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An Engineered Chorismate Mutase with Allosteric Regulation

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Abstract—Besides playing a central role in phenylalanine biosynthesis, the bifunctional P-protein in *Eschericia coli* provides a unique model system for investigating whether allosteric effects can be engineered into protein catalysts using modular regulatory elements. Previous studies have established that the P-protein contains three distinct domains whose functions are preserved, even when separated: chorismate mutase (residues 1–109), prephenate dehydratase (residues 101–285), and an allosteric domain (residues 286–386) for feedback inhibition by phenylalanine. By deleting the prephenate dehydrase domain, a functional chorismate mutase linked directly to the phenylalanine binding domain has been engineered and overexpressed. This manuscript reports the catalytic properties of the mutase in the absence and presence of phenylalanine.

Introduction

Among various regulatory mechanisms used by proteins to achieve functional control, allosteric interactions have been shown to play important roles in regulating substrate binding, product release, and other cooperative phenomena in enzyme catalysis.[1] In order to achieve similar types of control as part of the rational design of new proteins, it would be desirable to know whether specific regulatory motifs in naturally occurring systems might be adapted for use as self-standing (i.e., modular) domains in protein engineering. It would also be of interest if such elements could be tuned to respond either to specific biological substances, or perhaps even to synthetic compounds.

The biosynthesis of the aromatic amino acids phenylalanine (Phe) and tyrosine (Tyr) offers several opportunities to explore this question.[2] 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 catalyzed by prephenate dehydratase (PDT, EC 4.2.1.51), or decarboxylation/dehydrogenation catalyzed by prephenate dehydrogenase (PDH; EC 1.3.1.12). In *Escherichia coli*, two bifunctional proteins (the P- and T-proteins) have evolved for Phe and Tyr biosynthesis, respectively, and each is regulated by its respective pathway end-product.

Recent protein engineering studies from our laboratory established that the P-protein contained three distinct domains whose functions were preserved when the domains were expressed as fragments.[3] The two catalytic domains, CM (residues 1–109) and PDT (residues 101–285), retained full functional activity, even when separated. A separate allosteric domain (residues 286–386), which was responsible for feedback inhibition by Phe, was also expressed. Phe binding to that domain was shown by microcalorimetry to be substantially retained, inducing conformational changes comparable to those associated with allosteric regulation of the P-protein.[4] Although the detailed structural factors and domain interactions responsible for feedback inhibition in the P-protein remain unknown, it was of interest to investigate whether the Phe-binding domain of the P-protein might function as a 'modular' regulatory element when physically joined to an independent protein catalyst. If so, the allosteric domain of the P-protein might serve as a model for assembling unique allosteric regulatory elements or biosensors capable of functioning in conjunction with other target proteins. Here we disclose the properties of a hybrid CM, designated EcCM-R (for *E. coli* chorismate mutase, regulated), which was constructed by directly joining the CM

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(residues 1–101) and allosteric (residues 286–386) domains of the P-protein.[4]

Results and Discussion

Design considerations

The design of EcCM-R was guided by insights gained from an extensive body of knowledge on the *E. coli* P-protein. It has long been known that Phe binding to the C-terminal region led to the formation of higher-order oligomers in which PDT activity was more strongly inhibited than CM activity,[5] suggesting that allosteric control was influenced by proximity. Consistent with that finding, removal of C-terminal residues by limited proteolysis was shown to reduce the affinity of the N-terminal CM region for chorismate, indicating that the allosteric and CM domains interacted, either directly or indirectly.[6] We were therefore interested in deleting as much of the PDT domain as possible, so as to position the CM and R domains in close proximity.

With that goal in mind, EcCM-R was genetically engineered as a 203-residue protein. The mutase component was truncated to 101 residues, rather than the previously reported 109 residues, based on the following considerations. First, X-ray crystallographic analysis of the engineered 109-residue CM revealed significant disorder beyond residues 95–100.[7] Additionally, sequence alignment studies indicated that only the first 100 residues of the *E. coli* P-protein showed substantial homology with *S. cerevisiae* CM, whose overall 3-dimensional stucture was closely comparable.[8] Third, earlier subcloning studies[3] revealed that residues 101–113 of the P-protein constituted an integral part of the PDT domain. Therefore, those residues were presumed to be unnecessary for CM catalysis. The design of the allosteric domain was guided by earlier studies,[4] which established that residues 286–386 of the P-protein were required for full Phe binding activity, based on microcalorimetry. One additional glycine residue was introduced in the PCR-based cloning step when joining the catalytic and allosteric domains. The resulting 203-residue EcCM-R was well-expressed and stable.

