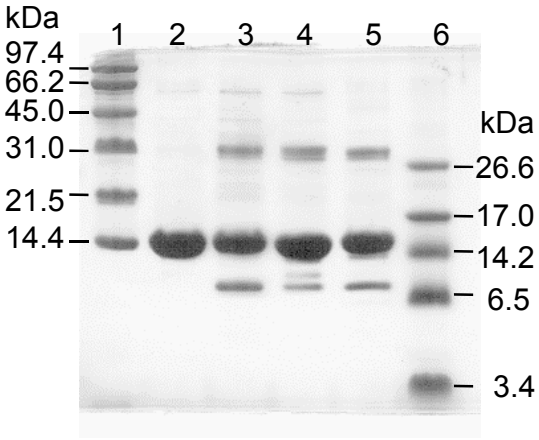
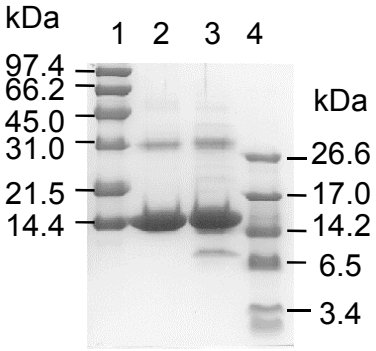
Supplemental Material

Supplemental Figure 1: Tris-tricine Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Analysis of Purified of Mutants. HA fractions are shown. A.) Lanes 1.) Molecular Weight Markers, BioRad, 2.) C93A mutant 3.) C109A mutant, 4.) C116A mutant, 5.) Native HisI 6.) Molecular Weight Markers, Sigma. B.) Lanes 1.) Molecular Weight Markers, BioRad, 2.) C109/116A 3.) Native HisI 4.) Molecular Weight Markers, Sigma.

A.)



B.)



Supplemental Table 1:MMTS Summary of inactivation results

MMTS (10^{-6}mol/L) (HisI@$1.9x10^{-6}$ mol/L)	1st order rate constant (min^{-1})
39 μM	0.0479
59 μM	0.1452
78 μM	0.2584
control (0μM)	nd
78 μM with 1.1 mM PRAMP	0.0171

Supplemental Table 2:DTNB Summary of inactivation results

DTNB(10^{-6}mol/L) (HisI@$1.9x10^{-6}$ mol/L)	1st order rate constant inactivation (min^{-1})	1st order Rate of TNB^{2-} formation (min^{-1})	Ratio of inactivation to TNB^{2-} formation
106 μM	0.0519		
127 μM	0.0611	0.0367*	1.7
212 μM	0.0854	0.0472*	1.8
296 μM	0.202	0.0573*	3.5
Control (0 μM)	0.0027		
212 μM with 1mM PRAMP	0.0182		

* These assays were performed under the same conditions with the same molar ratio of DTNB:enzyme. Second order plots for the TNB^{2-} formation and modification and gave values of 0.058 μM^{-1} min^{-1} 0.071 μM^{-1},min^{-1} respectively.

Supplemental Table 3: MALDI-MS Results PR-AMP Cyclohydrolase and Products from Cyanylation and Cleavage.

