Chorismate Mutase-Prephenate Dehydratase from *Escherichia coli*

STUDY OF CATALYTIC AND REGULATORY DOMAINS USING GENETICALLY ENGINEERED PROTEINS*

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The bifunctional P-protein, which plays a central role in *Escherichia coli* phenylalanine biosynthesis, contains two catalytic domains (chorismate mutase and prephenate dehydratase activities) as well as one R-domain (for feedback inhibition by phenylalanine). Six genes coding for P-protein domains or subdomains were constructed and successfully expressed. Proteins containing residues 1–285 and residues 1–300 retained full mutase and dehydratase activity, but exhibited no feedback inhibition. Proteins containing residues 101–386 and residues 101–300 retained full dehydratase activity, but lacked mutase activity. Fluorescence emission spectra and binding assays indicated that residues 286–386 were crucial for phenylalanine binding. The mutase (residues 1–109), dehydratase (residues 101–285), and regulatory (residues 286–386) activities were thus shown to reside in discrete domains of the P-protein. Both the mutase domain and the native P-protein formed dimers. Deletion of the mutase domain diminished phenylalanine binding to the regulatory site as well as prephenate binding to the dehydratase domain, both through cooperative effects. Besides eliminating feedback inhibition, removal of the R-domain decreased the affinity of chorismate mutase for chorismate.

Proteins employ a variety of regulatory mechanisms to achieve functional control, virtually all of which involve cooperative interactions between ligand binding sites (1). Understanding the detailed structural principles involved in allosteric interactions and other cooperative phenomena has become increasingly important in the rational design of new proteins with specifically engineered properties. A number of important applications can be envisioned for such proteins, including their use as novel molecular detection devices (2).

Several feedback control mechanisms are known in the biosynthesis of the aromatic amino acids, phenylalanine (Phe) and tyrosine (Tyr), via the shikimic acid pathway. Both Phe and Tyr are produced from chorismic acid, a key intermediate in the shikimate pathway whose metabolism branches in five different directions (3). Each of the five branch point enzymes must be carefully regulated to partition chorismate properly to various downstream intermediates.

In the first committed step to Phe and Tyr, chorismate undergoes a Claisen rearrangement to prephenate catalyzed by chorismate mutase (CM, EC 5.4.99.5). Prephenate can undergo either decarboxylation/dehydration to phenylpyruvate catalyzed by prephenate dehydratase (PDT, EC 4.2.1.51) or decarboxylation/dehydrogenation to p-hydroxyphenylpyruvate catalyzed by prephenate dehydrogenase. Transamination of each α-ketoacid produces the respective α-amino acid. In an alternative pathway, prephenate can first form arogenic acid and subsequently be converted to Phe and Tyr (4).

Procaryotes, eucaryotes, and higher plants may use either route to Phe and Tyr, and many organisms have achieved the desired levels of regulation by evolving multiple isozyme systems. For example, CMs have been identified that are monofunctional with allosteric control, monofunctional lacking allosteric control, or bifunctional (5). To date, three bifunctional CMs are known in which mutase activity is coupled with PDT, prephenate dehydrogenase, or 3-deoxyarabinoheptulosonate-7-phosphate synthase (5).

To understand the various domain interactions and allosteric effects in such bifunctional proteins, we elected to study the P-protein of *Escherichia coli*, in which CM and PDT are coupled (1, 6). Both activities are subject to feedback inhibition by Phe (7). The P-protein is encoded by the *pheA* gene and contains 386 amino acids with a molecular mass of 43 kDa (8, 9). The catalytically active form of the enzyme is a homodimer (10, 11); however, increasing Phe concentrations shift the P-protein from a dimer to a mixture of dimer, tetramer, and higher order species, suggesting cooperative homotropic interactions between Phe binding sites (10–12).

