Probing the Catalytic Mechanism of Prephenate Dehydratase by Site-Directed Mutagenesis of the *Escherichia coli* P-Protein Dehydratase Domain†

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ABSTRACT: The *Escherichia coli* bifunctional P-protein, which plays a central role in L-phenylalanine (Phe) biosynthesis, contains distinct chorismate mutase (CM) and prephenate dehydratase (PDT) domains as well as a regulatory (R) domain for feedback control by Phe. To elucidate the catalytic mechanism of PDT in the P-protein, 24 mutations of 15 conserved residues in the PDT domain were created, expressed in the *pheA*$^-$*E. coli* strain NK6024, and studied for their effect on PDT activity. Fourteen mutant enzymes were purified to homogeneity, tested for feedback inhibition by Phe, and characterized by kinetic analysis and circular dichroism spectroscopy. Selected mutant enzymes were further studied by gel filtration, fluorescence emission, and microcalorimetry. In addition, a monofunctional PDT domain (PDT20, residues 101−285) was cloned and overexpressed in plasmid pET with expression levels up to 200−250 mg/L. PDT20 retained full PDT activity, lacked CM activity, and was insensitive to feedback inhibition by Phe. Four residues (T278, N160, Q215, and S208) were shown to be important for PDT catalysis. The values of k_{cat}/K_m for the S208A/C and T278S mutant enzymes were 100-fold lower, and 500-fold lower for the N160A and Q215A mutant enzymes than the wild-type (WT) protein. The T278A and T278V mutant enzymes displayed no measurable catalytic activity, yet bound both prephenate and a competitive inhibitor (*S*-DNBA) comparably to the WT protein. These data, taken together with the normal CD spectra of the mutant enzymes, strongly suggested that T278 was involved in the catalytic mechanism. To establish whether acidic residues were involved in catalysis, all the conserved Glu and Asp residues in the PDT domain were mutated to Ala. None of these mutations significantly reduced PDT activity, indicating that the acidic residues of the PDT domain are not directly involved in catalysis. However, two mutant enzymes (E159A and E232A) displayed higher levels of PDT activity (2.2- and 3.5-fold, respectively), which was due to enhanced substrate binding. For the double mutant enzyme (E159A−E232A), k_{cat}/K_m was ca. 7-fold higher than for the WT enzyme, while its K_m was 4.6-fold lower.

The biosynthesis of most aromatic compounds produced by bacteria, fungi, and higher plants originates in the shikimic acid pathway (*1*, *2*). Chorismic acid, the key branchpoint pathway intermediate, is a central precursor of the essential aromatic amino acids L-Phe, L-tyrosine, and L-tryptophan, the folate coenzymes, and the iron-chelating agents such as enterobactin, as well as the ubiquinones, menaquinones, and plastoquinones involved in mitochondrial electron transport and oxidative phorphorylation.

All organisms produce Phe from chorismate in three enzyme-catalyzed reactions, although the sequence of steps may vary. In *Escherichia coli*, chorismate first undergoes a Claisen rearrangement to prephenate catalyzed by chorismate mutase (CM,[1] EC 5.4.99.5). Prephenate is then decarboxylated and dehydrated to phenylpyruvate in a reaction catalyzed by prephenate dehydratase (PDT, EC 4.2.1.51). Finally, phenylpyruvate is transaminated to Phe. In Gram-negative bacteria (*3*), a monofunctional CM transforms chorismate to prephenate, which subsequently undergoes transamination to arogenate. Arogenate is converted to Phe by the action of cyclohexadienyl dehydratase (CDT), a monofunctional decarboxylase/dehydratase that accepts either arogenate or prephenate as substrate (*1*, *4*).

In *E. coli*, the first two enzymes of Phe biosynthesis (CM, PDT) are combined in the bifunctional P-protein, whose activity is regulated by Phe-induced feedback inhibition. The P-protein contains 386 residues with a molecular mass (M_r) of 43 kDa (*5*, *6*) and exists as a homodimer in the catalytically active form (*7*, *8*). Recent work from our laboratory mapped the CM and PDT catalytic activities to distinct P-protein domains (residues 1−109 for CM; residues 101−285 for PDT) that could be expressed as discrete enzymes exhibiting full catalytic activity (*9*). Phe binding

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[1] Abbreviations: P-protein, *E. coli* bifunctional chorismate mutase/prephenate dehydratase; CM, chorismate mutase; PDT, prephenate dehydratase; CDT, cyclohexadienyl dehydratase; R domain, regulatory domain; ITC, isothermal titration calorimetry; PDT20, residues 101−285 of the P-protein; WT, wild-type; PDT32, residues 101−386 of the P-protein.

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and feedback inhibition were also shown to be mediated by an independent regulatory (R) domain, comprising residues 286−386 of the P-protein (*10*).