Protein expression and purification

Western blotting showed that the chimeric EcCM-R could be expressed and appeared to be stable in a cloned NK6024 strain after overnight growth. However, initial attempts to purify the protein from NK6024 using M9 medium failed. By switching to strain KS474 as a low-protease host, it was possible to obtain pure EcCM-R from M9 medium (1 mg/L culture). Although the yield of EcCM-R was lower than that of the previously prepared 109-residue CM (6 mg/L culture),[9] it was comparable to the yield of the allosteric domain when expressed alone (0.7 mg/L culture).[4] The diminished yield of EcCM-R might be due either to lower expression levels or to instability of the chimeric protein during purification. Since the low protease strain KS474 gave better results, degradation of EcCM-R during expression and purification seemed likely.

The EcCM-R protein was purified on a Q-Sepharose column using conditions that had previously been developed for the 109-residue CM domain[9] and allosteric domain (i.e., residues 286–386 alone).[4] As expected, EcCM-R bound more tightly to Q-Sepharose, requiring a 0–400 mM NaCl gradient, than did the 109-residue CM domain, which eluted in a 0–100 mM NaCl gradient.[9] As a further indication of its hybrid character, EcCM-R eluted more readily than did the allosteric domain alone, which required a 0–1.0 M NaCl gradient.[4] Analysis of EcCM-R by SDS–PAGE revealed the presence of minor impurities, and established a MW of 23 kDa (Fig. 1).

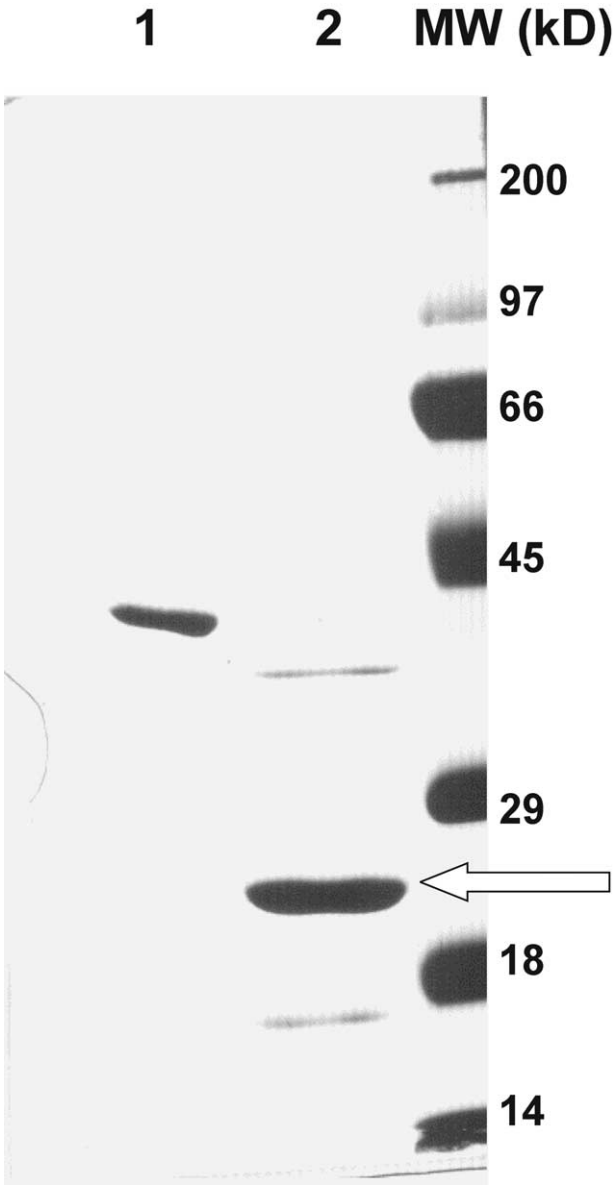

Figure 1. Electrophoresis of purified EcCM-R and P-protein on a 12% SDS gel. The molecular weight markers are indicated in kD at the right. Lane 1: 1 μg of P-protein and Lane 2: 2.5 μg of EcCM-R protein.

