Supplementary Material

Selective stabilization of the chorismate mutase transition state by a positively charged hydrogen bond donor

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11 pages

Experimental procedures:

Materials. Chemicals were purchased from Sigma, Acros, Merck, Aldrich or Fluka unless noted otherwise and used without further purification. Protected amino acids and resins were bought from Novabiochem. Reagents and solvents for peptide synthesis were obtained from Applied Biosystems. Chorismate was prepared by a previously published procedure.[1] Media and solutions for experiments other than purification of the intein-CBD fusion protein were prepared according to standard recipes.[2] Wild-type BsCM (BsCM) was produced and purified as previously described.[3]

Strains and plasmids. General cloning was done in the chorismate-mutase deficient *Escherichia coli* strain KA12. Protein production was carried out in *E. coli* strain KA13, a derivative of KA12 which carries the DE3 prophage in its chromosome, allowing IPTG-inducible expression of genes under the control of the T7 promoter. Both strains have been described previously.[4,5] All plasmids in this study carry an ampicillin resistance gene, and cells harboring them were grown in medium containing 150 μg/mL ampicillin (Amp). Plasmid pKET3-W (5766 bp), which encodes BsCM and served as template and acceptor for site-directed mutagenesis, has been described.[6] Plasmid pTXB1 (6706 bp), which contains the gene for the *Mxe* GyrA intein fused to a chitin binding domain (CBD) proximal to a multiple cloning site, was purchased from New England Biolabs.

DNA manipulations. All nucleic acid manipulations were according to standard procedures.[2] Oligonucleotides were custom-synthesized and purified by MicroSynth. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Polymerase chain reactions (PCRs) were performed using HotStarTaq polymerase (Qiagen). All PCR-amplified portions of the constructed plasmids were confirmed by DNA sequencing on an Applied Biosystems PRISM 310 Automated DNA Sequencer using the Terminator Ready Reaction Mix (BigDye™, PE Applied Biosystems) for chain termination chemistry.[7] DNA was prepared for sequencing using a QIAGEN Mini-prep kit.

HPLC and Mass spectrometry. RP-HPLC was performed on a Waters HPLC system with UV detection. For analytical runs a C_{18} column (Macherey-Nagel 250 mm x 4.6 mm, 100 Å, 5 μ) at a flow rate of 1 mL/min was used. Peptides were eluted with linear gradients of solvents A and B (A = acetonitrile containing 0.05% TFA, B = H_2O containing 0.1% TFA). Preparative RP-HPLC separations were performed using a C_8 column (Macherey-Nagel 250 mm x 21 mm, 300 Å, 7 μ) or a C_{18} column (Vydac 250 mm x 22 mm, 300 Å, 10 μ) at a flow rate of 10 mL/min. Linear gradients of solvents A' and B' (A' = acetonitrile, B' = H_2O containing 0.1% TFA) were used. Mass spectra were recorded on a Finnigan TSQ7000 Triple-Quad mass spectrometer (ESI-MS) in a 70:30 H_2O:CH_3CN mixture or in 0.1% aq. AcOH. Calculated masses are based on average isotope composition. If necessary, protein solutions were desalted prior to MS analysis on a NAP-5 column (Amersham-Pharmacia) that had been preequilibrated with 0.1% AcOH. Protein

concentration was determined by measuring the absorbance at 280 nm and using the calculated molar extinction coefficient ε_{280} = 8370 M^{-1} cm^{-1} for BsCM and variants.