Our approach, which involved expressing discrete CM, PDT, and R-domains from genetically engineered fragments of *PheA*, was guided by several considerations. First, we hoped to isolate and identify the individual binding sites for the P-protein’s substrates and products to better understand the interactions between CM, PDT, and their effectors. Second, while earlier chemical modifications (13,14), kinetic studies (15), and genetic mutations (16–18) suggested that the CM, PDT, and R-domains were relatively distinct, it was unclear to what extent the full, continuous polypeptide backbone would be necessary for correct folding, expression, and activity. Indeed, some degree of domain interconnection has been noted (16, 19), which could be probed in detail using the recombinant approach. Third, we expected that specifically engineered domains might shed light on the detailed molecular interactions...
involved in dimerization and in the formation of Phe-induced higher order aggregates. Earlier we successfully cloned, expressed, and crystallized the NH₂-terminal 109 residues of the E. coli P-protein, a fully active CM domain with no PDT or Phe binding activity (20, 21). Like the native P-protein, it formed a stable dimer. It was therefore of interest to create a smaller, monofunctional PDT domain to help understand the molecular interactions involved in feedback inhibition and higher order aggregation. Also of interest, from the perspective of designing biosensors for amino acids (2), was the possibility of excising a fully functional R-domain from the P-protein backbone. Here we report the use of genetic engineering to map the P-protein’s PDT and R-domains and describe how domain interactions affect higher order structure.

EXPERIMENTAL PROCEDURES

Materials—Unless indicated otherwise, all chemicals were purchased from Sigma, and biochemicals were obtained from New England Biolabs. L-(4-3H)-Phenylalanine (26.0 Ci/mmol) was obtained from Amersham Corp. and diluted with cold Phe. Polyethylene equilibrium dialysis cells having a volume of 0.2 ml were used for equilibrium dialysis (22).

Strains—E. coli strain KS474 (relevant genotype: degP41(D prostitution-Kan)) was used as the host for cloning, plasmid isolation and expression (23). Unless indicated, strains harboring plasmids were grown in either M9 medium, Luria broth, or Luria agar plates all containing 100 µg/ml of ampicillin. E. coli strain G1724, obtained from Invitrogen, Inc., was used as the host for making Thi6R.

Proteolysis of the P-protein—The P-protein was purified (24) and partially digested by adding 1 µl of 0.2 mg/ml elastase in reaction buffer (50 mM Tris, pH 8.3, 5 mM EDTA) to 25 µl of purified P-protein in 49 µl of reaction buffer with or without 1 mM Phe. Gel electrophoresis indicated that proteins of 34 and 52 kDa, respectively, were formed when digestion occurred in the presence or absence of Phe.

Recombinant DNA Manipulations and Plasmids—Six plasmids (pSZ41, pSZ42, pSZ44, pSZ50, pSZ70, and pSZ85) were constructed from pJS1, which carries the pheA gene in pUC18 as described previously (24). To delete the potential COOH-terminal domain, two oligonucleotides (33-mer and 31-mer) were synthesized that introduced a stop codon and a HindIII site after residue 300 or 285, respectively, and used as reverse primers for PCR. The M13/pUC universal primer 1224 (Biolabs) was used as the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with (from Biolabs) was the forward primer using pJS1 as a template. PCR was carried out at 95 °C (1 min), 55 °C (1 min), and 72 °C (1.5 min) with
concentration gave an a
(10).
tetramer, and higher order species, as had been noted earlier
shifted the P-protein from a dimer to a mixture of dimer,
caused PDT32 to dimerize (molecular mass 56 kDa), and
change the elution volumes of PDT22, P*300, and R12, but
[3H]Phe
buffer containing 400 pmol of [3H]Phe (specific activity: 2.5 pmol of
monomers. Gel filtration in the presence of Phe (1 mM) did not
the P-protein were dimers, while PDT32, PDT22, and R12 were
28, 65, 12, and 86 kDa, respectively, indicating that P*300 and
R12, and the P-protein were 32, 22, 33.5, 12, and 43 kDa,
electrophoresis (Fig. 1).
while a plot of the 1/v axis intercept obtained from Fig. 4 versus Phe
centrage gave an aKc = 1950 µM.

Thermostability Assay—Purified proteins (10 µmol/liter in 20 mM
Tris, pH 8.0, 10% glycerol, 50 mM NaCl, and 5 mM mercaptoethanol
with or without 1 mM Phe) were incubated at 25–65 °C for 1 h. The
temperature at which 50% (CM or PDT) activity remained after prein-
cubation for 1 h was measured to ascertain the stability of each enzyme.