Structural and mutagenesis studies on the P-protein's CM domain helped elucidate the mechanism of mutase catalysis in *E. coli* (*11*−*13*). However, the mechanism of decarboxylation and dehydration catalyzed by PDT (and, by analogy, CDT) is not currently known. While long presumed to involve a concerted, Grob-type fragmentation triggered by protonation or hydrogen bonding of the cyclohexadienyl hydroxyl group (*14*), no detailed mechanistic studies have been reported. A preliminary report of the crystallization of CDT has appeared (*15*). Despite their functional parallels, PDT and CDT show little similarity in their amino acid sequences.

Besides its importance as an essential amino acid, Phe is also a global commodity used to manufacture the nonnutritive sweetener known as aspartame (L-aspartyl-L-phenylalanine methyl ester) for which the worldwide market is currently estimated to be $1.5 billion (*16*). Phe production from D-glucose in the shikimate pathway is controlled primarily through end-product inhibition of three enzymes: 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase, CM, and PDT. Kinetic studies on the P-protein have further shown that the reaction catalyzed by PDT is rate limiting ($k_{cat}/K_m = 3.5$) compared to CM ($k_{cat}/K_m = 17$). Here, we report the results of site-directed mutagenesis studies designed to elucidate the mechanism of PDT in the P-protein, as well as the development of a double mutant exhibiting significantly improved PDT catalysis.

MATERIALS AND METHODS

Materials. Unless indicated otherwise, all chemicals were purchased from Sigma, and biochemicals were obtained from New England Biolabs. L-(4-^{3}H) Phe (26.0Ci/mmol) was obtained from Amersham.

Strains and Growth Conditions. Strain *E. coli* DH5α (*17*) was used for routine clone and plasmid isolation. *E. coli* NK6024 (relevant genotype: *pheA*$^-$ *tyrA*$^+$) (*12*) was used as the host for expression of all mutants and *E. coli* BL21-(DE3) (*18*) from Novagen were used for expression of PDT20. All *E. coli* strains harboring plasmids were grown in Luria broth or on LB plates containing either 100 μg/mL ampicillin or 30 μg/mL kanamycin. *E. coli* BL21(DE3) clones were grown in LB for seed cultures and in M9 medium for large-scale enzyme production (*19*). Unless indicated, strains harboring plasmids were grown in either M9 medium, Luria broth, or Luria agar plates with 100 μg/mL of ampicillin or 30 μg/mL kanamycin.

Mutagenesis and DNA Manipulations The parent vector pJS1, which carried the *pheA* gene in pUC18 (*20*), was used to construct P-protein mutants. All mutations were generated by the PCR overlap extension method (*21*). For multiple mutations in the same residue, two asymmetric mutagenic primers (24−31 mers) (*22*) were used and synthesized by the Cornell Biotechnology Facility. For single mutations, a modified PCR overlap extension approach was used. This approach requires only one forward internal mutagenic primer paired with a pUC18 universal primer 1224 (Biolabs) in the first round of PCR. The resulting PCR fragment was then directly overlapped to pJS1 cut with *Hin*dIII for eight cycles followed by further amplification with two added pUC18 universal primers 1224 and 1233 for 35 cycles. The PCR conditions were the same as described previously (*12*). The final PCR products were digested at two unique sites (*Asc*I and *Nco*I) and cloned back to the parent vector pJS1. To facilitate screening, all primers were designed to incorporate or destroy a restriction enzyme site at or near the site of the mutation. The PDT coding regions in each plasmid were sequenced by the Cornell Biotechnology Facility. For those plasmids that contained additional undesired mutations, the desired mutations were rescued by cloning using two additional unique sites (*Hpa*I and *Afl*III) present in the PDT domain gene. In the same manner, the double mutant E159A/E232A was constructed by cloning a 470 bp E232A DNA fragment from pSZ132 into pSZ121 containing the E159A mutation at *Hpa*I and *Nco*I sites. Plasmids having the desired mutation were isolated and confirmed by sequencing and then transformed into *E. coli* NK6024 for expression. To engineer an even smaller monofunctional PDT domain and improve its expression, a synthesized oligonucleotide that initiated translation at residue 101 with an introduced *Nde*I site was paired with 1224 primer for PCR using the pSZ70 plasmid as template (*9*). The resulting PCR product containing PDT20 (corresponding residues 101−285 of P-protein) gene was cloned into pET26b(+) vector at *Nde*I and *Hin*dIII sites to yield pSZ139 (Table 1). Two inactive mutations (T278A and T278V from pSZ99 and pSZ74, respectively)