Recombinant D102E BsCM (BsCM*). The D102E mutation was introduced into wild-type BsCM by replacing the wild-type 120 bp *Bsr*GI-*Xho*I fragment of *aroH* in pKET3-W with the corresponding 120 bp mutant fragment that was generated by PCR using SVSF-1-D102E (GATGACTGTACAGACAGATGTCCCTCAGGA**G**CAGATCAGACATGTA) and 04-T7TR (CAGCAGCCAACTCAGCTTCCTTTC) as the forward and reverse primers, respectively. The mutagenized position is shown in bold. The amplified 197 bp fragment was purified, digested with the restriction enzymes *Bsr*GI and *Xho*I, and ligated with the appropriate 5646 bp fragment of the acceptor vector pKET3-W. Strain KA13 was transformed with the resulting 5766 bp plasmid, pAK-D102E, and the encoded recombinant BsCM* was produced and purified as previously described for the wild-type enzyme.[3] The yield of purified protein was 51 mg per 1 L of culture. Protein for immediate use was stored at 4 °C under argon. The remainder was stored at -80 °C under argon to minimize oxidative damage. ESI-MS: 14,505 ± 2 Da (calcd 14,503 Da).

BsCM(1-87) thioester. The gene segment encoding residues 1-87 of BsCM was amplified by PCR from pKET3-W with the forward primer T7PRO2 (TAATACGACTCACTATAGGG) and the reverse primer AK-3-SAP-BsCM87 (GGTGGTGCTCTTCCGCACTTCTTAAGACCGCCTGT). The 366 bp PCR fragment was purified, digested with restriction enzymes *Sap*I and *Nde*I, and the 263 bp fragment was ligated with the 6653 bp *Sap*I-*Nde*I acceptor fragment from pTXB1. The resulting 6916 bp plasmid pTXB1-BsCM87 encodes BsCM(1-87) fused to the *Mxe* GyrA intein and a chitin-binding domain. The construct was verified by DNA-sequencing using the forward primer T7PRO2 and the reverse primer AK-1-RPMxeI (GGCACGATGTCGGCGATG). Gene expression was carried out in strain KA13. An overnight preculture (1 mL) was used to inoculate a 1 L LB-Amp^{150} culture that was grown at 37 °C, 230 rpm to an OD_{600} of ca. 0.6. Addition of 10 mL of a 50 mM isopropyl-1-thio-β-D-galactoside solution induced protein synthesis. The cells were incubated for another 24 h at 24 °C (230 rpm) and then harvested by centrifugation at 4 °C at 4,000 rpm (2,500×g) for 20 min. The supernatant was discarded and the pellet was frozen at -20 °C prior to purification. To isolate the fusion protein, the cell pellet was resuspended in 35 mL ice-cold Cell Lysis Buffer (20 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 20 μM phenylmethanesulfonyl fluoride, 0.1% Triton X-100, pH 8) and sonicated. After removal of the cell debris by centrifugation at 12,000 rpm (17,000×g) for 30 min at 4 °C, the clarified cell lysate was slowly loaded (less than 0.5 mL/min) onto a chitin column that had been equilibrated in 20 mM Tris-HCl buffer, pH 8, containing 300 mM NaCl and 1 mM EDTA (Column Buffer) at 4 °C. The column was washed with 100 mL Column Buffer and then incubated overnight at 4 °C with 30 mL of freshly prepared Cleavage Buffer (Column Buffer plus 50 mM 2-mercaptoethane sulfonate) to induce cleavage of the fusion protein. The BsCM(1-87) thioester was then eluted from the column in two batches with 5 and 10 mL Column Buffer. The first was purified directly by preparative RP-HPLC (C_{18}) using a gradient of 25 to 60% A' in B' over 45 min. The second was first concentrated by

ultrafiltration to 5 mL and then purified by preparative RP-HPLC under the same conditions. The fractions containing the thioester were pooled, lyophilized, and stored at –20 °C. The yield of purified BsCM(1-87) thioester was 3 to 5 mg per 1 L of culture. Analytical RP-HPLC (C_{18}, 0 to 60% A in B over 45 min): R_t = 39.3 min. ESI-MS: 9,977 ± 1 Da (calcd 9,976 Da). The mass spectrum showed an additional peak at 9,835 Da, corresponding to approximately 15% of the sample, which may be the lactam formed by intramolecular reaction of the thioester with the C-terminal lysine. A separate peak is not observed in the HPLC chromatogram, thus the contaminant either has the same R_t as the parent thioester or it is formed during ESI-MS analysis. Because the lactam is inert in the native chemical ligation reaction, further purification was not undertaken.