RESULTS

Expression, Purification, and Characterization of the Engineered Enzymes—Six new plasmids (Table I) were constructed,
sequenced, and expressed in E. coli KS474 and GI724 (Invitrogen),
and the corresponding expressed proteins were designated
with abbreviations indicating both function and size. Thus, PDT22 and PDT32 refer to 22- and 32-kDa proteins
exhibiting only PDT activity, whereas P*300 and P*285 designate COOH-terminal truncated P-proteins exhibiting both CM
and PDT activity, but no feedback inhibition by Phe.
Attempts to express 85- or 121-residue COOH-terminal fragments
of the P-protein to identify the putative regulatory domain failed, as the desired protein could not be detected by
Western blotting of crude cell extracts. A 30-kDa fragment of the P-protein containing residues 114–386 lacked both CM and
PDT activity, but retained Phe binding activity and was designated R30. A fusion protein containing residues 279–386
linked with thioredoxin, designated Thio-R, was successfully
processed and expressed in

| Clones | Protein       | Residues | Crude cell extract activitya | Crude cell extract activityb |
|--------|---------------|----------|-------------------------------|-----------------------------|
| pS81   | P-protein     | 1–386    | +                              | 1–386                       |
| pS9243 | PDT32         | 101–386  | –                              | 101–386                     |
| pS250  | PDT22         | 101–300  | –                              | 101–300                     |
| pS244  | P*300         | 1–300    | +                              | 1–300                       |
| pS270  | P*285         | 1–285    | +                              | 1–285                       |
| pS2741 | R30           | 114–386  | –                              | 114–386                     |
| pS2585 | Thio-R        | 279–386  | ND                            | 279–386                     |

a In all activity assays a crude cell extract from same host strain with
pUC18 was used as a negative control and pS81 was used as a positive control. ND, not determined.
b 20 µl of 5 µg/µl crude cell extract were incubated with binding buffer containing 400 pmol of [3H]Phe (specific activity: 2.5 pmol of
[3H]Phe = 1500 cpm/filter) at a final volume of 100 µl at 37 °C for 2 h. Triplicate 25-µl aliquots were filtered through nitrocellulose disc (13
mm, BA-85, Schleicher & Schuell) and washed three times with cold binding buffer.
c Ref. 24.

Amino-terminal sequence analyses of both PDT32 and PDT22 gave the NH2-terminal sequence MKIV, which matched the sequence of the P-protein starting at residue 101
(proline). The methionine residue had been added for translation
initiation.

Experimental pIs of purified proteins matched predicted pI
values except for PDT22, which had an experimental pI of 6.7 and a predicted pI of 7.2. Yields were typically 6–8 mg/liter for the purified P, P*285, and P*300 proteins and 2–3 mg/liter for the PDT32 and PDT22 proteins.

Phe-Protein Interactions—Fluorescence properties of the intact P-protein changed significantly in the presence of Phe. The
intrinsic fluorescence emission intensity increased and the emission maximum was shifted from 341 to 338 nm in the presence of 1 mM Phe (Fig. 2A). The increase in emission intensity at 340 nm was monitored in titration experiments.
Filtration binding assays revealed that PDT32 bound [3H]Phe P-protein. Earlier work (33) established a maximal stoichiometry of 639 ± 16.100, indicating much weaker cooperativity in Phe binding to each enzyme. To test whether the mutase active site was implicated in allosteric feedback inhibition, Phe concentrations between 0.025 and 0.2 mM, confirming that the substrate saturation curves were hyperbolic for both the CM and PDT activities of the P-protein. At a fixed concentration of chorismate, increasing the concentration of Phe up to 0.4 mM caused only a 10% inhibition of CM activity (data not shown), but resulted in over 90% inhibition of PDT activity (7). The same plot for the inhibition of PDT activity in the presence of the known mutase inhibitor 3-endo-S-exo-8-hydroxy-3-oxabicyclo(3.3.1)-non-6-ene-3,5-dicarboxylic acid (32). The inhibitor had no effect on Phe binding to either protein.

Equilibrium dialysis (31) was performed to determine Kd values for the binding of [3H]Phe to PDT32 and the P-protein. As shown in Table II, PDT32 had ~5-fold weaker binding to Phe than the P-protein, and the CM inhibitor did not change the affinity of the P-protein for Phe.

Activity and Kinetic Characterization—The specific activities with Phe and correlated well with the inhibition of PDT activity in the presence of 1 mM Phe, the emission spectra of PDT32 and R12 displayed a shift to higher wavelengths (335 and 344 nm, respectively; Fig. 2A). The relative fluorescence intensity increased as well. Phenylalanine had no effect on the fluorimetric properties of P*300, P*285, and P*300, which contained Trp226 as the only fluorescent amino acid (emission maxima 332–334 nm) (Fig. 2A). The fluorescence emission maximum of Thio-R (338 nm) was shifted up 0.5 nm after addition of 1 mM Phe. Control experiments with D-Phe and with increased concentrations of L-leucine and L-tyrosine had no effect on the fluorimetric properties of the P-protein and the different domains.