Characterization of the purified EcCM-R protein

Gel filtration analysis in the absence of Phe indicated that EcCM-R formed a noncovalent dimer. This finding was in agreement with earlier studies, which established that the 109-residue CM subcloned from the P-protein also formed a dimer in the absence of Phe.[9] The existence of wild-type P-protein as a dimer has been attributed to noncovalent interactions involving the N-terminal CM domain.

Gel filtration in the presence of Phe showed that EcCM-R remained dimeric, even at concentrations up to 2 mM. This result contrasted with earlier findings on the P-protein and a C-terminal fragment designated PDT32 (residues 101–386).[3] PDT32 was monomeric in the absence of Phe, but formed a dimer in the presence of Phe. Taken together, these data suggested that oligomerization of the P-protein in the presence of Phe involved Phe-induced conformational changes in the C-terminal region leading to noncovalent association. Radiolabelling experiments in which 4 μM EcCM-R was incubated with 400 μM labelled Phe confirmed that EcCM-R retained its ability to bind Phe. Using wild-type P-protein as a positive control (100% binding activity), EcCM-R exhibited 112% Phe binding and PDT32 exhibited 101% Phe binding. As expected, residues 1–109 of the P-protein showed no Phe binding activity.

Kinetic analysis of EcCM-R

The mutase specific activity in EcCM-R was determined by monitoring the conversion of chorismate to prephenate at 37 °C in 50 mM tris buffer (pH 7.8) and was compared to the wild-type P-protein and the 109-residue CM derived therefrom (Table 1). Since KS474 had the *pheA+ tyrA+* genotype, it was necessary to rule out any contaminating CM activity from adventitious P-protein and also from the bifunctional *E. coli* T-protein. Therefore, EcCM-R was assayed for both PDT and PDH activity using 5–20 times the usual quantity of protein. No detectable levels of either enzymatic activity were observed. In the absence of Phe, the specific activity of EcCM-R was substantial, comprising 74% of the CM activity of the P-protein and 78% of the 109-residue CM fragment (Table 1).

Steady-state kinetic evaluation of EcCM-R both in the absence and presence of Phe revealed substrate saturation curves that were hyperbolic for CM activity, giving rise to linear double-reciprocal plots. Values of K_m and V_{max} for each sample were then determined by fitting initial rate data to the Michaelis–Menten equation (Table 2). The kinetic results revealed significant differences in both k_{cat} and K_m values when EcCM-R was compared with the 109-residue CM.

In the absence of Phe, the value of k_{cat} for EcCM-R was about double that for the CM domain alone. Such a modest effect on turnover number upon attachment of the allosteric domain to the CM domain might be caused by conformational changes in the active site that would affect substrate binding and/or dissociation of the respective enzyme-product complexes. The K_m of EcCM-R was about 6-fold higher than that of the 109-residue CM, providing additional evidence that the hybrid enzyme had a reduced affinity for substrate. Overall, a comparison of k_{cat}/K_m, values for both enzymes indicated that EcCM-R was a less efficient mutase catalyst.

When EcCM-R was assayed in the presence of Phe, progressive reductions in the value of K_m were observed (Table 2). Likewise, the value of k_{cat} dropped at low concentrations of Phe, suggesting that steric and/or other interactions between the allosteric and catalytic domains could be modulated by the presence of Phe. While the allosteric domain affected access to the mutase active site, the effect was not reciprocal, since radiolabelling studies indicated that Phe binding was not diminished in EcCM-R. Addition of Phe at low concentrations apparently induced conformational changes in the allosteric domain that disengaged the two regions, thus relieving some of the active site distortions induced at the mutase catalytic pocket.

