding because β-alanine binds between two release steps. Generally, models for cooperativity in the absence of subunit interactions require that there are alternative modes of substrate binding and that the conversion of the enzyme-substrate complex into products is sufficiently fast to prevent equilibration between these modes (30). To explain the observed dependence of cooperativity toward β-alanine on pantoate levels, the above condition for kinetic cooperativity would specifically require that increasing levels of pantoate accelerate the rate at which the enzyme-panto toyl adenylate complex (F in Scheme 1) reacts with β-alanine to give products. In other words, increasing pantoate levels would be predicted to raise the apparent specificity constant for β-alanine, which is, however, essentially unaffected by pantoate (cf. supplemental Table 1).

Mutations in the Subunit Interface of AtPS—The kinetic properties of two AtPS enzymes carrying mutations in the putative dimerization region (Fig. 1B) were determined as described for wild type AtPS. Substrate inhibition by pantoate could not be detected in AtPS-Mut1 and was reduced in AtPS-E132A (Fig. 6). Also, neither mutant showed negative cooperativity for β-alanine at cosubstrate levels that caused strong negative cooperativity in wild type AtPS. AtPS-E132A still showed substrate inhibition by pantoate, but the inhibitory effect of this substrate was reduced as shown, for example, by the observation that the inhibitory pantoate concentrations required to achieve 50% inhibition relative to v_{opt} increased by at least 7-fold. The stepwise reduction of substrate inhibition by pantoate in the wild type, E132A, and Mut1 series of AtPS enzymes was accompanied by a stepwise increase of substrate inhibition by ATP (supplemental Fig. 1). Substrate inhibition by ATP was suppressed by pantoate and not detectable above 0.05 mM pantoate in AtPS or above 0.2 mM pantoate in AtPS-E132A, whereas it was apparent in AtPS-Mut1 at pantoate concentrations up to 20 mM.

The apparent kinetic parameters displayed by AtPS-E132A and AtPS-Mut1 reveal that the respective mutations in the dimerization region of AtPS consistently led to increased maximum rates, whereas the corresponding substrate affinities and specificities were lowered. This effect was observed for both pantoate (Table 2) and β-alanine (Table 3) as the variable substrate and was generally stronger in AtPS-Mut1 than in AtPS-E132A. Given that the reaction rates at low and high substrate concentrations are determined by the specificity constant and k_{cat}, respectively, AtPS will be more active than the AtPS mutants at low concentrations of pantoate and β-alanine, whereas AtPS-E132A and AtPS-Mut1 will have superior turnover rates at intermediate and high levels of these substrates, respectively.

The notion that AtPS is optimized for catalysis at low pantoate levels is supported by the effect of pantoate on the appar-
ent specificity constant for ATP ($k_{ATP/app}$) (Fig. 7). When pantoate was varied between 0.01 and 20 mM, $k_{ATP/app}$ passed through a maximum in the AtPS, AtPS-E132A, and AtPS-Mut1 enzymes, and each form of AtPS had superior values for $k_{ATP/app}$ in a different range of pantoate concentrations. Notably, wild type AtPS had the highest specificity for ATP at pantoate levels below 50 mM. The pantoate concentration at which $k_{ATP/app}$ was maximal shifted from 0.05 to 0.2 and 10 mM in AtPS, AtPS-E132A, and AtPS-Mut1, respectively. Concomitantly, the maximal values for $k_{ATP/app}$ increased from ~0.3

\[ \text{mm}^{-1} \text{s}^{-1} \] in AtPS to 0.5 mm$^{-1}$ s$^{-1}$ in AtPS-E132A and 1.3 mm$^{-1}$ s$^{-1}$ in AtPS-Mut1. This pattern of $k_{ATP/app}$ values is consistent with a stepwise reduction of competitive substrate inhibition by pantoate on mutation of the dimerization region in AtPS. Also, it suggests that the heterotropic interactions between ATP and pantoate are balanced to achieve maximal specificity for ATP at low pantoate levels.

**FIGURE 5.** Kinetic response of AtPS to $\beta$-alanine. Initial rates were obtained by using the coupled enzyme assay with $\beta$-alanine as the variable substrate (0.02–10 mM) in the presence of 2 mM ATP (A) or 10 mM ATP (B). Pantoate was present at fixed concentrations of 0.5 mM (●), 5 mM (○), and 50 mM (□). Hill plots were constructed using the estimates of $V_{max}$ defined in supplemental Table 1 and show that AtPS exhibits negative cooperativity for $\beta$-alanine at elevated pantoate concentrations. $h$, Hill coefficient.