Table 1: Specific Activity of P-Protein Mutants

	PDT[a]			CM[a]	
clones	mutant	units/mg[b]	(%)	units/mg	(%)
pJS1	P-protein	18.2	100	44.0	100
pSZ139	PDT20wt	37.6	98	0	0
pSZ133	E129Ac		93		100
pSZ134	D141Ac		96		100
pSZ135	D152Ac		63		100
pSZ121	E159A	40.4	222	43.7	99
pSZ122	N160A	0.07	0.38	44.4	101
pSZ130	N160Dc		0.16		100
pSZ127	D171A[c]		98		100
pSZ124	E183A[c]		25		100
pSZ178	S208A	0.27	1.5	43.8	99.5
pSZ180	S208C	0.27	1.5	43.9	100
pSZ179	S208D[c]		0.30		100
pSZ125	H209A[c]		9		100
pSZ125	Q215A	0.062	0.34	44.1	100
pSZ77	C216A	10.3	56.6	43.3	98
pSZ78	C216S	10.8	59.3	41.4	94
pSZ76	W226A	3.2	17.6	41.6	95
pSZ75	W226L	3.9	21.4	43.6	99
pSZ136	E229Ac		112		100
pSZ132	E232A	42.9	236	43.7	99
pSZ131	E255Ac		110		100
pSZ99	T278A	∼0.18	<0.01	43.3	98
pSZ73	T278S	0.45	2.5	43.2	98
pSZ74	T278V	0[d]	0	43.9	100
pSZ137	E159A/E232A	56.9	313	44.9	102

[a] Specific activities were assayed in 0.4 mL reaction mixtures using either 500 μM prephenate/0.3 μg of enzyme (for PDT) or 1000 μM chorismate/0.15 μg of enzyme (for CM) (*24*). [b] A unit of enzyme was defined as the quantity of enzyme that catalyzed the conversion of 1 μmol of substrate to product in 1 min under the assay conditions. [c] These mutant proteins have not been purified. The PDT activities of crude cell extracts for each mutant were assayed as percent activity of pJS1 crude cell extract by adjusting each CM activity as 100% of pJS1 crude extract. [d] No PDT activity was detected with 1000× the usual quantity of protein.

were then cloned into pSZ139 at *Asc*I and *Hin*dIII. These pET-based plasmids were transformed into *E. coli* BL21-(DE3) for expression.

Expression and Crude Extract Preparation. Cell pellet and crude cell extracts were screened by Western blotting to confirm expression in *E. coli* as described (*12*). All P-protein mutants were produced in *E. coli* NK6024 in M9 medium following a published procedure (*20*). For PDT20 and its mutant enzymes, a 20 mL LB culture with *E. coli* BL21-(DE3) transformants was grown up at 37 °C to OD_{600} =1.0 and used to inoculate 1 L of M9 medium. After growth for about 5 h to OD_{600} =1.0, the culture was induced with IPTG (1 mM) and grown at 30 °C for 16 h and harvested. The cell extracts for each sample were prepared as before (*20*), and tested for CM and PDT activities using a pUC18 cell extract as a negative control. Expression levels were measured by both Western blotting and activity assays.

Purification of P-Protein Mutants and Engineered PDT20s. P-Protein and P-protein mutants were purified from cell crude extracts by a published protocol (*20*). Purification of PDT20 and PDT20 mutant enzymes was carried out as described (*9*). Enzyme purity was assessed on SDS gels, and protein concentrations were determined by the Bradford method (*23*) using bovine serum albumin (BSA) as a standard.

Activity and Phe Feedback Inhibition Assays. Chorismate mutase was assayed by monitoring the conversion of chorismate to prephenate. PDT activity was measured by the conversion of prephenate to phenylpyruvate as described previously (*24*). One unit of enzyme was defined as the amount of enzyme required to form 1 μmol of product per minute at 37 °C. Substrate and effector concentrations were described in the individual experiments. The kinetic parameters were determined with the curve fitting options in the KaleidaGraph program (Abelbeck Software). Feedback inhibition of PDT activity by Phe was measured at 0−3.2 mM Phe concentrations, and I_{50} values for each protein were determined as the Phe concentration causing 50% inhibition of PDT activity.

[^{3}H]Phe Binding Assay and Gel Filtration. Both analytical methods were performed as described previously (*9*). Five selected P-protein mutants (T278V, T278S, C216A, N160A, and W226A) were tested for Phe binding with 4 μM enzyme and 20 μM [^{3}H]Phe. Gel filtration of two inactive mutants (T278A and T278V) was performed in the presence and absence of 1 mM. The effect of Phe on the molecular masses of each enzyme was determined from the elution volume.

Fluorescence Measurements. Fluorescence measurements were performed at room temperature on an AMINCO SCM 8000C spectrofluorimeter equipped with a xenon light source, using an excitation wavelength of 295 nm. Fluorescence of W226 was monitored. Emission was scanned from 305 to 385 nm with the slit widths for excitation and emission set at 4 nm. Protein concentrations were kept at 2.5 μM in 10 mM Tris, pH 8.0. Dilution effects from prephenate titrations were negligible. All spectra were corrected for background and Raman scattering by subtracting the buffer plus prephenate signal.