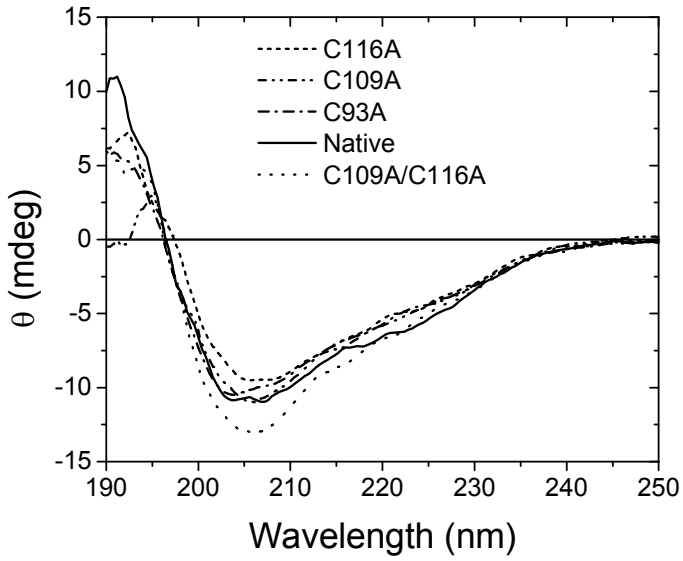
Potential Fragments	Calculated mass	ITC calculated fragment mass(+25)**	β-elimination calculated product mass(-34)***	NTCB/TCEP Reaction Set (1)	TCEP/NTCB Reaction Set (2)	Control unmodified protein
2*-136	15,490.1	na	15,456.1(-1) 15,422.1(-2) 15,388.1(-3)	no	no	15,486.0 (7752.9, 2+)
2-115	13,217.5	na	13,183.5(-1) 13,149.5(-2)	no	no	no
2-108	12,409.6	na	12,375.6(-1)	no	no	
2-92	10,689.7	na	na	10,684.8 (5339.3, 2+)	10,677.6 (5345.3, 2+)	10,697.6 (5351.2, 2+)
93-136	4818.4	4843.4	4784.4(-1) 4750.4(-2)	4825.8	4765.6	4817.5
109-136	3098.4	3123.4	3064.4(-1)	3024.8 (3094.7) 3153.2 3345.4	3003.3 3022.1 (3091.6) 3151.1	no
93-115	2545.9	2570.9	2511.9(-1)	2790.3 2893.2	no	no
116-136	2290.5	2315.5	na	2319.1	2317.2	no
93-108	1737.9	17629	na	1766.2	1763.9	no
109-115	825.9	849.9	na	no	no	no

Bold italic underlined values are cysteines. na-not applicable; no-not observed. Masses for peptide fragments were calculated using the average molecular weights in the GP-MAW program. *N-terminal methionine was processed, it was not observed in N-terminal sequence

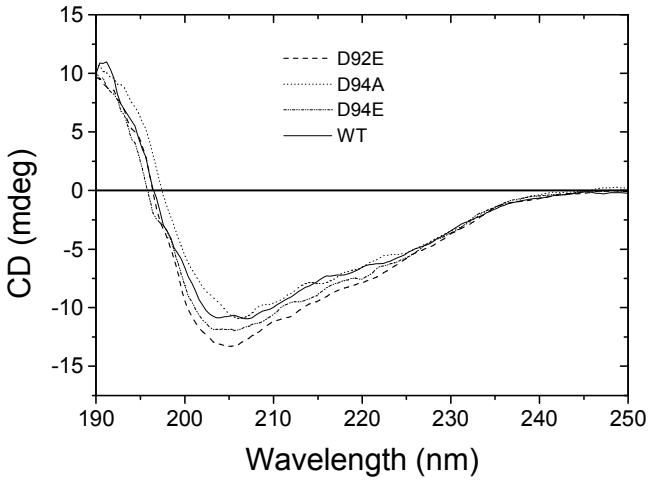
analysis consistent with subunit molecular mass as determined by MALDI-MS above. **ITC fragment +25; ***β-elimination -34 resulting in an un-cleaved peptide.

Circular Dichroism Spectropolarimetry. The CD spectra of both native and mutant forms of PR-AMP cyclohydrolase were recorded in 15 mM $KH_2 PO_4$ pH 7.3 at 20 °C with a Jasco Model 600 Spectropolarimeter (calibrated with (+)-D-10-camphorsulfonic acid) (*1*). The reported spectra (θ, mdeg, average of two scans) are uncorrected and were collected from 190 nm to 250 nm at 0.4 nm intervals with time constant, 2 s; band width, 1 nm; scan speed 20 nm min^{-1}. Quartz cells of 2.0 mm path length and 500 μL volume were used with protein concentrations of 4.5-5.0 μM.

Supplemental Figure 2a: CD Spectra of Native and Mutant Forms of PR-AMP Cyclohydrolase