Solid-Phase Peptide Synthesis. Peptides were synthesized in a stepwise fashion on the NovaSyn TGA resin using an ABI 433A peptide synthesizer (Applied Biosystems) either on a 0.25 or 0.1 mmol scale.[8] Standard HBTU/HOBt/NMP activation protocols for Fmoc chemistry (FastMoc® protocol, Applied Biosystems)[9] were applied. For peptides longer than 10 amino acids, a capping step with acetic anhydride was performed. Amino acid side chains were protected as follows: Arg(Pbf), Asn(Trt) or Asn(Tmob), Asp(O*t*Bu), Cys(Trt), Gln(Trt), Glu(O*t*Bu), Lys(Boc), Ser(O*t*Bu), Thr(O*t*Bu), Tyr(O*t*Bu). After drying under high vacuum, peptides were cleaved from the solid support by shaking in TFA for 3 h in the presence of scavengers [91% (v/v) TFA, 4% (v/v) UPW, 1% (v/v) TIPS, 2.5% (v/v) phenol (melted), 1.5% (v/v) EDT]; 40 mL of the cleavage cocktail was typically used per g of resin. The peptide was subsequently precipitated by adding cold Et_2O. After centrifugation at 3,000 rpm (1,600×g) for 20 min, the Et_2O was decanted and the procedure repeated twice. Crude peptides were purified by RP-HPLC.

BsCM(108-127) (YLEKAVVLRPDLSLTKNTEL). The BsCM(108-127) fragment was synthesized on a 0.25 mmol scale. The synthesizer was loaded with 1.19 g NovaSyn TGA resin preloaded with Fmoc-Leu (substitution: 0.21 mmol/g, Wang linker). With the exception of Asn124, which was double coupled (2 h each coupling), the first ten amino acids in the sequence were single coupled. Double coupling was employed for the remainder of the sequence. In order to avoid β-sheet formation, amino acids Ser120 and Leu121 were introduced as the pseudo proline dipeptide Fmoc-Leu-Ser(psiMe,Mepro)-OH. After drying, the loaded resin was stored at –20 °C until it was split and further elongated (*vide infra*).

BsCM(88-127)* (<u>CIRVMMTVQTDVPQEQIRHV</u>YLEKAVVLRPDLSLTKNTEL). The target peptide, which contains Glu in place of wild-type Asp102 to avoid aspartimide formation, was synthesized on a 0.1 mmol scale. 0.82 g NovaSyn TGA resin preloaded with the protected BsCM(108-127) fragment (substitution: 0.122 mmol/g) were elongated with the underlined amino acids. With the exception of Cys88, Arg90 and Arg105 which were triple coupled (4 h each coupling), each amino acid was double coupled (2 h each). After drying, 1.124 g of resin loaded with the sidechain-protected peptide were obtained (expected for 100% coupling yield: 1.213 g). Following deprotection and cleavage from the resin, the crude product was purified by preparative HPLC (C_8, 20 to 50% A' in B'

over 50 min) in several batches of 15 to 20 mg each. Under these conditions, BsCM(88-127)* eluted with a retention time of 28.8 min. The desired peptide corresponded to 25% (w/w) of the crude product. ESI-MS: 4,668 ± 2 Da (calcd 4,668.4 Da). Analytical RP-HPLC (C_{18}, 0 to 60% A in B over 45 min): R_t = 34.2 min.

