Regulation of Phenylalanine Biosynthesis. Studies on the Mechanism of Phenylalanine Binding and Feedback Inhibition in the *Escherichia coli* P-Protein†

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ABSTRACT: Isothermal titration calorimetry (ITC) and site-directed mutagenesis were used to study the interaction of Phe with (a) the *Escherichia coli* P-protein, a bifunctional chorismate mutase/prephenate dehydratase that is feedback inhibited by Phe, (b) PDT32, a 32 kDa P-protein fragment (residues 101−386) containing the prephenate dehydratase and regulatory domains, and (c) R12, a C-terminal 12 kDa P-protein fragment (residues 286−386) containing the regulatory domain. ΔH_{total} values for PDT32, which included the heats of Phe binding, conformational change, and dimerization, established that in developing a mechanism for end product feedback inhibition, the P-protein has evolved a ligand recognition domain that exhibits Phe-binding enthalpies comparable to those reported for other full-fledged amino acid receptor proteins. Sequence alignments of R12 with other Phe-binding enzymes identified two highly conserved regions, GALV (residues 309−312) and ESRP (residues 329−332). Site-directed mutagenesis and ITC established that changes in the GALV and ESRP regions affected Phe binding and feedback inhibition to different extents. Mutagenesis further showed that C374 was essential for feedback inhibition, but not for Phe binding, while W338 was involved in Phe binding, but not in the Phe-induced conformational change required for feedback inhibition.

Chorismic acid is a key branchpoint intermediate in the biosynthesis of phenylalanine (Phe),[1] tyrosine, and tryptophan, as well as other aromatic metabolites in the shikimic acid pathway. In *Escherichia coli*, chorismate metabolism is regulated, and physiological levels of aromatic amino acids are maintained, through feedback inhibition mechanisms for each branchpoint enzyme (*1*).

Regulation of Phe biosynthesis is achieved through control of the *E. coli* P-protein, a bifunctional catalyst that converts chorismic acid to phenylpyruvate (*2*). The first step, the Claisen rearrangement of chorismate to prephenate, is catalyzed by chorismate mutase (CM, EC 5.4.99.5). The second step, decarboxylation and dehydration of prephenate to phenylpyruvate, is catalyzed by prephenate dehydratase (PDT, EC 4.2.1.51). A separate enzyme catalyzes the transamination of phenylpyruvate to Phe.

Regulation of both CM and PDT activities in the P-protein is achieved through feedback inhibition by Phe. The wild-type (WT) P-protein, which forms a dimer at the N-terminal CM domain (*3*, *4*), binds Phe with positive cooperativity at elevated ionic strengths (>0.1 mM), and with both positive and negative cooperativity at low ionic strengths (*5*). Inhibition of PDT activity is more pronounced (*2*). Allosteric binding of Phe also results in self-association of P-protein dimers, leading to less active tetrameric and higher-oligomeric species (*6*).

Recent work mapped the CM and PDT catalytic activities to distinct P-protein domains that could be expressed as discrete enzymes with full catalytic activity (*3*). Fluorescence assays involving tryptophan 338 further established that the interaction of Phe with the P-protein was localized in a C-terminal 12 kDa fragment designated R12, comprising residues 286−386. Deletion of R12 led to a fully bifunctional CM−PDT dimer that was insensitive to Phe (*3*). Earlier work had shown that chemical modification of glycine 309 reduced the extent of feedback inhibition by Phe (*7*). Taken together, these data indicated that allosteric regulation was mediated largely by the P-protein's C-terminal regulatory domain (R-domain).

To better understand the nature of feedback inhibition, the interaction of Phe with the P-protein has now been studied using both isothermal titration calorimetry (ITC) and site-directed mutagenesis. Calorimetric titrations of the WT P-protein, and of a 32 kDa fragment comprising residues 101−386 of the P-protein (PDT32), gave ΔH_{total} values for the interaction of Phe with each protein. To pinpoint the locus of Phe interactions, the R12 domain was subcloned and overexpressed, and shown by ITC to possess a fully functional Phe binding site.

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[1] Abbreviations: P-protein, *E. coli* bifunctional chorismate mutase/prephenate dehydratase; Phe, L-phenylalanine; CM, chorismate mutase; PDT, prephenate dehydratase; WT, wild type; R domain, regulatory domain; R12, residues 286−386 of the P-protein; ITC, isothermal titration calorimetry; PDT32, residues 101−386 of the P-protein.