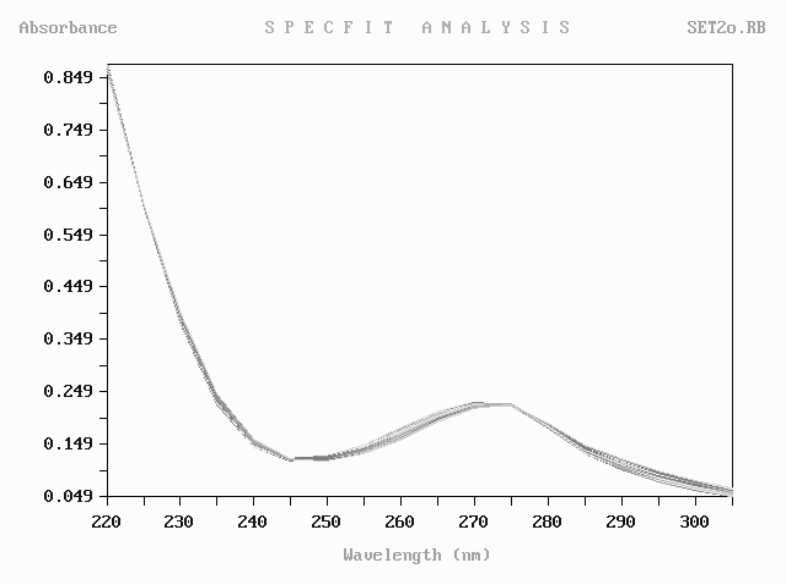


Supplemental Figure 2b: CD Spectra of aspartate mutants and WT enzyme.

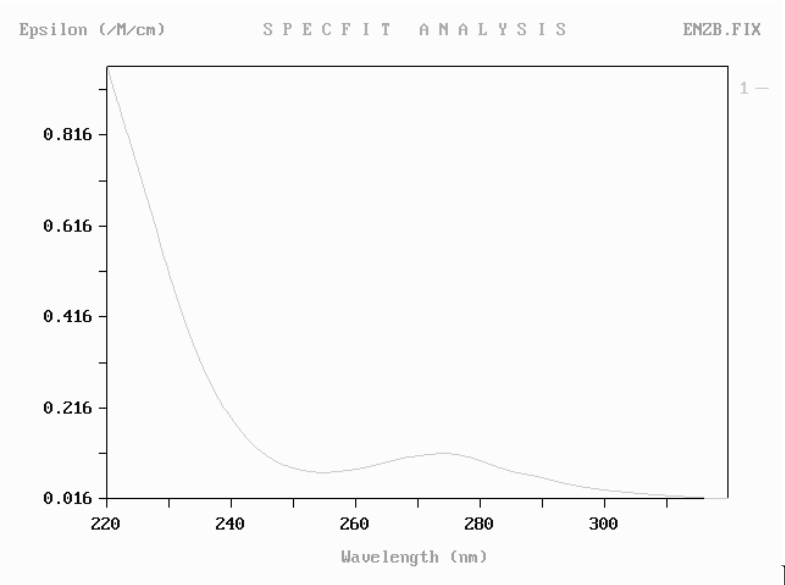


Supplemental Figure 3A-H: Stopped–Flow Evidence for an Intermediate in the PR-AMP Cyclohydrolase Reaction: The UV profile for the PR-AMP cyclohydrolase enzymatic reaction was monitored from 220 nm to 340 nm at ambient temperature (23°C) using the Hi-Tech Stopped-Flow in single-mixing mode. To perform a single turnover reaction, syringes contained solutions of PR-AMP (4μM) with Mg^{2+} (5mM) and PR-AMP cyclohydrolase (4μM) in 50mM Tris-HCl pH 7.5 1 mM EDTA. Spectra for the enzyme, product and substrate starting solutions were acquired toward the potential identification and deconvolution of an intermediate species. Shown below are some representative data from these experiments. The output is from Specfit (Ver 2.1). Data were handled as described in Binstead (*2*) and Bracher (*3*).