Binding assays using [3H]Phe according to the method of Shiman et al. (29) were performed on PTD32, P*285, P*300, R300, and R12 and compared with controls with the P-protein. Earlier work (33) established a maximal stoichiometry of one Phe binding site per P-protein subunit and showed that Phe regulated the mutase and dehydratase activities. Filtration binding assays revealed that PDT32 bound [3H]Phe at levels comparable to the P-protein, whereas no Phe binding was detected with PTD22, P*285, P*300, or R12.

Scatchard plots for Phe binding to both the P-protein and PTD32 exhibited downward curvature, indicating positive cooperativity in Phe binding to each enzyme. To test whether the mutase active site was implicated in allosteric feedback inhibition, Phe binding assays were performed on both the P-protein and PTD32 in the presence of the known mutase inhibitor 3-endo-S-exo-8-hydroxy-3-oxabicyclo(3.3.1)-non-6-ene-3,5-dicarboxylic acid (32). The inhibitor had no effect on Phe binding to either protein.

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Activity and Kinetic Characterization—The specific activities of both CM and PDT were determined for PTD22, PTD32, P*285, and P*300 at 1 mM chorismate and 0.5 mM prephenate (Table III) and were slightly lower than previously reported (CM = 45 units/mg; PDT = 22 units/mg) (24). All four enzymes retained about 90% of the PTD activity observed in the P-protein. As expected, the PTD32 and PTD22 proteins lacked CM activity.

Phenylalanine had markedly different inhibitory effects on the CM and PDT activities of the P-protein. At a fixed concentration of chorismate, increasing the concentration of Phe up to 0.4 mM caused only a 10% inhibition of CM activity (data not shown), but resulted in over 90% inhibition of PDT activity (Fig. 3A). This result agreed well with a previous report (7). The PTD32 protein showed markedly weaker inhibition of PDT activity by Phe (40% inhibition at 0.4 mM Phe and 85% inhibition at 3.2 mM Phe). Consistent with the Phe binding data, neither P*300 nor PTD22 displayed feedback inhibition. A Hill plot for P-protein inhibition was linear (Fig. 3B, slope = 1.6) at the PTD concentrations between 0.025 and 0.2 mM, confirming that Phe showed cooperative binding in its inhibition of PDT activity (7). The same plot for the inhibition of PDT activity in PTD32 revealed a linear region (slope = 1.1) for Phe concentrations from 0.1 to 3.2 mM, indicating much weaker cooperative interactions.

Steady-state kinetic evaluation of all enzymes in the absence of Phe revealed that the substrate saturation curves were hyperbolic for both the CM and CM activities, giving a linear double-reciprocal plot. The kinetic parameters for the CM and PDT activity of each protein were determined by fitting the

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**TABLE II**

| Samples          | Ke value (mean ± S.D.)* (µM) | n (measurement) | Relative binding (%) |
|------------------|------------------------------|-----------------|----------------------|
| P-protein        | 2.013 ± 0.344                | 7               | 100                  |
| P + CM inhibitor | 1.983 ± 0.298                | 3               | 101                  |
| PDT32            | 9.366 ± 0.412                | 4               | 21                   |
| PDT32 + CM inhibitor | 9.393 ± 1.170          | 3               | 21                   |

* 12 µM enzyme was added to the plus compartment and 36 µM [3H]Phe (0.25 µCi) was added to the minus compartment at a final volume of 100 µl for both, and the chamber was incubated at 30 °C overnight with rotation. 10 µl of sample was withdrawn from each compartment for scintillation counting.

| TABLE III

| Protein          | PTD (SA) Units/mg | CM (SA) Units/mg | % units/mg | % units/mg |
|------------------|-------------------|-----------------|------------|------------|
| P-protein        | 14.81 ± 0.49      | 36.7 ± 1.8      | 1581 ± 78 100 |
| P*300            | 16.85 ± 2.4       | 88.4 ± 1.3      | 1392 ± 42 88.0 |
| P*285            | 17.80 ± 0.3       | 89.0 ± 1.1      | 1348 ± 36 85.3 |
| PTD32            | 18.96 ± 0.5       | 94.2 ± 0.0      | 0          |
| R12              | 24.90 ± 1.5       | 93.0 ± 0.0      | 0          |

* One unit of enzyme was defined as the quantity of enzyme that catalyzed the conversion of 1 mol of substrate to product in 1 min under the assay conditions.