Circular Dichroism Analysis and Isothermal Titration Microcalorimetry Study. CD spectra of the P-protein, PDT20, and each mutant (30 μg/mL) were measured on a modified Cary model 14 (*25*) at room temperature in 10 mM potassium phosphate buffer (pH 7.0) using a 1 cm path-length cell as described (*12*). Isothermal titration microcalorimetry (ITC) was performed using an ω titration microcalorimeter from MicroCal, Inc. (Northampton, MA) as described (*10*). Titrations on PDT20 and mutants thereof with a PDT inhibitor (*S*-DNBA) were carried out in 20 mM Tris at pH 8.2 + 0.1 M NaCl using 113−220 μM protein with 5−10 mM *S*-DNBA (*26*). Protein solutions were dialyzed against 1 L of the assay buffer using Slide A Lyzer cassettes (Pierce, IL). Protein concentrations were determined after dialysis. The inhibitor *S*-DNBA was dissolved in the dialysis buffer in concentrations resulting in a molar ratio of inhibitor:protein (calculated as monomer) = 2 at the end of the titration. The solutions were degassed for 15 min prior to each experiment. In each run, 20 or 30 injections of 2 μL *S*-DNBA solution were added to the protein solution via a rotating (300 rpm) stirrer-syringe. The heat of dilution was negligible in separate titrations of the ligand solution into the buffer solution. The data were analyzed with manufacturer-supplied software (ORIGIN, MicroCal, WA).

RESULTS

Mutations in the E. coli P-Protein. Multiple sequence alignments of known PDT proteins or domains found in the NIH protein database and Swiss database (12 total) identified residues of interest for site-directed mutagenesis in the P-protein. Three mutations (A,S,V) were introduced at T278, a residue whose importance was underscored by the identification of a highly conserved ^{278}TRF^{280} motif in all 12 PDT sequences. Alanine mutations were introduced at residues E159, N160, D171, E183, S208, H209, Q215, and T278, which were conserved in all 12 sequences, and at E129, D141, D152, E229, E232, and E255, which were conserved in at least 5 of the 12 sequences searched. Additional mutants S208C and S208D were prepared to probe position 208 further. Mutations at C216 (A and S) and W226 (A and L) of the P-protein were prepared to confirm and explore earlier chemical modification studies (*27*, *28*).

Enzyme Production and Purification. All P-protein mutants were expressed at levels comparable to the WT P-protein and were purified as for the WT protein. The final yields of the purified enzymes ranged from 3 to 6 mg/L of culture. Expression of the monofunctional PDT domain PDT20 in the pET system led to a 50-fold improvement compared to the pUC18 expression systems. The yields of PDT20 and its T278A/V mutants were 140−180 mg/L.

Activity and Kinetic Characterization. The specific activities of both CM and PDT were determined for all mutant enzymes at 1 mM chorismate and 0.5 mM prephenate (Table 1). For the freshly prepared WT P-protein, CM activity was 45 units/mg, and PDT activity was 22 units/mg (*20*). All P-protein mutants in this study (from residues 129 to 278) retained full CM activity, consistent with previous findings indicating nonoverlapping CM and PDT domains (*9*). As expected, PDT20 retained full PDT activity, but lacked CM activity. Mutations in residues T278, N160, Q215, and S208 of the P-protein led to substantial losses in PDT activity. Activity in two mutants (T278A/V) was undetectable the usual assay conditions. Consistent with those findings, the T278A/V mutations in PDT20 also abolished PDT activity (data not shown).

No significant decreases in PDT activity were observed when mutations were introduced at conserved acidic residues

Table 2: Kinetic Parameters of PDT Mutants

	Prephenate				Chorismate			
protein	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} mM^{-1})		k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})	
P protein	1932 ± 95	559 ± 78	3.46	(100%)	2051 ± 84	127 ± 19	16.1	(100%)
C216A	*a*	>3000	0.89	25	2013 ± 38	159 ± 11	12.7	79
C216S	2096 ± 192	2087 ± 255	1.0	29	1947 ± 62	113 ± 13	17.2	107
E159A	2528 ± 100	250 ± 41	10.11	292	2053	124	16.5	102
E232A	2401 ± 91	162 ± 17	14.8	428	ND	ND	ND	
E159A/E232A	2962 ± 24	123 ± 3	24.0	694	ND	ND	ND	
N160A	13	1649	0.008	0.23	2013	151	13.3	83
Q215A	*a*	>10000	0.009	0.26	2108	154	13.7	85
S208A	*a*	>5000	0.03	0.87	ND	ND	ND	
S208C	*a*	>3000	0.03	0.87	ND	ND	ND	
T278A	n/a	n/a	n/a		2131 ± 61	123 ± 12	17.3	107
T278S	24.6 ± 1.1	565 ± 75	0.04	1.2	2047 ± 71	123 ± 13	16.6	103
T278V	n/a	n/a	n/a		1936 ± 50	115 ± 13	16.8	104
W226A	*a*	>10000	0.18	5.2	1956 ± 44	139 ± 12	14.1	88
W226L	1227 ± 228	~3000	0.47	13.6	ND	ND	ND	
PDT20	1893 ± 113	648 ± 86	2.92	84	ND	ND	ND	

[a] Saturation was not achieved even at 3200 μM substrate. As a result, k_{cat} and K_m values could not determined. k_{cat}/K_m was determined by a linear fit of initial velocity vs low substrate concentration [$v = S(V_{max})/K_m$] at less than 400 μM substrate. n/a = not available. ND = not determined.