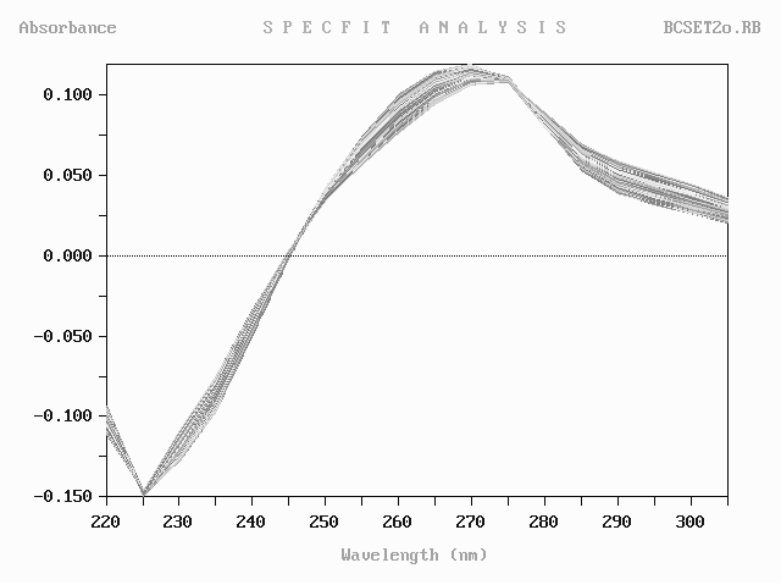
The figures below show the full spectral view of a set of these experiments. From top to bottom there is: A) the raw data for the reaction, B) the enzyme absorbance spectrum and C) the difference spectra views respectively. Below are plots of the time domain at D) 300 nm (product formation) and E) 260 nm (substrate consumption). Analysis of the full spectral view of this reaction previously has demonstrated the presence of three isosbestic points at 223, 247, and 274 respectively. From this data set analysis of the rate of change at 225, 245, and 275 was performed and. from top to bottom the figure shows the change with respect to time at F) 225, G) 245, and H) 275 respectively. These data show that there are multiple processes (increases and decreases in absorbance) taking place at the isosbestic points indicating that there is an intermediate on the path from substrate to product. The results of global fitting of the data to a two-step model A>B->C in which three species including one intermediate gave the best global fit are shown in Figure 5 in the paper.


Absorbance
S P E C F I T A N A L Y S I S
SET2o.RB
0.849
0.749
0.649
0.549
0.449
0.349
0.249
0.149
0.049
220
230
240
250
260
270
280
290
300
Wavelength (nm)

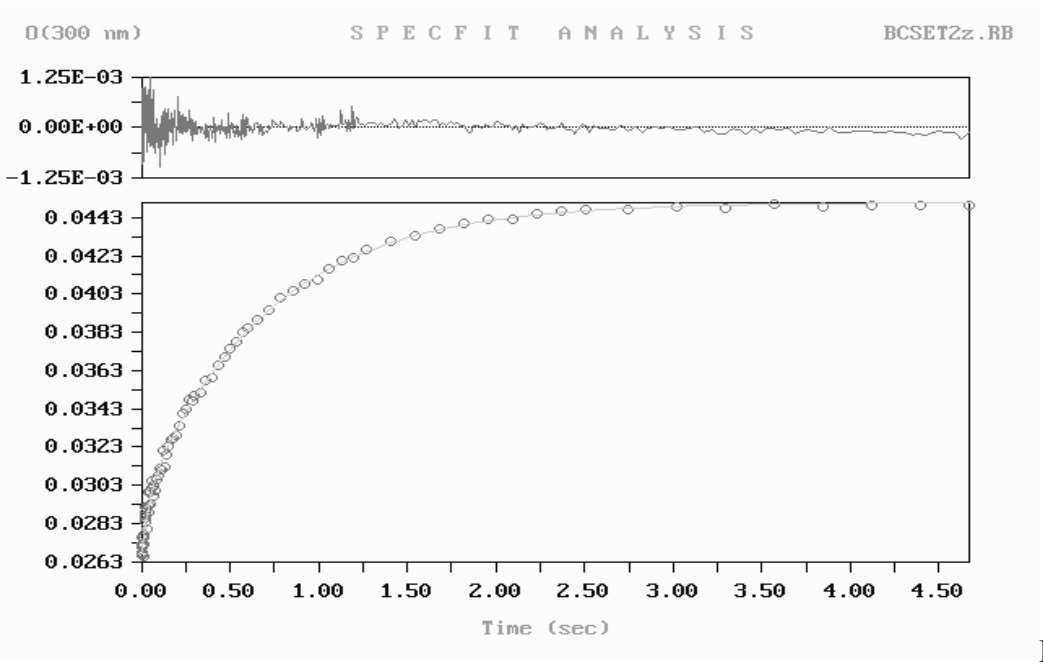
A)


Epsilon (/M/cm)
S P E C F I T A N A L Y S I S
ENZB.FIX
1
0.816
0.616
0.416
0.216
0.016
220
240
260
280
300
Wavelength (nm)