Arg90Cit BsCM(88-127)* (CI-**Cit-**VMMTVQTDVPQEQIRHVYLEKAVVLRPDLS-LTKNTEL). Peptide synthesis was carried out on a 0.1 mmol scale using 0.82 g of the NovaSyn TGA resin preloaded with the protected BsCM(108-127) fragment (substitution: 0.122 mmol/g). The underlined amino acids were coupled as described for BsCM(88-127)*. Citrulline (instead of Arg90) was introduced without side chain protection. After drying, 1.079 g of resin loaded with the full-length, sidechain-protected peptide were obtained (expected for 100% coupling yield: 1.213 g). Deprotection and cleavage from the resin gave 180 mg of crude product, which was purified by preparative HPLC (C_{18}, 20 to 50% A' in B' over 50 min) in several batches of 15 to 20 mg each. The desired peptide eluted with a retention time of 22.6 min. Purified Arg90Cit BsCM(88-127)* was obtained as approximately 30% (w/w) of the crude material. ESI-MS: 4,669.6 ± 2 Da (calcd 4,669.4 Da). Analytical RP-HPLC (C_{18}, 0 to 60% A in B over 45 min): R_t = 34.2 min.

Native chemical ligation.[10] Peptide fragment condensations were performed in degassed 6 M GdmHCl, 100 mM Tris-HCl, pH 8.0, containing 2.5% (v/v) PhSH for 24 h at r.t. under argon. The C-terminal BsCM(88-127)* or Arg90Cit BsCM(88-127)* fragments (1 mM) were reacted with a slight excess (10 to 20%) of the N-terminal BsCM(1-87) thioester. After the ligation reaction was complete as judged by HPLC, tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added (10 mg/mL reaction mixture) to reduce any disulfides that had formed. After 2 h, the ligated protein was renatured by diluting the reaction mixture (10 µl at a time, in order to keep the concentration of denatured protein low) with gentle stirring at r.t. 100-fold into 50 mM glycine-NaOH, pH 8.9, containing 5% (v/v) 2-propanol and 10% (v/v) glycerol. The diluted protein was reconcentrated to the original volume by ultrafiltration over a membrane with a molecular mass cut-off of 10 kDa and purified by ion-exchange chromatography on a Mono Q column. Column fractions were analyzed by SDS-PAGE. Those containing the desired protein were pooled, concentrated by ultrafiltration, and dialyzed into 50 mM potassium phosphate, pH 7.5. For all operations, separate columns and membranes were used for BsCM* and Arg90Cit BsCM*

Semisynthetic BsCM*. The N-terminal BsCM(1-87) fragment activated as a thioester with 2-mercaptoethane sulfonate (4 mg) and the synthetic C-terminal BsCM(88-127)* fragment (2.4 mg) were coupled by native chemical ligation to afford 1.9 mg (35%) of purified semisynthetic enzyme (Fig. 1). ESI-MS: 14,502 ± 2 Da (calcd 14,503 Da; Fig. 2). Analytical RP-HPLC (C_{18}, 0 to 60% A in B over 45 min): R_t = 40.2 min.

Arg90Cit BsCM*. The N-terminal BsCM(1-87) fragment activated as a thioester with 2-mercaptoethane sulfonate (17.8 mg) and the synthetic C-terminal BsCM(88-127)* fragment (8 mg) were coupled by native chemical ligation to afford 7 mg (28%) of purified

Arg90Cit BsCM* (Fig. 1). ESI-MS: 14,503 ± 2 Da (calcd 14,504 Da; Fig. 2). Analytical RP-HPLC (C_{18}, 0 to 60% A in B over 45 min): R_t = 40.5 min.