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Sequence alignments with Phe hydroxylase were carried out to identify residues in R12 that might be involved in Phe binding. Appropriate P-protein mutants were prepared by site-directed mutagenesis, and the interaction of Phe with each recombinant mutant protein was monitored by microcalorimetry, fluorimetry, and radioactive binding assays. Certain mutants, which selectively affected either Phe binding or feedback inhibition, shed light on the nature of allosteric effects in the P-protein.

EXPERIMENTAL PROCEDURES

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

Recombinant DNA Manipulations. Plasmid pGP3 encoding the R12 fragment of the P-protein (residues 286−386) was constructed by PCR using pJS1, which carried the *pheA* gene in pUC18 (*8*) as a template. An oligonucleotide, TTGTGGTGTTGG*TCATG*AAAGCCATTAACGTGTC, that initiated translation at residue 286 (underlined) was synthesized containing an initiation codon preceded by a *Bsp*HI site, and was used as a forward primer in PCR with the reverse primer TGTTATTG*CTGCAG*GCGAATGACA containing a *Pst*I site.

A 292 bp trc promoter fragment was obtained by PCR from pBsCM$_2$ (*9*) using the reverse primer CGAATCA*CCATGG*TCTGTTTCCTGTGTG with an introduced *Nco*I site and the forward primer CC*GAATTC*TCATGTTTGACAGGTTA located upstream of the trc promoter in pBsCM$_2$. The resulting PCR products were cut with *Bsp*HI and *Pst*I for the R12 fragment, and *Nco*I and *Eco*RI for the trc promoter fragment. Both were ligated into the pBsCM$_2$ vector (*9*) at *Eco*RI and *Pst*I sites to give pGP3. Sequencing the structural gene and promoter region confirmed the identity of the clone.

Eleven P-protein mutants were created by site-directed mutagenesis using the PCR overlap extension method on pJS1 (*8*) following a published protocol (*10*). Oligonucleotides were synthesized by the Cornell University Biotechnology Facility to mutate A310 to S and V, L311 to S and F, E329 to A, S330 to A, R331 to A and K, C374 to A and S, and W338 to A. The designed primer and universal primer were annealed with a pJS1 template and extended by Expand polymerase (from Boehringer). The amplified DNA was cloned into the parent vector pJS1 at two unique restriction sites and transformed into the host strain NK6024 for expression. A new restriction site was introduced for each of the mutant primers and used for screening the desired clones. All mutant clones were shown by sequencing to possess only the single expected mutation in their structural gene and promoter regions.

Expression and Purification of R12 and P-Protein Mutants. The R12 fragment was overexpressed in LB medium using NK6024 as the host strain. All P-protein mutants were overexpressed in M9 medium following a published procedure (*8*). Expression levels were measured by both Western blotting and activity assays. Purification of R12 was achieved using a variation of the protocol for the P-protein (*11*) as follows. After phenyl-Sepharose chromatography, the protein was loaded on a HPLC porous HQ column (0.5 cm × 5 cm) preequilibrated with 25 mM Tris (pH 8.2) and 10% glycerol, and eluted with a linear gradient (30 column volumes) of 0 to 1.0 M NaCl. Final purification was achieved using a Waters 300 HPLC gel filtration column using an elution buffer, which consisted of 50 mM Tris-HCl (pH 8.2) and 150 mM NaCl. Purification of PDT32, P-protein WT, and P-protein mutants followed the protocol described for truncated P-protein fragments (*3*, *10*).

Table 1: Activities of the WT P-Protein and Mutants

enzyme	catalytic activity: CM (units/mg)	catalytic activity: PDT (units/mg)	[^{3}H]Phe binding activity (pmol of Phe/100 pmol of protein)	feedback inhibition IC_{50} (μM)
WT	36.7 ± 1.8	14.8 ± 0.4	30.3 ± 1.4	70
A310S	36.3 ± 2.0	15.8 ± 0.9	20.6 ± 2.2	336
A310V	32.8 ± 0.8	13.3 ± 0.4	3.1 ± 0.3	na[a]
L311F	36.1 ± 1.2	15.0 ± 0.5	3.2 ± 0.3	na
L311S	33.9 ± 1.1	14.3 ± 0.6	3.4 ± 0.3	na
E329A	33.0 ± 2.3	13.9 ± 1.1	12.3 ± 1.4	>1500
S330A	ND[b]	14.9 ± 0.8	16.1 ± 1.3	780
R331A	35.0 ± 1.8	14.8 ± 1.2	2.8 ± 0.4	>1500
R331K	31.4 ± 2.1	14.3 ± 0.9	12.9 ± 1.3	100
W338A	35.7 ± 0.9	15.8 ± 0.7	3. 5± 0.4	200
C374A	34.5 ± 1.3	14.0 ± 0.9	16.9 ± 1.3	na
C374S	35.6 ± 2.1	14.1 ± 1.5	25.3 ± 1.5	70

[a] Not available. [b] Not determined.