Values of k_{cat}/K_m for EcCM-R continued to rise up to 2 mM Phe, demonstrating the classic profile of non-essential allosteric activation (Fig. 2). Ultimately, the mutase activity of EcCM-R increased 220% in the presence of Phe (Fig. 2), achieving half-maximal activation at 200 μM Phe under assay conditions with 1 mM chorismate as substrate. Although the catalytic efficiency of EcCM-R did not achieve that of the wild-type P-protein, EcCM-R was nearly as efficient the 109-residue CM domain in the presence of 2 mM Phe. These results suggested that Phe binding caused an allosteric change that restored catalytic efficiency to the CM

Table 1. Specific activity of EcCM-R protein

Clone	Protein	PDT (SA)			CM (SA)		
		u/mg[a]	u/μmol	(%)	u/mg	u/μmol	(%)
pJS1	P protein	14.8±0.4[b]	639±16	100	36.7±1.8	1581±78	100
pJS47	EcCM-109	0[c]	0	0	124±19	1476±126	94
pSZ84	EcCM-R	0	0	0	53.1±0.7	1168±16	74
pSZ84	EcCM-R + 100 μM Phe	0	0	53.0±0.8	1166±17	74	
pSZ84	EcCM-R + 400 μM Phe	0	0	91.8±8.9	2020±196	128	
pSZ84	EcCM-R + 1600 μM Phe	0	0	106.2±6.7	2336±147	148	

[a]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.
[b]The values represent the mean±SD of three to five measurements.
[c]No detectable level of PDT activity was observed with 20 times the usual quantity of protein.

Table 2. Kinetic parameters of P-protein and EcCM-R

Protein	Prephenic acid			Chorismic acid[a]			
	k_{cat} (min^{-1})[b]	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})	
P protein	1569±59	549±58	2.9	2342±258	226±25	10.4	(100%)
CM(109)	n/a	n/a		2482±287	296±10	8.4	81
EcCM-R	n/a	n/a		6094±522	2547±296	2.4	23
EcCM-R +50 μM Phe		n/a		3902±373	1036±175	3.8	36.5
EcCM-R +500 μM Phe		n/a		4223±267	628±84	6.7	64.4
EcCM-R +2000 μM Phe		n/a		4299±120	591±36	7.3	70

n/a = not available.
[a]The concentration of chorismate was limited to 3 mM in the kinetic assays to reduce UV absorbance at high substrate concentrations.
[b]In calculating k_{cat}, protein concentrations were measured by the Bradford assay, and calculated mass was used as MW.

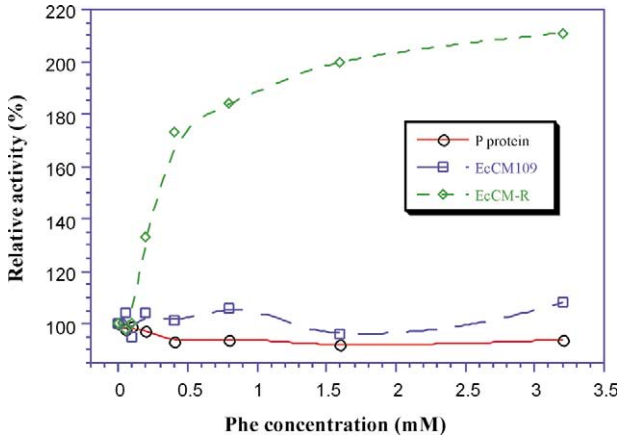

Figure 2. Effects of phenylalanine on the chorismate mutase activity. A relative activity of 100% corresponds to 0.008 unit of activity.

domain. Control experiments (Fig. 2) gave the expected results when Phe was added to the P-protein (10% inhibition of CM; 90% inhibition of PDT) and to the 109-residue CM (no change).

Conclusion

The inhibition or activation of enzymes by means of allosteric effects usually involves a distinct regulatory site, either elsewhere on the same polypeptide chain, or on a separate subunit or chain. Efforts to probe the structural factors that contribute to such allosteric control have utilized both random and site-directed mutagenesis of regulatory sites. In some instances, chimeric enzymes have been assembled in which the catalytic and regulatory subunits and chains have been reconfigured or hybridized.[10,11] To the best of our knowledge, however, the present work represents the first attempt to graft a well-characterized regulatory element directly to the C-terminal of an enzyme with which it had co-evolved. The resulting hybrid protein, EcCM-R, displayed the classic profile of allostery, although the activation of mutase activity by Phe was unexpected.