Figure 1. SDS-polyacrylamid gel of Arg90Cit BsCM*, semisynthetic BsCM*, and BsCM

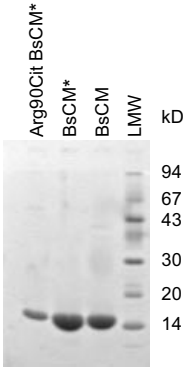
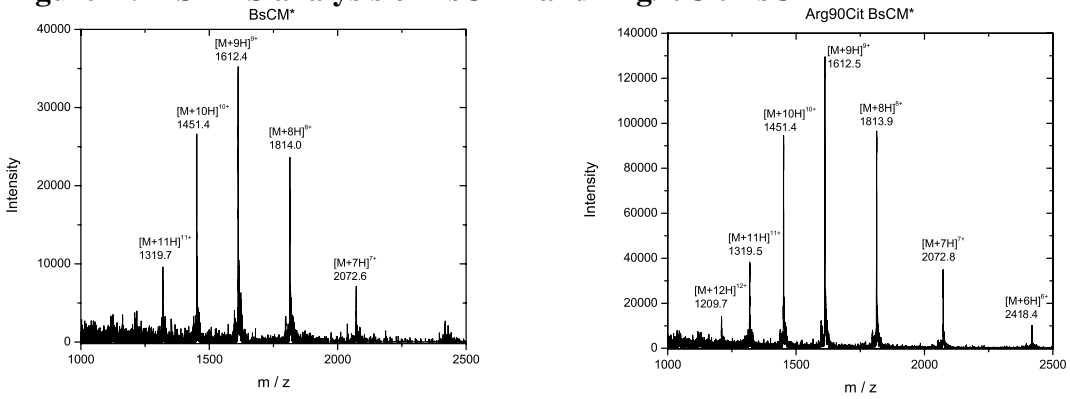
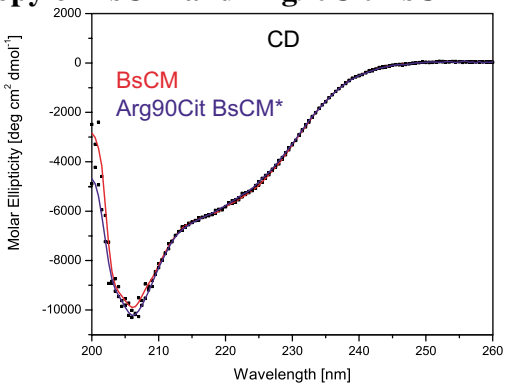


Figure 2. ESI-MS analysis of BsCM* and Arg90Cit BsCM*



Circular Dichroism (CD) Spectroscopy. Spectra (Fig. 3) were recorded on an Aviv Circular Dichroism Spectrometer 202 or on a Jasco J-715 at 25 °C. CD spectra were measured at a protein concentration of ~ 4 µM in degassed phosphate buffered saline (PBS; 10 mM phosphate, 160 mM NaCl, pH 7.5) by averaging at least 3 wavelength scans from 200 nm to 260 nm in 0.5 or 1 nm steps (signal averaging time 3 s; bandwidth 1 nm). The samples were measured in quartz cuvettes with a path length of either 0.1 or 0.2 cm.

Figure 3. CD spectroscopy of BsCM and Arg90Cit BsCM*

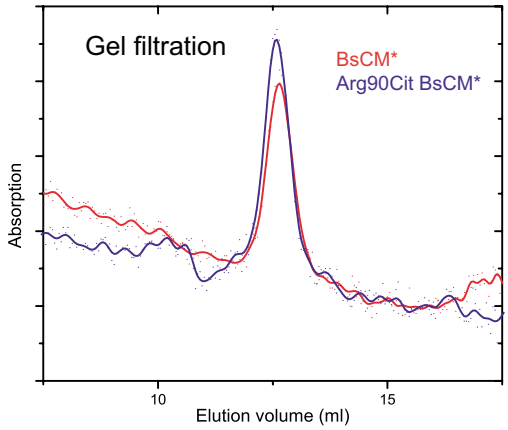


Analytical Size Exclusion Chromatography. The aggregation state of the BsCM* variants was determined by analytical size exclusion chromatography using a Superose 12 (HR 10/30) FPLC column from Amersham-Pharmacia (Fig. 4). Chromatography was performed at 4 °C using PBS as the running buffer (flow rate: 0.3 mL/min, detection at 280 nm) and a 500 µl injection loop. Prior to analytical runs, the column was calibrated according to the method outlined in the instruction manual for the LMW Gel Filtration Calibration Kit from Amersham-Pharmacia using the supplied protein standards (ribonuclease A, M_W 13,700; trypsinogen, M_W 24,000; ovalbumin, M_W 43,000; bovine serum albumin, M_W 67,000) plus aprotinin, M_W 6,500; cytochrome *c*, M_W 12,400; and carbonic anhydrase, M_W 29,000 (from Sigma). The void volume, V_o (6.84 mL), and the total bed volume, V_t (26.01 mL), were determined using blue dextrane 2000 and DTT. The elution parameter, K_{AV}, was calculated for each protein using equation (1):