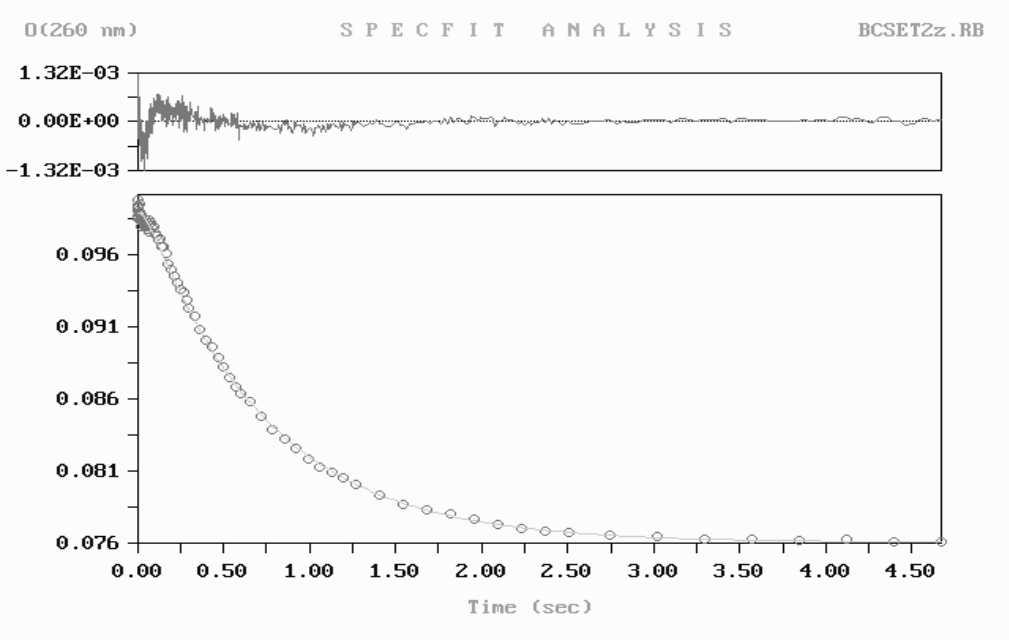
B)


Absorbance
S P E C F I T A N A L Y S I S
BCSET2o.RB
0.100
0.050
0.000
-0.050
-0.100
-0.150
220
230
240
250
260
270
280
290
300
Wavelength (nm)

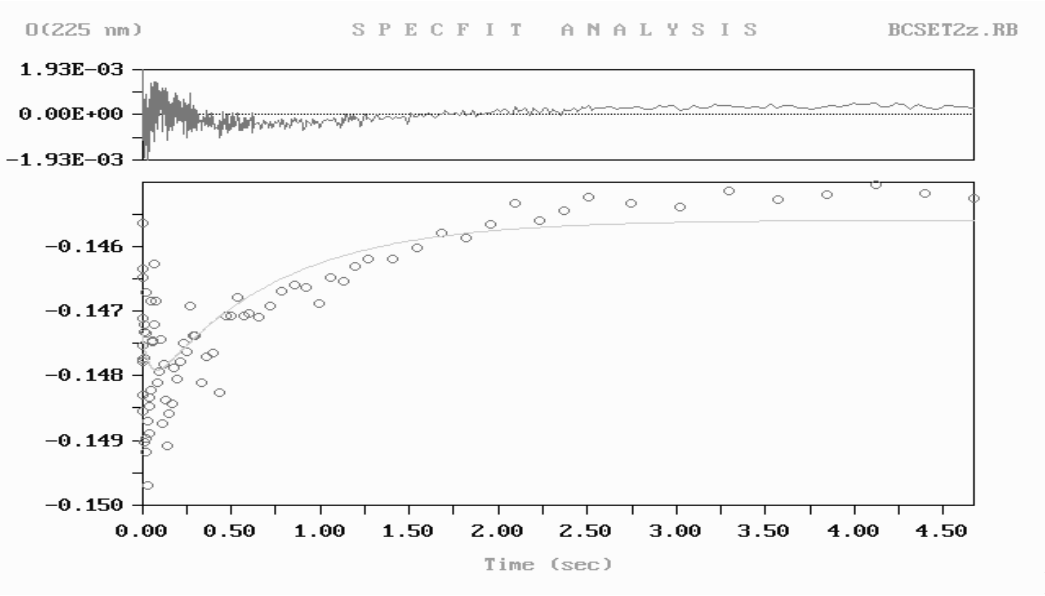
C)