**FIGURE 6.** Initial rate plots for AtPS-E132A (A) and AtPS-Mut1 (B). Initial rates were obtained as described for wild type AtPS by using the coupled enzyme assay with pantoate as the variable substrate (0.01–20 mM) in the presence of 5.0 mM $\beta$-alanine. ATP was present at fixed concentrations of 1.0 mM (●), 2 mM (○), 5 mM (□), and 10 mM (□). Individual sets of rate data were fitted to Equations 1–4, and the curve corresponding to the preferred rate law is shown (cf. supplemental Table 1).

| AtPS     | $S_{0.5/app}$ | $k_{cat/app}$ | $k_{app}$ |
|----------|---------------|---------------|-----------|
| Wildtype | 0.088         | 0.16          | 4.4       |
| E132A    | 0.48          | 0.59          | 1.2       |
| Mut1     | 4.7           | 1.3           | 0.27      |

*Pantoate concentration where $V_0 = 0.5 V_{max}$.
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FIGURE 7. The effect of pantoate on the apparent specificity constant for ATP ($k_{\text{ATP/app}}$) in wild type AtPS (●), AtPS-E132A (○), and AtPS-Mut1 (□). The plotted values for $k_{\text{ATP/app}}$ were estimated from double-reciprocal plots for ATP as the variable substrate (cf. supplemental Fig. 1) and are for a fixed $\beta$-alanine concentration of 5 mM. Wild type AtPS gave essentially identical values for $k_{\text{ATP/app}}$ in the presence of 0.5 mM $\beta$-alanine (not shown).

DISCUSSION

Allosteric of AtPS—The pantothenate synthetases from E. coli and M. tuberculosis catalyze a two-step reaction that proceeds via a pantoyl adenylate intermediate as shown in Scheme 1 (6, 7). These enzymes can be assumed to be non-allosteric because they obeyed Michaelis-Menten kinetics, and crystal structures of M. tuberculosis PS indicated that the active sites located on each of the two subunits are catalytically independent (10). Judging by the conservation of functional residues in PS sequences from bacteria and eukaryotes (Fig. 1A), the active site and the reaction mechanism are conserved within the pantothenate synthetase protein family. A conserved reaction mechanism is also supported by our analysis of AtPS and E. coli PS, which indicated that pantoyl adenylate is the key reaction intermediate in both enzymes since they catalyzed the isotope exchange between pantothenate and $\beta$-alanine in an AMP-dependent fashion (Figs. 2 and 3). However, initial rate studies of AtPS revealed several deviations from hyperbolic kinetics. Most obviously, pantoate caused strong substrate inhibition (Fig. 4) and, at higher levels, negative cooperativity for $\beta$-alanine (Fig. 5). Both effects were suppressed by ATP, which, conversely, caused weak substrate inhibition that was suppressed by pantoate (supplemental Fig. 1). As argued under “Results,” these observations cannot fully be accounted for by kinetic cooperativity, leaving allosteric interactions as the preferred explanation for the non-Michaelis-Menten behavior of AtPS. The E132A and Mut1 mutations in the dimerization region of AtPS partially and fully removed substrate inhibition by pantoate, respectively, which was associated with a stepwise increase of substrate inhibition by ATP. The mutations also eliminated the ability of pantoate to induce negative cooperativity for $\beta$-alanine. Homotropic and heterotropic effects of pantoate, therefore, appear to be tightly coupled, i.e., they cannot be changed independently, which is a classic tenet of allosteric systems (31).

Interactions between active sites on an oligomeric enzyme have no bearing on its ability to discriminate among competing substrates but may significantly enhance turnover by utilizing the enzyme-substrate binding energy more than once during catalysis (13). A general mechanism that can explain rate enhancement through interacting sites was proposed based on half-of-the-sites reactivity where one subunit has little or no catalytic activity but, instead, is used to modulate catalysis on the other subunit (32). Scheme 2 illustrates how this mechanism could account for key aspects of the kinetics of AtPS. The basic assumption in Scheme 2 is that the formation of F-PPi (F represents the enzyme-bound pantoyl adenylate intermediate) from ATP and pantoate is energetically unfavorable, causing its steady-state concentration to be low. Strong binding of a second molecule of pantoate (Pt*) to F-PPi, would then shift the equilibrium toward F-PP, and increase turnover. Substrate inhibition at elevated pantoate levels would follow from Scheme 2 if binding of Pt* to the second site significantly retards the release of PPi from F-PPi ($k' < k$). With these constraints, negative cooperativity for $\beta$-alanine can be explained provided that the two forms of the pantoyl adenylate intermediate, F and F-Pt*, are not in equilibrium. Assuming that Pt* competes with ATP for binding to the second site, Scheme 2 also provides a basis to explain the heterotropic interactions between these substrates.