$$K_{AV} = (V_e - V_o)/(V_t - V_o) \qquad (1)$$

where V_e is the elution volume of the protein. A plot of K_{AV} vs. log(M_W) for the standard proteins gave the calibration curve (log M_W = -3.982 K_{AV} + 5.778). The oligomeric state of the mutant variants was assigned by dividing the experimentally determined molecular weights by the molecular weight of the monomeric species as calculated from the sequence and confirmed by mass spectroscopy. Apparent molecular weights for recombinant BsCM* and Arg90Cit BsCM* were 37,250 and 38,520, corresponding to 2.6 and 2.7 times the molecular weight of the corresponding polypeptides, respectively.

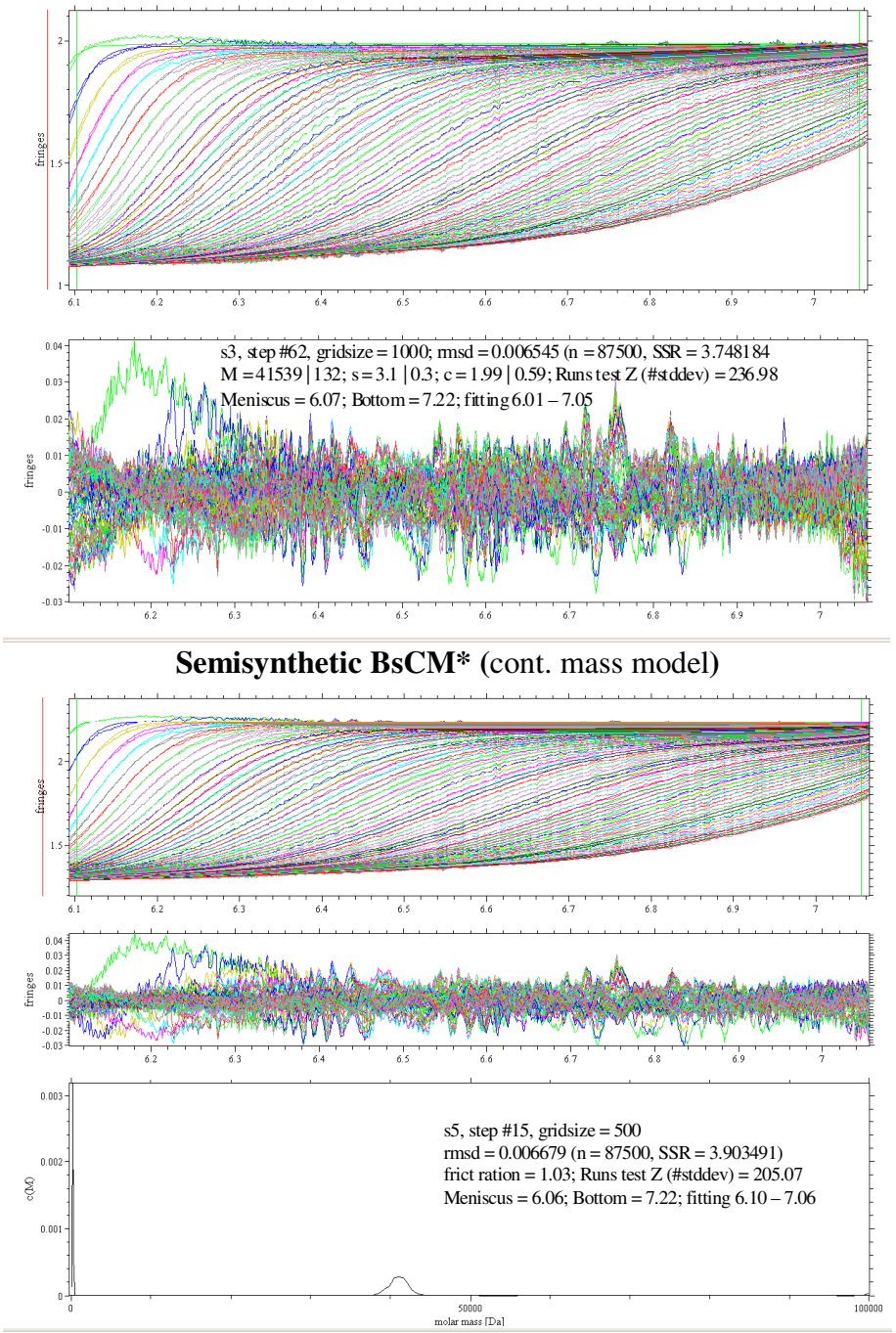
Figure 4. Analytical size exclusion chromatography of recombinant BsCM* and Arg90Cit BsCM*