Table 3: Comparison of Prephenate and Phenylpyruvate Produced from Chorismate by P-Protein Mutants

	prephenate + phenylpyruvate[a]		phenylpyruvate[b]		calculated prephenate
enzyme	(μmol min^{-1} mg^{-1})	fold	(μmol min^{-1} mg^{-1})	fold	(μmol min^{-1} mg^{-1})
WT P-protein	48.7	1	2.4 (5%)	1	46.3 (95%)
E159A	48.1	0.99	11.2 (23%)	4.7	36.9 (77%)
E232A	48.4	0.99	11.0 (23%)	4.7	37.4 (77%)
E159A/E232A	49.9	1.02	24.7 (50%)	10.3	25.2 (50%)

[a] Regular CM activity assay was conducted using 1 mM chorismate as substrate as described in Materials and Methods. [b] After 5 min incubation, the reaction was directly terminated by adding 1.5 M NaOH.

(E129, D141, D152, E159, D171, E183, E229, E232, and E255). Two point mutations (E159A and E232A) each resulted in >2-fold increased in PDT activity. PDT activity in the corresponding double mutant (E159A/E232A) displayed a greater than 3-fold increase.

Kinetic evaluation of all P-protein mutants revealed that the substrate saturation curves were hyperbolic for both the PDT and CM activities. Kinetic parameters for the CM and PDT activities in each mutant P-protein were subsequently obtained by fitting the initial rate data to the Michaelis−Menten equation as shown in Table 2. As predicted, all P-protein mutants retained comparable K_m and k_{cat} values for chorismate. Mutations at both W226 and C216 resulted in increased values of K_m for prephenate, while values of k_{cat} were largely unchanged. In the case of the T278S mutant, which displayed ~2% WT PDT activity, k_{cat} was 100-fold lower than in the WT P-protein, while K_m was largely unchanged. Mutations at Q215A and S208 in the PDT domain resulted in dramatically increased K_m values, so that saturation of PDT activity could not be achieved at high concentrations of prephenate. Mutagenesis of N160 affected k_{cat} much more strongly than K_m, suggesting that N160 was important for PDT catalysis. Introduction of an alanine mutation at E159 led to a nearly 3-fold enhancement of k_{cat}/K_m resulting from a modest increase in k_{cat} and a substantial reduction in K_m. Similarly, more than a 4-fold increase in k_{cat}/K_m was noted in the E232A mutant, arising from a >3-fold lowering of K_m and a slight increase in k_{cat}. Furthermore, the double mutant E159A/E232A displayed a 7-fold elevation in k_{cat}/K_m for the PDT-catalyzed reaction. The K_m for prephenate was reduced from 560 to 123 μM, making it comparable to the K_m of chorismate in the CM-catalyzed reaction.

To quantify the overall change in metabolic flux from chorismate to phenylpyruvate in the E159A/E232A P-protein double mutant, as well as in both single mutations, a modified CM assay for measuring only prephenate (the CM product) was performed concurrently with the standard CM assay, in which prephenate and phenylpyruvate (the PDT product) were both converted to phenylpyruvate anion for spectrophotometric determination. Using the WT P-protein as a control (Table 3), the dual CM assays indicated a 19:1 ratio of prephenate:phenylpyruvate, confirming that PDT catalysis represented the slow step in the bifunctional enzyme. For the E159A/E232A P-protein double mutant, a 1:1 ratio of prephenate:phenylpyruvate was observed. For the relevant single mutants (E159A and E232A), a 3:1 ratio of prephenate:phenylpyruvate was determined in each case. Besides confirming that alanine mutations at residues 159 and 232 significantly improved the overall turnover of prephenate, these results also established that the double mutant, when assayed in vitro, was capable of achieving a 10-fold rate acceleration in production of phenylpyruvate from chorismate.

Feedback Inhibition of P-Protein Mutants by Phe. Using the standard PDT activity assay to monitor feedback inhibition, five mutants having significantly decreased PDT activity were found to be more sensitive to Phe-induced feedback inhibition (I_{50} = 10−25 μM) than was the WT P-protein (~80 μM), as shown in Figure 1a. Values of I_{50} for four