O(300 nm)
S P E C F I T A N A L Y S I S
BCSET2z.RB
1.25E-03
0.00E+00
-1.25E-03
0.0443
0.0423
0.0403
0.0383
0.0363
0.0343
0.0323
0.0303
0.0283
0.0263
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
Time (sec)

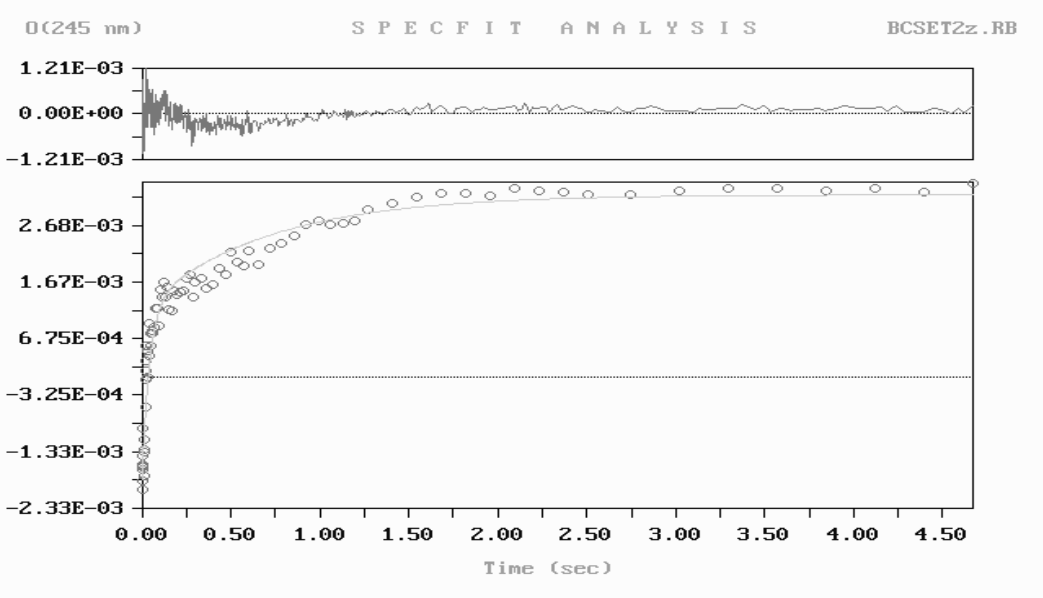
D)


O(260 nm)
S P E C F I T A N A L Y S I S
BCSET2z.RB
1.32E-03
0.00E+00
-1.32E-03
0.096
0.091
0.086
0.081
0.076
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50
Time (sec)

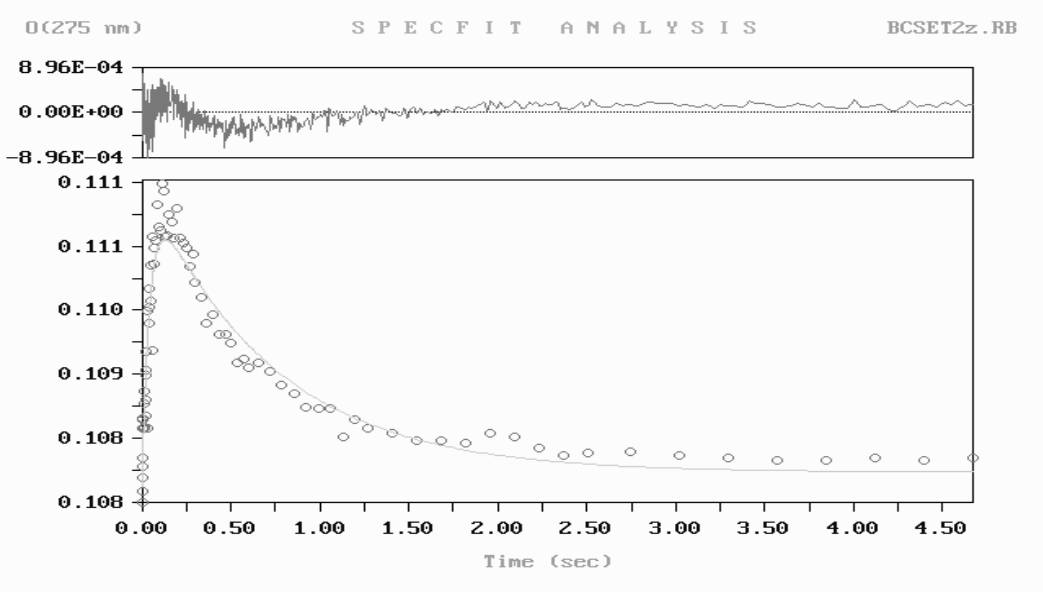
E)