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**Fig. 3.** A, effects of phenylalanine on the PDT activity. A relative activity of 100% corresponds to 0.005 unit of activity. Symbols: ○, P-protein; □, P*300; ◯, PTD32; ×, PTD22; ○, P*285. B, Hill plot for Phe inhibition of the PDT activity. Symbols: ○, P-protein; ◯, PTD32.
initial rate data to the Michaelis-Menten equation (Table IV). Chorismate and prephenate $K_m$ values were comparable with those for the *Alcaligenes eutrophus* bifunctional P-protein ($K_m$ 0.2 mM for CM; 0.67 mM for PDT) (34), but differed from these reported for the *E. coli* P-protein ($K_m$ 0.045 mM for CM; 1.0 mM for PDT) (7). The mutase $k_{cat}$ values for P*285 and P*300, as well as the dehydratase $k_{cat}$ values for all four engineered proteins were close to those measured for the respective activities in the P-protein. Both P*285 and P*300 displayed a larger increase in the $K_m$ for chorismate than for prephenate, resulting in a 3–4-fold decrease in $k_{cat}/K_m$ for CM compared with the P-protein and a 1.5-fold decrease in $k_{cat}/K_m$ for PDT.

Substrate saturation curves for the P-protein at several different concentrations of Phe gave a set of sigmoidal curves with an increase in sigmoidicity as Phe increased. Hill plots of these data gave a Hill constant for prephenate of 1.1 in the absence of Phe, 1.5 at 20 μM Phe, 2.7 at 50 μM Phe, and >3.0 at 200 μM. These results are in good agreement with earlier observations (7), suggesting that a cooperative interaction with prephenate occurred only in the presence of Phe. The same experiments on PDT32 gave hyperbolic curves with increasing apparent $K_m$ values and decreasing apparent $V_{max}$ values as Phe concentrations increased. Double-reciprocal plots of $1/V$ versus $1/S$ were linear for all reactions of PDT32 as shown in Fig. 4. Hill plots of these data were also linear over the prephenate concentration range used and had slopes of 0.97–1.07, respectively. These results showed that unlike the P-protein, PDT32 had no cooperative effects in prephenate binding even in presence of Phe.

The type of noncompetitive inhibition observed with Phe on PDT32 indicated that while prephenate and Phe were bound at separate sites, each affected the affinity of PDT32 for the other.

**TABLE IV**  
Kinetic parameters of cloned P-proteins

| Protein (or + Phe) | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|--------------------|----------|-------|---------------|----------|-------|---------------|
| P-protein          | 1569 ± 59| 549 ± 58| 2.9           | 2342 ± 258| 226 ± 25| 10.4          |
| P*300              | 1484 ± 106| 896 ± 88 | 1.7           | 2440 ± 80 | 705 ± 65 | 3.5           |
| P*285              | 1587 ± 123| 860 ± 89 | 1.8           | 2655 ± 263| 1171 ± 232 | 2.3          |
| PDT32              | 1340 ± 86 | 710 ± 67 | 1.9           |          |       |               |
| PDT32 (no Phe)     | 1362 ± 45 | 625 ± 57 | 2.2           |          |       |               |
| PDT32 + 0.2 mM     | 1213 ± 75 | 822 ± 135| 2.2           |          |       |               |
| PDT32 + 0.5 mM     | 1018 ± 70 | 1264 ± 190| 10.4         |          |       |               |
| PDT32 + 2.0 mM     | 767 ± 77  | 1828 ± 242| 22.6         |          |       |               |

a The concentration of both chorismate and prephenate was limited to 3 mM in the kinetic assays to reduce UV absorbance at high substrate concentrations.

b Purified protein, 100–150 ng, was used in each CM assay and 200–300 ng in each PDT assay.

Fig. 4. Double-reciprocal plots for the inhibition of the dehydratase activity of PDT32 by Phe. Details of the assays are given under "Experimental Procedures." For each assay, 0.20 g of enzyme was used with the concentration of Phe shown. Symbols: E, PDT32 alone; M, PDT32 + 0.2 mM Phe; L, PDT32 + 0.5 mM Phe; △, PDT32 + 2 mM Phe.

Fig. 5. A, thermal inactivation of PDT activity. Symbols: O, P-protein; □, PDT32; ◯, P*300; ×, PDT22; △, P*285. B, thermal inactivation of CM activity. Symbols: O, P-protein; □, P-protein + 1 mM Phe; ◯, P*300; △, P*285; C, effects of Phe on the thermostability of the P-protein and PDT32. Symbols: O, P-protein; □, P-protein + 1 mM Phe; ◯, PDT32; △, PDT32 + 1 mM Phe.