Activity and Phe Feedback Inhibition Assays. Chorismate mutase and PDT activities were assayed as described previously (*3*). The extent of feedback inhibition of PDT activity by Phe was measured at Phe concentrations of 0−3 mM, and IC_{50} values for each protein were determined as the Phe concentration causing 50% inhibition of PDT activity (Table 1).

Western Blotting, Fluorimetry, [^{3}H]Phe Binding Assay, and Gel Filtration. These analytical methods that were used were performed as described previously (*3*). Phe binding assays employed 20 μM [^{3}H]Phe (2.5 Ci/mmol).

Circular Dichroism Analysis. CD spectra of the WT P-protein and each mutant (30 μg/mL) were recorded on a modified Cary model 14 instrument at room temperature (*10*) in 10 mM potassium phosphate buffer (pH 7.0) using a 1 cm path length cell. Spectra were recorded two to four times for each sample from 290 to 190 nm at a scanning rate of 0.5 Å/s. Data were digitized with an integration time of 1 s and stored on a SUN IPC computer.

Titration Microcalorimetry Study. Isothermal titration microcalorimetry was performed using an Omega titration microcalorimeter from MicroCal, Inc. (Northampton, MA). Calorimetry on the WT P-protein and two mutants (A310S and C374A) was performed over the temperature range of 10−35 °C. ITC measurements on PDT32 and R12 were performed over the temperature range of 10−30 °C to minimize thermal decomposition of those proteins. Since the extent of Phe binding to the P-protein was known to be strongly dependent on ionic strength (*3*), 0.1 M NaCl was used in all experiments. Titrations on PDT32 were carried out in (a) 20 mM Tris at pH 8.2 and 7.4, (b) 20 mM phosphate buffer at pH 7.4, and (c) 20 mM HEPES buffer at pH 8.2 using either 88 or 110 μM protein. Unless otherwise stated, all titrations were conducted in 20 mM Tris at pH 8.2. Titrations of the A310S and C374A P-protein mutants were performed using 44 μM protein calculated as a monomer. Protein solutions were dialyzed against 1 L of the assay buffer using Slide A Lyzer cassettes (Pierce, IL).