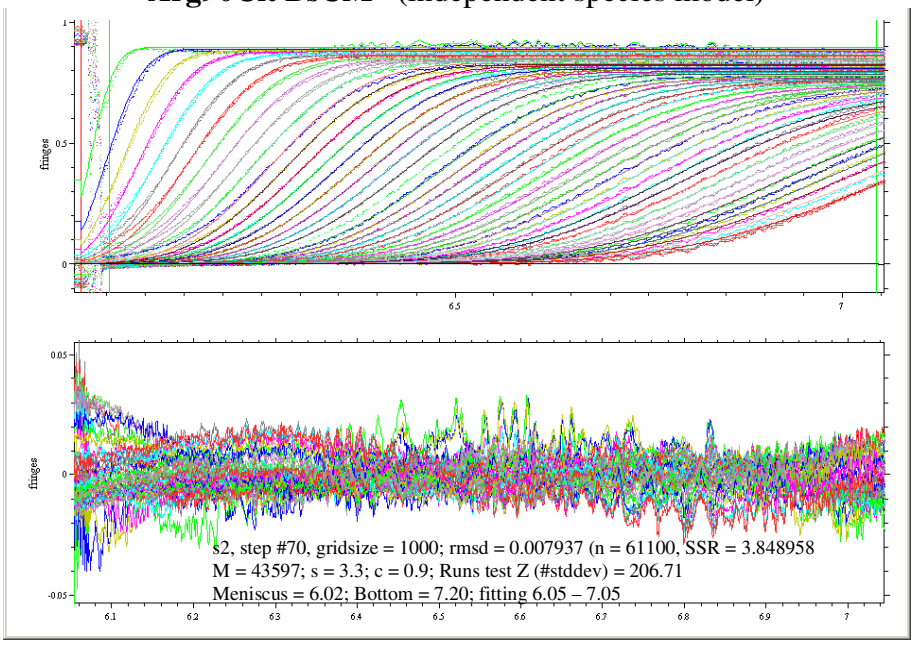
Analytical Ultracentrifugation. Sedimentation velocity experiments were performed with a Beckman XL-I analytical ultracentrifuge at 20 °C and 42,000 rpm. The sedimentation velocity was determined by measuring the sedimentation status every 3 min during the 420 min run (140 times) with an interference detector. The sedimentation coefficient was determined to be 3.56 svedberg (3.56 10^{-13} s) and the partial specific volume ν_{bar} to be 0.745 cm^3/g by the program Hydropro 5.a[11] using the pdb data (2CHS) as input file. The data was evaluated using sedfit as shown in Fig. 5,[12] and the results are

summarized in Table 1. To account for differences in the buffer of the sample and the reference a small component was included into the fit.