The fact that EcCM-R displayed both mutase activity and Phe binding indicated that at least the molecular recognition capabilities of each subunit were retained in the engineered protein. Moreover, the ready association of EcCM-R into a stable noncovalent dimer also indicated that the attached allosteric site did not disrupt the native conformation of the mutase domain. While the mechanism of interaction of Phe with EcCM-R remains unclear at this time, of particular interest was the finding that allosteric activation, and not inhibition, was observed. Phe-induced association of the regulatory sites in EcCM-R was clearly compromised, which may explain the failure to observe Phe-induced inhibition in EcCM-R. That result suggests that the inclusion of additional residues within the PDT domain might be essential for dimer/oligomer formation. X-ray crystallographic analysis should provide additional useful insights into the organization of subdomains in EcCM-R.

Experimental

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 and diluted with cold Phe.

Strains

E. coli NK6024 (relevant genotype: pheA$^-$ tyrA$^+$) was used as the host for cloning and plasmid isolation.[9] *E. coli* strain KS474 (relevant genotype: *degP*41{D*Pst*I-*Kan*I}) was used as the host for expression and purification.[9] Unless indicated, strains harboring plasmids were grown in either M9 medium, Luria broth or Luria agar plates all containing 100 μg/mL of ampicillin.

Recombinant DNA manipulations and plasmids

Plasmid pSZ84 encoding the EcCM-R hybrid protein (residues 1–101 and 286–386 of P protein) was constructed by PCR using vector pJS1, which carried the *pheA* gene in pUC18 as a template.[12] For amplifying the EcCM domain, a DNA fragment was synthesized containing the *pheA* gene promoter region linked to a 33 mer oligonucleotide, 5′-GATGCGTGCTGA*CCCGG*-*G*ATTAATTTTATTGAG -3′ that introduced a *Sma* I site (italized with the mismatched bases being highlighted) after residue #101, and was used as a reverse primer for PCR. While the M13/pUC universal primer

#1233 (Biolabs) was used as the forward primer. For amplifying the regulatory fragment (R), a DNA fragment was synthesized containing a 31 mer primer, 5′-TGTGGTGTTG*CCCGGG*AAAGCCATTAACGTG-3′, that initiated translation at residue 286 (underlined) preceded by a *Sma* I site, and was used as forward primer in PCR with the synthesized reverse primer 5′-CATCATCCGG*AAGCTT*TTCATCAGG-3′. The reverse primer contained an introduced *Hind*III site and annealed to the downstream of *pheA* gene. The PCR was carried out at 95 °C (1 min), 55 °C (1 min) and 72 °C (1 min) with 35 cycles. The resulting 990 bp and 340 bp PCR products were cut with *Eco*RI and *Sma* I for EcCM fragment, and *Sma* I and *Hind*III for the regulatory fragment respectively. The desired DNA fragments were isolated on a 1.5% agarose gel, ligated into pUC18 at *Eco*RI and *Hind*III sites, and transformed into NK6024 by the TSS method.[13] The resulting transformants were screened by restriction site analysis and confirmed by western blot using P protein antibody. The positive plasmid was designated pSZ84.

The PCR amplified fragment in pSZ84 clone was further sequenced on an ABI Model 373A DNA Sequencer in the Cornell Biotechnology Facility. The nucleotide sequences and deduced peptide sequences were analyzed using the DNASTAR Program. The pSZ84 clone was expected to produce a 203-residue EcCM-R protein containing residues 1–101 for EcCM domain and residues 286–386 for R domain with an extra glycine residue (introduced by part of *Sma* I site) between two domains. The calculated mass of EcCM-R was 22832.56 Da and the calculated isoelectric point was 6.17.

Expression and crude extract preparation

Cell pellets and crude cell extracts were screened by Western blotting to confirm expression in *E. coli*. Proteins were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and detected with anti-P-protein antibody as described.[9] Cell extracts were prepared as before,[12] and were tested for CM, PDT, and Phe binding activities using a pUC18 cell extract as a negative control.