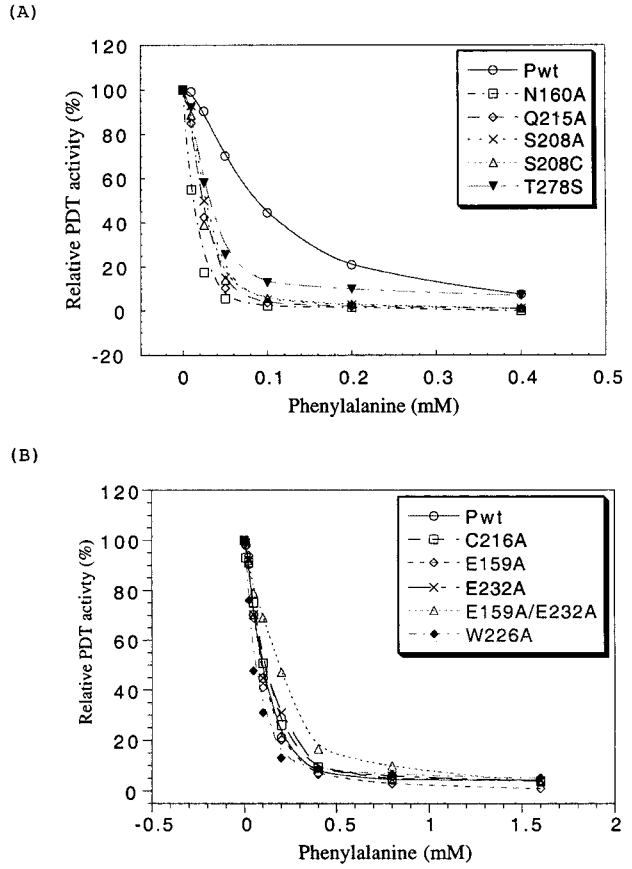

FIGURE 1: (A) Effect of phenylalanine on the activity of active site mutant PDT enzymes; (B) Effect of phenylalanine on the activity of other mutant PDT enzymes.

other mutants were similar to WT (Figure 1b), while the double mutant exhibited somewhat weaker feedback inhibition. These results provide further evidence that the mutations in the PDT domain have little effect on Phe-induced allosteric regulation. Mutant enzymes that became more sensitive to inhibition by Phe likely induced active-site alterations that caused substrate binding to be more strongly affected by Phe-induced conformational changes.

[^{3}H]Phe Binding Assay and Gel Filtration. Gel filtration of both P-protein inactive mutant enzymes (T278A/V) showed that both behaved the same as WT. In absence of Phe, both enzymes were dimeric while in the presence of 1 mM Phe, both formed tetramers and higher oligomers. The [^{3}H]Phe binding experiments showed that all five P-protein mutant enzymes (T278S/V, C216A, N160A, and W226A) tested retained the same binding activity with range of 27−30 pmol of Phe bound/100 pmol of enzymes, which is 90−100% of the reported binding activity for the P-protein (*10*). These data suggest that mutations in the PDT domain do not affect the interaction between the R-domain and Phe, confirming the previous finding that the PDT and the regulatory domains in the P-protein were essentially independent.

Fluorescence Analysis. Kinetic measurements indicated that prephenate binding was unaffected in the weakly active T278S mutant PDT enzyme. To ascertain whether prephenate binding was similarly unaffected in the catalytically inactive T278V and T278A mutants, fluorescence assays based on PDT20 were devised. These assays were based on kinetic results with the W226A mutant enzyme, which indicated that