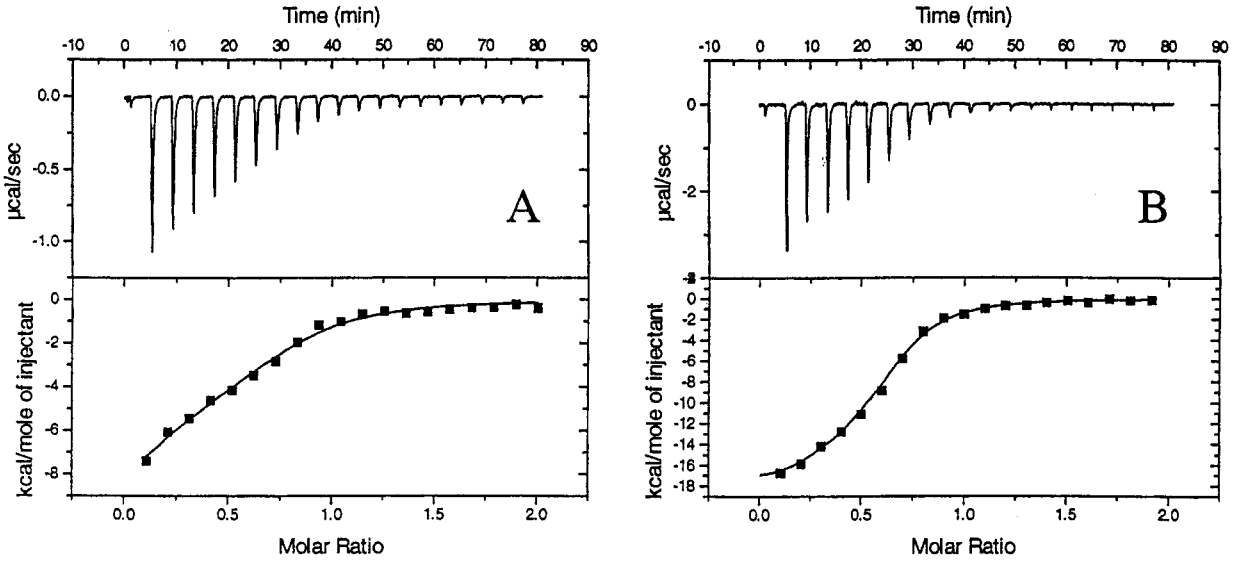

FIGURE 1: Titration calorimetry of the binding of Phe to the P-protein at (A) 15 and (B) 35 °C. Each peak represents the heat resulting from the injection (4 μL) of Phe (3.04 mM) into the P-protein (1.38 mL of an 88 μM solution, calculated as a monomer). Each point in the bottom panel represents the integrated area of the corresponding peak in the upper panel. The solid line represents the best fit to the data as described in Experimental Procedures.

Protein concentrations were determined after dialysis by the Bradford method using bovine serum albumin as a standard (*12)*. Phe was dissolved in the dialysis buffer in concentrations that resulted in a Phe:protein molar ratio of 2 (calculated as a monomer) 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 4 or 2 μL of Phe 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, Inc.).

In titrations of the P-protein and PDT32 with Phe, the cumulative amount of heat that was released that is associated with (a) binding, (b) conformational changes, and (c) oligomerization was integrated to give ΔH_{total} (Figure 1). In titrations of the R12 dimer with Phe, values of ΔH_1 and ΔH_2, representing the enthalpy changes associated with the stepwise binding of Phe to R12, were calculated from the heat of the *i*th injection ΔQ_i. Overall thermodynamic parameters for the interaction of Phe with the P-protein, C374A, and A310S were determined according to a one-set-of-sites model (*13*) utilizing a site concentration which equals 1/P-protein unit. Thermodynamic parameters were calculated from the equation $\Delta G = \Delta H - T\Delta S = -RT \ln K_a$, where ΔG, ΔH, and ΔS represent the changes in free energy, enthalpy, and entropy of binding, respectively, T is the absolute temperature, and $R = 1.989$ cal mol^{-1} K^{-1}.

RESULTS

Expression of R12 and P-Protein Mutants. The expression of R12, a monofunctional Phe-recognizing P-protein domain (residues 286−386), was previously achieved as a thioredoxin fusion protein (*3*). Because of difficulties in obtaining sufficient R12 from the fusion protein, a nonfused R12 coding sequence was genetically truncated from the WT P-protein and overexpressed under the trc promoter, a system that proved to be suitable for the expression of other truncated P-protein fragments (data not shown). While expression levels of R12 were high (~10 mg/L), judging from Western blots, the yield of pure R12 was only 0.7 mg/L of culture because of precipitation during purification and concentration. All P-protein mutants were overexpressed, and the final yields of the purified proteins ranged from 3 to 6 mg/L of culture.

Identification of GALV and ESRP Sites for Mutagenesis. Earlier work established that nitrous acid modification of G309 reduced the extent of feedback inhibition by Phe, and suggested the importance of that region of the P-protein's R-domain (*7*). To ascertain what other residues in R12 might be involved in Phe binding, the NIH protein database was searched for additional Phe-binding proteins. Sequences were found for several mammalian Phe hydroxylases, which belong to a larger class of biopterin-dependent aromatic amino acid hydroxylases that include tyrosine and tryptophan hydroxylases. Sequence homologies indicated that the N-terminal third of each hydroxylase determined substrate specificity, while the remaining two-thirds of the protein constituted the common oxidation apparatus (*14*).

Gel Filtration Studies of R12 and P-Protein Mutants. Gel filtration studies showed that R12 was predominantly dimeric at protein concentrations of <0.4 mM (calculated as a dimer), both in the absence and in the presence of 2 mM Phe. Gel filtration experiments confirmed earlier findings that the WT P-protein formed tetramers and higher oligomers in the presence of Phe (*6*). All mutant P-proteins prepared in this study were dimeric in the absence of Phe, and most formed tetramers and higher oligomers in the presence of Phe (*6*). Three mutants (A310V, L311F, and L311S) remained dimeric even in the presence of 2 mM Phe.