The E132A and Mut1 mutations in the dimerization region of AtPS decreased the enzyme’s specificity for pantoate and $\beta$-alanine and increased the corresponding values for $k_{\text{cat/app}}$ (Tables 2 and 3). Thus, AtPS-E132A and AtPS-Mut1 are tuned for optimal activity at increasingly higher concentrations of pantoate and $\beta$-alanine, respectively. Fine-tuning of an enzyme that catalyzes a two-step reaction to different ranges of substrate concentration can be achieved by adjusting the stability of the reaction intermediate, and this type of fine-tuning was demonstrated to occur in tyrosyl tRNA-synthetase (13). Tyrosyl tRNA-synthetase could be optimized for different ATP concentrations by mutating a specific active site residue that stabilizes the reaction intermediate, tyrosyl adenylate (33). In contrast, the residues in the dimerization region of AtPS that were mutated in this study would not be expected to directly interact with pantoyl adenylate or any other reaction intermediate because all active site residues are conserved (Fig. 1A). Also, the dimerization domain and the active site cavity are about 15 Å apart in the structures of bacterial PS (8, 9). Thus, the observed changes in the kinetic constants for pantoate and $\beta$-alanine in AtPS, AtPS-E132A, and AtPS-Mut1 are more likely to be due to changes in long-range, allosteric interactions. Again, this can be rationalized in terms of Scheme 2. Utilizing
the additional binding energy of Pt* solely for stabilization of the F-PP\textsubscript{i} intermediate will inevitably increase the activation energy of the subsequent step on the reaction pathway. The increased rate of F-PP\textsubscript{i} formation is, thus, achieved at the expense of a slowed downstream reaction (13). Consequently, stabilization of F-PP\textsubscript{i} through binding of Pt* at the second site will benefit the overall rate of pantothenate synthesis only under conditions where the formation of F-PP\textsubscript{i} is rate-limiting, i.e. at low pantoate levels. Mutations that reduce the amount of binding energy of Pt* that is realized in F-PP\textsubscript{i} will then decrease the enzyme’s affinity for pantoate and increase $k_{cat}$, i.e. destabilization of F-PP\textsubscript{i} will tune the enzyme for optimal activity at higher pantoate levels. In summary, we propose that optimization of AtPS for low pantoate concentrations relies on an allosteric mechanism that facilitates stabilization of the F-PP\textsubscript{i} intermediate through binding of a second molecule of pantoate.

**Evolution of Allostery in Plant Pantothenate Synthetases**—Two lines of evidence support our conclusion that the allosteric properties of AtPS are conserved in all plant pantothenate synthetases. First, the characteristics of substrate inhibition by pantoate are similar for the PS enzymes from *Arabidopsis* (this study), *L. japonicus* (11), and the more distantly related bryophyte *P. patens*. Second, all available plant PS sequences contain a dimerization region characterized by an insertion relative to *E. coli* PS and the $^{131}$HETWIRVER$^{139}$ motif, both of which are important for allosteric interactions in AtPS. PS arose in the bacterial domain and was inherited, presumably independently, by plants and fungi (2). Subsequent selection for and maintenance of a divergent dimerization region in the plant lineage of the PS protein family indicate that the associated allosteric properties have an important role in the biosynthesis of pantothenate in plants. This leads to the question in which way allosteroy of plant pantothenate synthetases might be physiologically relevant. As discussed above, allosteroy of AtPS has two important consequences for catalysis, i.e. increased catalytic efficiency at low pantoate levels and substrate inhibition at elevated pantoate levels. Although pantoate has not been determined in plants, it can be argued that the latter enzymatic feature is not important in *vivo*. If the concentration of pantoate in the cytosol, where AtPS is localized (3), were within the range where substrate inhibition is significant (*i.e.* above 0.1 mM), it would need to change by at least 10-fold to cause a 2-fold change in AtPS activity (cf. Table 1). Thus, inhibition of AtPS by pantoate is unlikely to constitute an efficient regulatory mechanism. On the other hand, if cytosolic pantoate levels were well below 0.1 mM, substrate inhibition would be negligible, whereas the high specificity of AtPS for pantoate would benefit the overall rate of pantothenate synthesis. Pantothenate biosynthesis in plants is more complex than in bacteria in that the pathway is compartmentalized, and it is tempting to speculate that allosteroy arose in plant pantothenate synthetases as an adaptation to this compartmentation. The committed step of pantothenate biosynthesis in plants, the formation of keto-pantoate from 2-ketovalerate, takes place in the mitochondria (3). Even though the reductase responsible for convert-

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