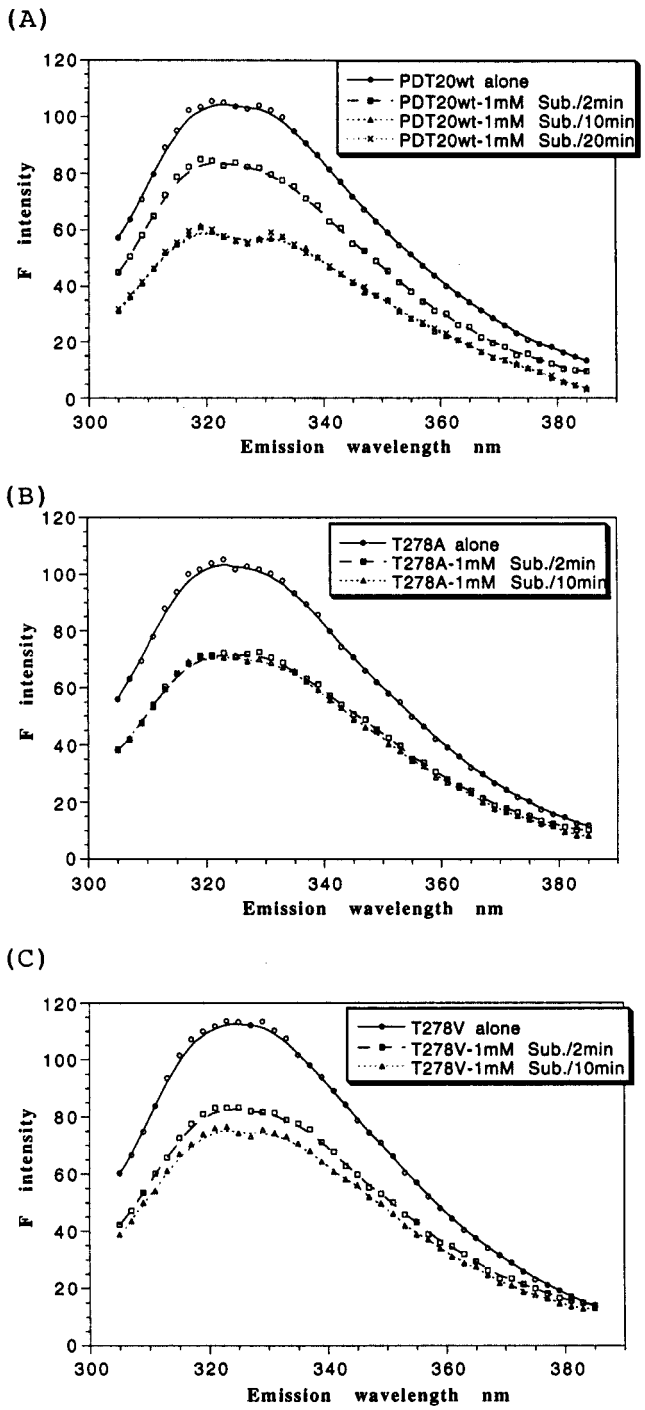

FIGURE 2: (A) Effect of prephenate on the relative fluorescence intensity of WT PDT20; (B) Effect of prephenate on the relative fluorescence intensity of the PDT20-T278A mutant enzyme; (C) Effect of prephenate on the relative fluorescence intensity of the PDT20-T278V mutant enzyme.

W226 was involved in prephenate binding. Specifically, in the presence of 1 mM prephenate, WT PDT20 displayed decreasing intrinsic fluorescence intensity and a slight shift in the emission maximum from 322 to 321 nm. Therefore, the relative fluorescence intensity of the T278A and T278V mutant enzymes was determined with and without prephenate (Figure 2). In the presence of 1 mM prephenate, both mutant enzymes exhibited changes similar to WT PDT20. As noted previously, control experiments with all three proteins in the presence of 1 mM Phe displayed no fluorescence changes. The data showed that prephenate bound to the catalytic sites

of the inactive mutants, causing the observed changes in their fluorescence properties. Since *S*-DNBA completely quenched fluorescence, it was not possible to perform comparable measurements on the T278A/V mutants using this competitive inhibitor.

CD Spectra and ITC Analysis. The CD spectra of the purified mutant enzymes were essentially superimposable with that of the WT P-protein (data not shown), indicating that no significant disruption in global conformation was caused by the point mutation. Moreover, CD spectra of the PDT20 domain and its two inactive mutant proteins indicated that the A and V mutations at T278 caused no disruption of overall protein secondary structure. When the binding of *S*-DNBA to PDT20 was monitored using ITC, a weak-binding exotherm was observed, but no quantifiable data were obtained. The detection limit for ligand binding using ITC has been estimated to be $K_a < 5 \times 10^{-3}$ M^{-1} (*29*), which is consistent with the inhibition constant reported earlier for *S*-DNBA when assayed against a slightly larger dehydratase domain (PDT22; $K_i = 300$ μM) (*26*). Both T278A and T278V mutants of PDT20 showed similar weak-binding exotherms when titrated with *S*-DNBA (data not shown), indicating that the mutations had not significantly changed the binding affinity of the PDT20 active site for either substrate or inhibitor.

DISCUSSION

The goal of the present work was to gain insight into the mechanism of PDT by identifying the amino acid residues in the protein that are responsible for substrate binding and catalysis. Earlier chemical modification, random mutagenesis, and peptide-mapping studies suggested that one threonine residue in the *E. coli* P-protein was catalytically or structurally essential for dehydratase activity (*30*). Using multiple sequence alignments of 12 known mono- or bifunctional dehydratases, we established that T278 in the P-protein belonged to a ^{278}TRF^{280} tripeptide motif that was conserved in all 12 sequences. P-Protein mutants were prepared in which T278 was substituted with alanine or valine, and these proteins exhibited no detectable dehydratase activity. Like the WT P-protein, both mutants were dimeric, retained full CM activity, and formed higher-order aggregates in the presence of Phe. Circular dichroism spectra further indicated that the mutants were properly folded, while fluorescence measurements on the mutants confirmed that substrate binding had not been disrupted. The corresponding serine mutant enzyme, T278S, displayed ca. 2% of WT activity. Further kinetic evaluation established that this conservative mutation had little effect on substrate binding (unchanged K_m), whereas k_{cat} was100-fold lower than for the WT P-protein. Taken as a whole, the data on T278 mutations clearly implicated this residue as important in catalysis, perhaps involving hydrogen bonding to prephenate or to other catalytic residues.

The enzymatic conversion of prephenate or arogenate to phenylpyruvate has long been presumed to involve acid catalysis (*14*). When prephenic acid was first isolated and characterized in 1953, its spontaneous aromatization was qualitatively observed to be faster in aqueous acid (*31*, *32*). In the most widely accepted mechanism, protonation of the ring hydroxyl group triggers the concurrent loss of H_2O and CO_2 in a Grob-type fragmentation of the ionized prephenate or arogenate dianion.

It was therefore of interest that the *E. coli* PDT domain contained several acidic residues that might potentially contribute to enzymatic catalysis. Sequence alignments indicated that three glutamate or aspartate residues (E159, D171, and E183) were conserved in all 12 sequences. Moreover, six additional acidic side chains (E129, D141, D152, E229, E232, and E255) were conserved in nearly half the sequences. However, alanine mutations in the P-protein in any of the nine conserved acidic residues caused no significant decrease in PDT activity.