Characterization of P-Protein Mutants. The CD spectra of the purified proteins could essentially be superimposed with that of the wild-type P-protein (data not shown), indicating that no significant disruption in overall secondary structure had occurred. Data in Table 1 indicate that in the absence of Phe, all 11 mutants exhibited levels of CM and

Table 2: Thermodynamic Parameters for Phe Binding at pH 8.2 in Tris Buffer

P-Protein				
T (°C)	$K_{a(av)}$ ($\times 10^5$ M^{-1})	ΔH_{total} (kcal/mol)	ΔG_{total} (kcal/mol)	$T\Delta S_{total}$ (kcal/mol)
10	2.0 ± 0.2	−6.8 ± 0.3	−6.9 ± 0.2	0.1 ± 0.2
15	2.5 ± 0.4	−9.0 ± 0.3	−7.1 ± 0.2	−1.9 ± 0.2
20	4.2 ± 0.3	−10.8 ± 0.3	−7.6 ± 0.06	−3.2 ± 0.1
25	8.4 ± 0.5	−13.1 ± 0.3	−8.1 ± 0.2	−5.0 ± 0.2
30	8.9 ± 0.6	−15.8 ± 0.4	−8.3 ± 0.2	−7.5 ± 0.3
35	10.1 ± 0.7	−16.7 ± 0.4	−8.5 ± 0.3	−8.2 ± 0.3

PDT32			
T (°C)	ΔH_{total} (kcal/mol)	ΔG_{total} (kcal/mol)	$T\Delta S_{total}$ (kcal/mol)
10	−10.4 ± 0.4	−6.9 ± 0.1	−3.5 ± 0.4
20	−14.0 ± 0.2	−7.1 ± 0.02	−6.9 ± 0.2
30	−17.1 ± 0.3	−7.0 ± 0.1	−10.1 ± 0.3

R12		
T (°C)	ΔH_1 (kcal/mol)	ΔH_2 (kcal/mol)
10	−2.8 ± 0.8	−14.5 ± 1.2
20	−2.1 ± 0.4	−8.8 ± 1.1
30	−1.2 ± 0.2	−6.9 ± 0.6

PDT activity comparable to those of the WT P-protein. Representative determinations of K_m and k_{cat} for the PDT activity in the L311F, C374A, and W338A mutants (data not shown) matched values reported for the WT P-protein (*3*), confirming that the catalytic and regulatory domains in the P-protein were essentially independent. As judged from the [^{3}H]Phe binding results for each mutant (Table 1), which were consistent with fluorimetric and calorimetric assays, four mutants (A310V, L311F, L311S, and W338A) lost all or most Phe binding ability.

Analysis of Phe Binding in the P-Protein, PDT32, and R12 by ITC. Values of ΔH_{total} (Table 2), the overall enthalpy (*15*) measured in titrations of the P-protein with Phe, were composed of (a) the heat of Phe binding, (b) the heat of conformational changes, and (c) the heat of tetramer and higher-oligomer formation. Standard deviations were calculated from three measurements at each temperature. The sigmoidal shape of the titration curves at pH 8.2 in Tris buffer (Figure 1) confirmed the positive cooperative homotropic interactions in Phe binding to the P-protein noted earlier by Baldwin et al. using ultracentrifugation techniques (*6*).

Similar titrations were also performed with PDT32, which lacked the CM domain and was thus monomeric (Table 2). In defining ΔH_{total} for PDT32, a dimerization term replaced the heat of tetramer and higher-oligomer formation. ITC titrations on PDT32 were conducted in three buffers over the pH range of 7.4−8.2, and the energies proved to be independent of buffer at a given pH (Table 3). Values of ΔH_{total} for PDT32 were more negative at pH 7.4 than at pH 8.2 and, as for the P-protein, became more negative with increasing T.

ITC experiments on Phe binding to R12 were carried out at a protein concentration of 0.11 mM (calculated as a dimer) with 10.1 mM Phe, and established a 2:1 interaction of Phe with the R12 dimer. Gel filtration data showed that R12 remained dimeric under these conditions. The derived thermodynamic values for stepwise binding of Phe to the R12 dimer (Table 2) indicated negative cooperativity that was more pronounced at elevated temperatures (*16*).

Analysis of Phe Binding in P-Protein Mutants by ITC. Titrations of two mutant proteins (A310S and C374A) with Phe were monitored by ITC, and gave ΔH_{total} values listed in Table 4. None of the other mutants exhibited quantifiable levels of Phe binding, for which the detection limit (K_a) was estimated to be $<5 \times 10^{-3}$ M^{-1} (*13*). Using ΔH_{total}, a model for binding with a 1:1 stoichiometry (*13*) was developed to determine the average binding constant $K_{a(av)}$ for binding of Phe to one receptor site of the P-protein or the mutants (Table 4). While the A310S and C374A mutants exhibited somewhat weaker binding, the temperature dependence of ΔH_{total} (Table 4) paralleled that displayed by the P-protein, suggesting similar mechanisms of binding and oligomerization in the P-protein mutants.