Purification of EcCM-R

EcCM-R was purified by the published procedure[9] with several modifications. Freshly transformed colonies containing the desired plasmid in KS474 were inoculated into 40 mL of LB culture and grown with rotary shaking at 37 °C until the $OD_{600\ nm}$ reached 1.0. The LB culture was used to inoculate 2 L of M9 medium supplemented with 0.8% of glucose. After growth at 37 °C for about 4–5 h ($OD_{600\ nm}$ around 0.5) the culture was induced with 0.5 mM IPTG and allowed to grow overnight. Cells were harvested by centrifugation, the pellet was resuspended in 20 mL of 100 mM tris (pH 8.2), 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride and French pressed at 10,000 lb/sq. inch. The extract was centrifuged at 14,000 rpm for 30 min and 0.15 volume of 30% streptomycin sulfate was added to the supernatant. After centrifugation, the supernatant was adjusted to 0.6 M with NaCl and loaded on a Phenyl-Sepharose column (2×15 cm) equilibrated previously with 20 mM tris pH 8.2, 0.5 mM EDTA, 5 mM β-mercaptoethanol, and 0.6 M NaCl. The column was then washed with 20 mM tris pH 8.2, 0.5 mM EDTA, 5 mM β-mercaptoethanol buffer. The interesting EcCM-R protein was eluted with water and 50% ethylene glycol. The resulting fractions containing CM activity were combined and loaded on a Q-Sepharose column (2×10 cm) equilibrated previously with 20 mM tris pH 8.2, 0.5 mM EDTA, 5 mM β-mercaptoethanol and 10% glycerol. The EcCM-R protein was eluted with a linear gradient (2×300 mL) of 0–400 mM NaCl in above equilibration buffer. The protein purity of active fractions from Q-column were assessed on a 15% SDS-gel. The fractions with above 90% purity were combined and concentrated. Protein concentration was measured by the Bradford assay using BSA as the standard.[14]

Molecular weight estimation

The molecular weight of purified EcCM was estimated by gel-filtration on a Waters Protein Pak Glass 300SW HPLC column.[3] Samples were run in 25 mM Tris pH 7.8 containing 50 mM NaCl with or without 1 mM Phe at the flow rate 0.1 mL/min. Molecular weights of each protein were estimated from elution volumes (relative to standards of known molecular weight run under the same conditions).

Phe binding assay

Filtration binding assays were performed by measuring enzyme bound Phe adsorbed to a nitrocellulose filter following the published procedure[15] used in earlier work,[3] but adapted to a higher Phe concentration. The binding and wash buffer were 20 mM Tris pH 8.2, 10 mM EDTA, 0.01% BSA, 20 mM mercaptoethanol and 100 mM KCl. The binding of the proteins with ^{3}H-Phe was run at a constant enzyme concentration of 4 μM incubated with a constant ratio of ^{3}H-Phe/protein from 100 to 1. Purified proteins (400 pmol) were incubated with the binding buffer containing 40 nmol of Phe (39.6 nmol cold Phe and 0.396 nmol hot Phe) at a final volume of 100 μL (400 μM labelled Phe) at 37 °C for 2 h. Triplicate 25 μL aliquots were filtered through NC disc (13 mm BA) and washed three times with cold binding buffer. The binding activity was determined by scintillation counting of the filters for each sample.

Enzyme assays and kinetic studies

Chorismate mutase activity was assayed by monitoring the conversion of chorismate to prephenate 37 °C for 5 min in 50 mM tris (pH 7.8), 2.5 mM EDTA, 20 mM mercaptoethanol and 0.01% BSA with 1 mM chorismic acid; PDT activity was measured by the conversion of prephenate to phenylpyruvate as described previously.[16] One unit of enzyme was defined as the amount of enzyme required to form 1 μmol of product per min at 37 °C. For kinetic assays, 125 ng of mutase protein was used with chorismate at concentrations from 0 to 3 mM. Kinetic parameters were determined by fitting initial

rate data to the Michaelis–Menten equation using the KaleidaGraph software program (Abelbeck Software).

Phe feedback inhibition assay

Feedback inhibition of CM activity by Phe was measured at 0–3.2 mM Phe concentrations. As controls, both the wild-type P-protein and the subcloned 109-residue CM domain were assayed concurrently with EcCM-R. In each assay, 240 ng of P-protein, 50 ng of EcCM109 and 125 ng of EcCM were used. The activities in the absence and presence of Phe were used to determine the relative activity of each protein. The specific activity and kinetic assays were also run for all proteins in the absence and presence of the Phe at several different concentrations.

Acknowledgements

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