The observation that no aspartate or glutamate residue plays a key catalytic role in catalysis by PDT is of interest in conjunction with ongoing studies of CDT, which catalyzes a very similar decarboxylation/dehydration. At present, however, more differences than similarities are evident in the two enzymes. For example, CDT can accelerate the aromatization of arogenate as well as prephenate, whereas PDT can only aromatize prephenate. The basis of substrate discrimination is not known. Moreover, alignment studies indicate that known PDT sequences share less than 10% identity with CDT, suggesting that different mechanisms may have evolved to promote the aromatization of arogenate and prephenate.

Observations on the in vitro aromatization of arogenate and prephenate raise the distinct possibility that different catalytic potencies may have been designed into CDT and PDT. Zamir et al. noted several years ago that arogenate undergoes nonenzymic acid-catalyzed aromatization 10 times more slowly than prephenate (*33*). That finding suggests that, for proper catalytic function, CDT may have evolved a stronger acid in its active site (e.g., an aspartate or glutamate) to aromatize arogenate. The more acid-sensitive prephenate would also be a reactive substrate for CDT. By contrast, PDT might need a weaker acid, or perhaps only hydrogen bonding, to promote the dehydration-decarboxylation of prephenate. Besides rationalizing the mutagenesis studies, that hypothesis presents a plausible explanation as to why the less acidic active-site functionality of PDT might not catalyze the aromatization of arogenate. Further insights into this hypothesis will be gained from detailed structural information on the CDT and PDT active sites.

Additional studies on the P-protein implicated one cysteine residue in the PDT reaction, whose modification led to dehydratase inactivation with only 5% loss of mutase activity (*27*, *28*). Three cysteines occur in the *E. coli* PDT domain. Alignment studies indicated that Cys136, Cys191, and Cys216 were conserved in seven, five, and eight sequences, respectively, of the 12 known dehydratases. Substitution of alanine or serine at Cys216 led to P-protein mutants in which substrate binding was significantly diminished, with little or no loss of mutase activity. In the case of the C216A enzyme, saturation was not achieved even at 3.2 mM prephenate, so that k_{cat} could not be determined. However, kinetic parameters could be determined for the C216S enzyme, which displayed a 4-fold increase in K_m, but little difference in k_{cat} compared to WT P-protein. Mutation of the highly conserved adjacent residue, Q215, diminished substrate binding even more, suggesting that both adjacent residues interacted with prephenate. Besides being involved in substrate binding, Q215 may also be involved in stabilizing the PDT transition

state since k_{cat}/K_m for Q215A fell ~500-fold while K_m was too high to be determined experimentally.

Two additional residues (N160 and S208) were shown to be important in PDT catalysis. Compared to the WT protein, values of k_{cat}/K_m were 100-fold lower for S208A/C mutant enzymes and 500-fold lower for the N160A mutant enzyme. Mutations at S208 in the PDT domain resulted in dramatically increased K_m values, making it impossible to achieve saturation, and suggesting that S208 was mainly involved in prephenate binding. By contrast, replacing N160 with alanine lowered k_{cat} more than 150-fold but caused only a 3-fold increase of K_m, suggesting that N160 is a catalytic residue.

Modification of one or both tryptophan residues (Trp226 and Trp338) in the P-protein has long been known to result in partial loss of both mutase and dehydratase activities, as well as desensitization to Phe-induced feedback inhibition (*28*). In the presence of 2 mM Phe, both enzyme activities were protected against chemical modification. Fluorescence changes in the R-domain (residues 286−386) upon Phe binding clearly implicated Trp338 in Phe-induced conformational changes leading to higher-order P-protein aggregates (*9*). Therefore, the role of Trp226 in PDT catalysis was probed by mutating this residue to Leu or Ala. The latter mutant enzyme could not be saturated with substrate, while the former exhibited a 6-fold increase in K_m, and a slight drop in k_{cat}, indicating that Trp226 was likely involved in prephenate binding.

Unexpectedly, two point mutations at acidic residues (E159A and E232A) each more than doubled PDT activity in the expressed P-protein. When both mutations were combined, the resulting double mutant enzyme E159A/E232A displayed a greater than 3-fold increase in PDT activity. Kinetic analysis indicated that the increased PDT activity in each mutation was mainly achieved by improving substrate binding. How mutations enhance prephenate binding to the PDT active site is presently unclear, but at least three possibilities seem plausible. The removal of charged carboxylate groups might diminish unfavorable charge−charge repulsions with prephenate. Alternatively, replacement of E159 and E232 with sterically less demanding side chains might make the active site more accessible to prephenate. Last, one or both mutations may cause a conformational change in the active site leading to improved substrate binding. Although additional structural and/or kinetic information will be required to understand this effect, the higher activity mutants reported herein suggest that further engineering of PDT may significantly increase Phe production in vivo.

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