Analysis of Feedback Inhibition in P-Protein Mutants. Using the standard PDT activity assay to monitor feedback inhibition, three mutants in the hydrophobic GALV region (A310V, L311F, and L311S) were shown to be insensitive to feedback inhibition by Phe at concentrations up to 3 mM, whereas A310S was weakly regulated by Phe (Figure 2A). Four mutants in the ESRP region (E329A, S330A, R331A, and R331K) retained some or all of their capacity for feedback inhibition (Figure 2B). In the case of the C374A mutant, the extent of feedback inhibition by Phe was significantly reduced, although Phe binding was largely unaffected (Figure 2C). Interestingly, mutation of C374 to serine had no effect on Phe binding or feedback inhibition. In the case of the W338A mutant, feedback inhibition by

Table 3: Effect of Buffer and pH on the Thermodynamic Parameters of Phe Binding to PDT32

	T (°C)	$K_{a(av)}$ ($\times 10^5$ M^{-1})	ΔH_{total} (kcal/mol)	ΔG_{total} (kcal/mol)	$T\Delta S_{total}$ (kcal/mol)
pH 8.2, HEPES	20	0.8 ± 0.1	−14.3 ± 0.2	−6.6 ± 0.07	−7.7 ± 0.2
pH 8.2, HEPES	30	1.2 ± 0.06	−17.4 ± 0.2	−7.1 ± 0.03	−10.3 ± 0.2
pH 7.4, Tris	20	1.3 ± 0.1	−15.1 ± 2.8	−6.9 ± 0.07	−8.2 ± 2.8
pH 7.4, Tris	30	0.9 ± 0.1	−20.2 ± 3.5	−6.9 ± 0.07	−13.3 ± 3.5
pH 7.4, phosphate	20	2.6 ± 0.2	−15.9 ± 0.3	−7.3 ± 0.04	−8.6 ± 0.3
pH 7.4, phosphate	30	0.9 ± 0.03	−23.5 ± 1.5	−6.9 ± 0.02	−16.6 ± 1.4

Table 4: Thermodynamic Parameters for Phe Binding to Two P-Protein Mutants

	T (°C)	$K_{a(av)}$ ($\times 10^5$ M^{-1})	ΔH_{total} (kcal/mol)	ΔG_{total} (kcal/mol)	$T\Delta S_{total}$ (kcal/mol)
A310S mutant	12	0.75 ± 0.2	−4.3 ± 0.8	−6.4 ± 0.3	2.1 ± 0.7
A310S mutant	35	5.5 ± 0.5	−8.7 ± 0.5	−8.2 ± 0.2	0.53 ± 0.1
C374A mutant	10	1.3 ± 0.3	−7.6 ± 0.2	−6.7 ± 0.1	−0.9 ± 0.4
C374A mutant	15	1.7 ± 0.5	−9.6 ± 0.5	−7.0 ± 0.3	−2.6 ± 0.6
C374A mutant	25	2.2 ± 0.4	−12.1 ± 0.4	−7.4 ± 0.2	−4.7 ± 0.5