O(225 nm)
S P E C F I T A N A L Y S I S
BCSET2z.RB
1.93E-03
0.00E+00
-1.93E-03
-0.146
-0.147
-0.148
-0.149
-0.150
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50
Time (sec)

F)

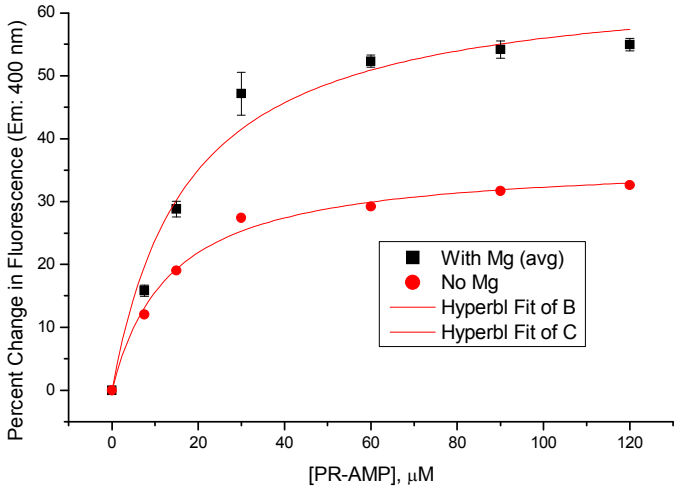

O(245 nm)
S P E C F I T A N A L Y S I S
BCSET2z.RB
1.21E-03
0.00E+00
-1.21E-03
2.68E-03
1.67E-03
6.75E-04
-3.25E-04
-1.33E-03
-2.33E-03
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50
Time (sec)

G)


O(275 nm)
S P E C F I T A N A L Y S I S
BCSET2z.RB
8.96E-04
0.00E+00
-8.96E-04
0.111
0.111
0.110
0.109
0.108
0.108
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50
Time (sec)

H)

Supplemental Figure 4: Mg^{2+} Fluorescence titration experiments: Various fluorescence titration experiments were performed with PR-AMP cyclohydrolase in the presence and absence of Mg^{2+}. Experiments involved the titration of a solution of PR-AMP cyclohydrolase (0.43 μM) in 50 mM Tris-HCl pH 7.5 with or without 2 mM Mg^{2+}. Experiments were executed on a Spex FluoroMax spectrophotometer with excitation at 290 nm and observation at 337 and 400 nm. Successive titration of PR-AMP cyclohydrolase solution (2.0 mL sample equilibrated at 30°C) by substrate ((No Mg 6) or product (No Mg 7 and 8) (0-150 μM) was followed by a 3 min incubation before reading the fluorescence change. Figure 4 below summarizes the data from these experiments using fits to hyperbolic functions in Origin 5.0. The K_d observed was estimated to be 12.6- 17.2 μM depending on if Mg^{2+} was present in the titration mixture. These data are empirically corrected for the inner filter effect by subtraction of the change fluorescence for a sample of tryptophan titrated with substrate under similar conditions.



Equation	y = P1*x/(P2 + x)		
Adj. R-Square	0.97547	0.99123	
		Value	Standard Error
With Mg2+	Vmax	65.79575	3.99169
With Mg2+	Km	17.55711	3.75808
No Mg2+	Vmax	36.62999	1.15949
No Mg2+	Km	13.47167	1.6654

References

1. Pain, R. (1996) *Determining the CD Spectrum of a Protein Unit 7.6*, Vol. 1, John Wiley and Sons, Inc, NY.
2. Binstead, R. A., and Zuberbuhler, A. D. (1996) SPECFIT, a global analysis system with expanded factor analysis and Marquardt least squares minimization, 2.1 ed., Spectrum Software Associates, Chapel Hill, NC.
3. Bracher, A., Schramek, N., and Bacher, A. (2001) Biosynthesis of pteridines. Stopped-flow kinetic analysis of GTP cyclohydrolase I, *Biochemistry 40*, 7896-7902.