In fact, the PDT32-Phe complex had a 9-fold lower affinity for prephenate than did free PDT32. Moreover, the PDT32-prephenate complex displayed a 9-fold lower affinity for Phe than
did free PDT32. The decrease in $V_{\text{max}}$ with increasing Phe was thus attributed to a decreased abundance of the PDT32-prephenate complex.

**Thermal Inactivation Assay**—All four engineered enzymes, whether mono- or bifunctional, displayed somewhat better thermal stability than the P-protein. Fifty percent inactivation in 1 h was observed at 54 °C for the P-protein, 58 °C for P*285 and P*300 and 62 °C for PDT32 and PTD22. Studies with P, P*285, and P*300 revealed little difference in the thermal inactivation of CM and PDT domains (Figs. 5, A and B). In the case of PTD32, the presence of Phe retarded thermal inactivation, although not as effectively as with the P-protein (Fig. 5C). The presence of Phe also improved the thermostability of the P-protein’s CM domain (Fig. 5B).

**DISCUSSION**

Our results indicate that the PDT domain of the P-protein, like the CM domain (20, 21), could be expressed as a fully active, monofunctional enzyme. Kinetic data on PDT22 (residues 101–300 and P*285 (residues 1–285), together with the absence of PDT activity in R30 (residues 114–386), located the P-protein’s dehydratase domain in residues 101–285. When separated, the individual CM and PDT domains displayed greater robustness, as noted in thermostability assays (Fig. 5). Since smaller domains may refold more easily than larger, multiodomain proteins, this apparent increase in stability might be attributed to more rapid renaturation of the smaller fragments upon cooling.

Both the CM and PDT domains, in their smallest active forms, were unaffected by Phe. This finding was consistent with earlier work of Backman and Ramaswamy (35), who noted that deletion of residues 338–386 in the E. coli P-protein also reduced feedback sensitivity to Phe without destroying catalytic activity. Xia et al. (18) reported that a truncated pheA construct from *Erwinia herbicola* lacking residues 301–387 lost allosteric control, but retained bifunctional catalytic competence.

Both the CM and R-domains exerted discrete effects on the overall structure of the P-protein. In the absence of Phe, proteins lacking the CM domain (PDT32, PTD22, R12) formed monomers, whereas proteins containing the CM domain (P-protein, P*285, P*300) formed dimers. The P*300 protein remained dimeric in the presence of Phe, implicating residues 301–387 lost allosteric control, but retained bifunctional catalytic competence.

Using fluorimetric analysis to elucidate allosteric effects, a specific interaction of the R-domain with Phe was noted, although Phe binding was diminished in the absence of the catalytic domains. Earlier spectrophotometric studies had indicated changes upon Phe binding in the environment of the P-protein’s tryptophan residues, Trp$^{226}$ and/or Trp$^{338}$ (36). More specific and sensitive fluorimetric measurements (Fig. 2A), demonstrated that the conformational changes were localized in the vicinity of Trp$^{338}$. The interaction was specific for L-Phe and led to higher order aggregates of the P-protein, which were not observed with PDT32 and R12.

Feedback inhibition by Phe was more pronounced on PDT than on CM, with inhibition of dehydratase activity in the P-protein increasing sigmoidally with Phe concentration (7). Likewise PDT32 displayed a mixed type of noncompetitive inhibition, with binding of Phe to the R-domain causing a 9-fold lowering in the affinity of the dehydratase active site for prephenate ($\Delta K_I = 1950 \mu M$). The addition of a CM inhibitor to the P-protein did not change Phe binding (Table II), suggesting that the mutase site had little effect on the allosteric site. The value of the intrinsic Phe binding constant ($K_I$) was $\Delta K_I$ folds higher for PDT32 than for the P-protein, which was attributed to the fact that the P-protein formed a mixture of aggregate states in the presence of Phe, each with a potentially different binding affinity.

Several P-protein domain interactions were noted. Removal of the CM domain diminished cooperative binding and affected both the R-domain (through weakened Phe binding) and the PDT domain (through weakened prephenate binding). Removal of the R-domain not only eliminated feedback inhibition, but also decreased the affinity of CM for chorismate. The full effect of feedback inhibition by Phe required a binding domain consisting of the P-protein’s COOH-terminal 101 residues.

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