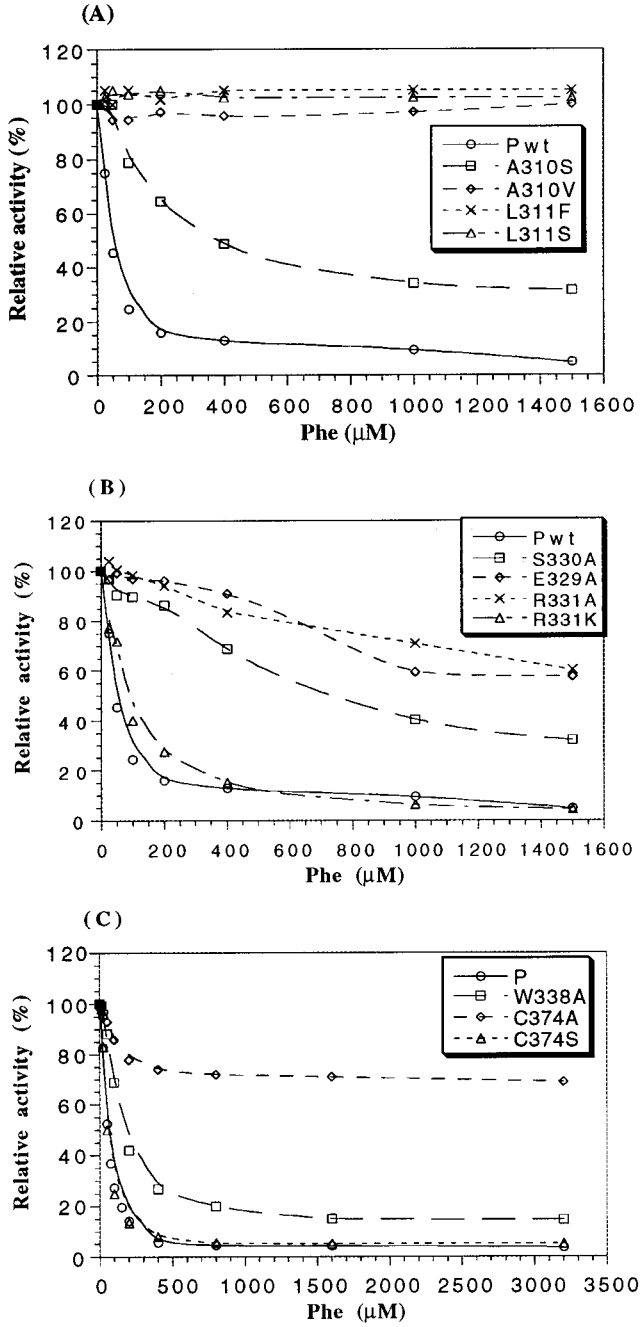

FIGURE 2: Effects of phenylalanine on PDT activity. A relative activity of 100% corresponds to 0.005 unit of activity for the WT P-protein and all mutants: (A) GALV mutant and WT P-protein data, (B) ESRP mutant and WT P-protein data, and (C) C374S, C374A, W338A, and WT P-protein data.

Phe was still observed despite a 90% drop in the level of Phe binding.

DISCUSSION

The interactions of Phe with the WT P-protein and with the subcloned, overexpressed PDT32 and R12 domains were monitored by ITC to gain quantitative thermodynamic information about ligand binding leading to feedback inhibition. Calorimetric measurements with PDT32 were performed in several different buffers and at two different pHs (Table 3). The results, which were consistent and reproducible, established that the system was well-behaved and not sensitive to subtle pH or buffer ion effects.

The calorimetric data further revealed similar thermodynamic profiles for the interaction of Phe with the WT P-protein and with PDT32, suggesting that the CM domain had little effect on the overall interaction of Phe with the P-protein. Values of ΔG were slightly more exergonic for the P-protein, as might be expected since this dimeric structure was capable of higher-order multimerization.

Values of ΔH_{total} for the WT P-protein and PDT32 (Table 2) included the heat of Phe binding, the heat of any conformational changes, and the heat of association into dimers (PDT32) or higher oligomers (P-protein). Interestingly, ΔH_{total} values for titrations of the smaller PDT32 fragment with Phe were similar to ΔH values for the interaction of dedicated amino acid-binding proteins with their ligands. For example, ΔH for serine binding to its cognate receptor in *E. coli* at 27 °C was determined to be −18 kcal/mol (*17*). At 25 °C, ΔH for binding of valine and isoleucine to *E. coli* threonine deaminase was found to be −18.4 and −12.3 kcal/mol, respectively (*15*). Thus, in developing a mechanism for end product feedback inhibition, the P-protein has evolved a ligand recognition domain that exhibits Phe-binding enthalpies comparable to those reported for other full-fledged amino acid receptor proteins.

Fluorescence assays and chemical modification studies implicated a C-terminal 100-residue fragment of the P-protein in Phe binding and oligomerization (*3*). The R12 domain was therefore subcloned and overexpressed in an effort to investigate Phe binding to this region by ITC, and to gain insight into the mechanism of feedback inhibition. Unexpectedly, the R12 domain spontaneously dimerized in the absence of Phe, suggesting that its native conformation had been altered by truncation. Nevertheless, ITC was used to obtain thermodynamic parameters for the interaction of Phe with the R12 dimer (Table 2). Stepwise binding of Phe occurred with negative cooperativity ($K_2 < K_1$), which was more pronounced at elevated temperatures (data not shown). Such allosteric effects are well-known in tyrosyl-tRNA synthase (*18*) and glyceraldehyde-3-phosphate dehydrogenase (*19*), which are oligomeric enzymes composed of identical subunits. Although spontaneous dimerization of R12 precluded any study of Phe-induced oligomerization, ITC established that the R12 fragment played a significant role in binding the amino acid ligand.

When the BLAST search algorithm was used to perform sequence alignments of R12 and the N-terminal domain of Phe hydroxylase, two highly conserved sequences were identified. The first was a hydrophobic GALV sequence comprising residues 309−312. This region of R12 represented the locus of maximum hydrophobicity. To evaluate its importance in Phe binding and/or feedback inhibition, four mutations in the GALV region were introduced. Mutants A310S and L311S decreased the hydrophobicity, whereas A310V and L311F increased the hydrophobicity, according to the solution scale of Engelman et al. (*20*). The A310S mutant retained some residual Phe binding and feedback inhibition activity, whereas the A310V mutant exhibited no Phe binding and no feedback inhibition activity. These findings suggested that a side chain that was too large, however hydrophobic, might impair Phe binding. Mutation of L311 to either serine or phenylalanine disrupted all Phe binding and feedback inhibition. Taken as a whole, the mutagenesis data indicated that besides hydrophobicity, the

GALV region exhibited other structural requirements that are important for Phe binding.

The second conserved region was the ESRP (residues 329−332) sequence, which comprised the hydrophilic maximum within R12. Four site-directed mutants in this polar locus (E329A, S330A, R331A, and R331K) affected Phe binding and feedback inhibition to different extents. The R331A mutant protein exhibited binding only using the most sensitive fluorimetric assay, and feedback inhibition only at elevated Phe concentrations. In contrast, R331K exhibited only a slightly reduced level of binding, indicating the importance of the positive charge at position 331. The long-known requirement for a carboxylic acid group in Phe for inducing feedback inhibition (*2*) suggested that R331 might directly interact with this acidic group. Alanine mutations at E329 and S330 also reduced the level of, but did not completely abolish, Phe binding.

ITC studies of the A310S mutant revealed that *T*-dependent enthalpy changes in the presence of Phe followed the same trend observed for the WT P-protein, suggesting similar mechanisms of binding and oligomerization in the P-protein mutants. However, significantly lower $K_{a(av)}$ values were noted for A310S (Table 4). These results suggested that at least one mutant in which Phe binding was moderately attenuated could still achieve the requisite conformational changes leading to oligomerization.

The correlation between Phe binding and feedback inhibition noted in the WT P-protein and several mutants (Table 1) suggested a linkage between the two phenomena. However, studies of two additional mutants led to a different mechanistic picture. Mutations at C374 were investigated because chemical modification studies of the P-protein using 5,5′-dithiobis(2-nitrobenzoate) (*21*) had established the importance of one or more cysteine residues in feedback inhibition. Calorimetric measurements on the C374A mutant revealed only a slightly diminished ability to bind Phe, with the expected positive cooperativity. However, feedback inhibition of PDT activity was largely absent in this mutant. In the case of the C374S mutant, both Phe binding and feedback inhibition were retained. The behavior of the C374A mutant suggested that a discrete structural element distinct from the Phe-binding domain was required for feedback inhibition, perhaps involving some Phe-induced conformational change. Such a coupling of Phe binding and feedback inhibition might be mediated by a hydrogen bond involving either cysteine or serine (but not alanine) at residue 374.

Tryptophan 338, which had been implicated in Phe binding by fluorescent assays (*3*), was also examined by site-directed mutagenesis. In the case of the W338A mutant, the level of Phe binding was diminished by 90% compared to that of the WT P-protein, with little effect on feedback inhibition. Moreover, the W338A mutant formed Phe-induced oligomers as extensively as the WT P-protein. These findings suggested that residue 338 was involved in the recognition and binding of Phe, but was not a critical structural element in the Phe-induced conformational change required for feedback inhibition and Phe-mediated oligomerization.

In summary, the combination of site-directed mutagenesis and ITC provides evidence for an independent regulatory domain in the P-protein that is spatially and functionally distinct from the catalytic (CM and PDT) domains. Two conserved sequence motifs in this R-domain, GALV and ESRP, are required for Phe binding. Additional mutagenesis studies have identified P-protein mutants that are defective either in Phe binding or in feedback inhibition, suggesting that the process of Phe-induced feedback inhibition involves distinct structural elements within the R-domain that are coupled, perhaps by an obligatory Phe-induced conformational change.

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SUPPORTING INFORMATION AVAILABLE

Experimental titration curves depicting Phe binding to R12 as monitored by ITC and derivation of a two-site binding model. This material is available free of charge via the Internet at http://pubs.acs.org.

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