Figure 5. Analytical ultracentrifugation of BsCM* and Arg90Cit BsCM*



Arg90Cit BsCM* (independent species model)



Arg90Cit BsCM* (cont. mass model)

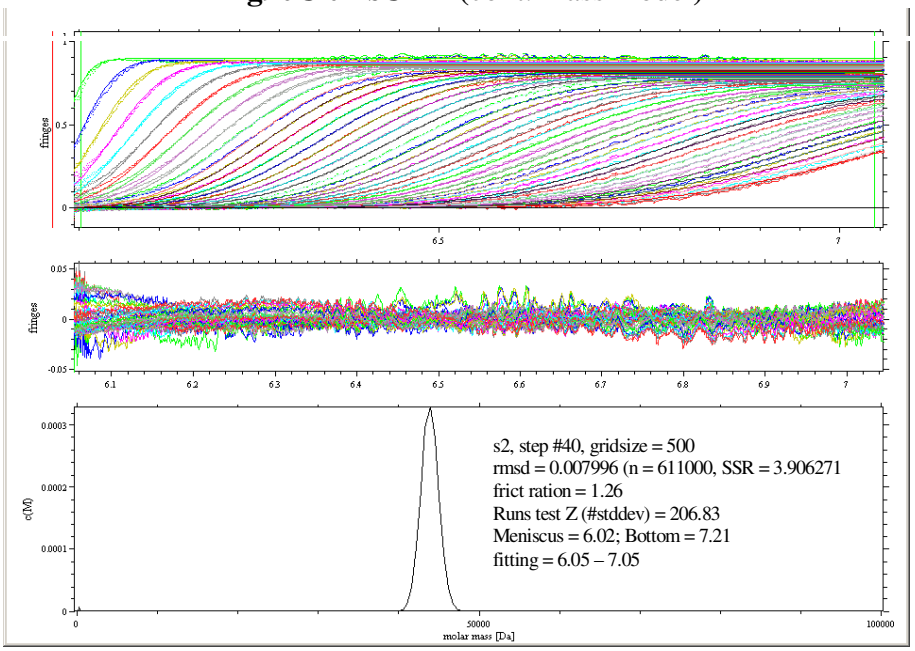


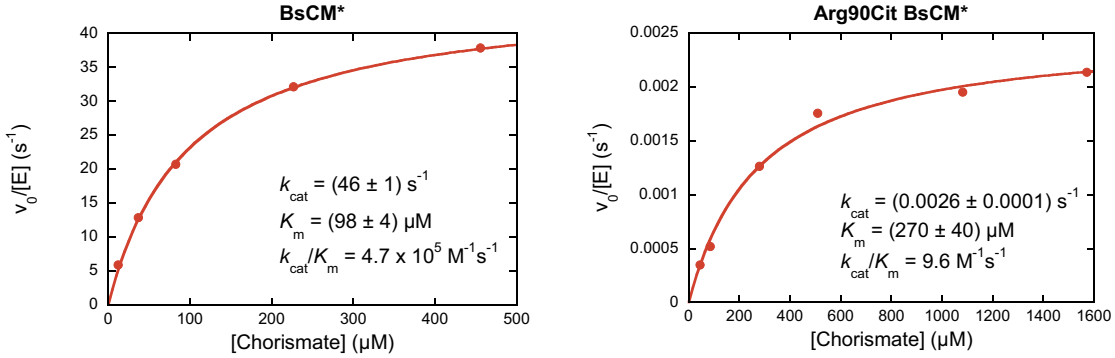
Table 1. Analytical ultracentrifugation of BsCM* and Arg90Cit BsCM*

	Independent species model	Continuous mass model
Recombinant BsCM*	39,200 Da	38,400 Da
Semisynthetic BsCM*	41,500 Da	41,000 Da
Arg90Cit BsCM*	43,600 Da	44,000 Da

Expected mass for a trimer of BsCM*: 43,509 Da; of Arg90Cit BsCM*: 43,512 Da

Kinetic Assays. All kinetic measurements were performed in 50 mM potassium phosphate buffer, pH 7.5, at 30 °C. Initial rates were determined by monitoring the disappearance of chorismate spectrophotometrically at 274 nm ($\Delta\varepsilon_{274\ nm}$ = 2630 M^{-1} cm^{-1}) or 310 nm ($\Delta\varepsilon_{310\ nm}$ = 370 M^{-1} cm^{-1}) using a Lambda series UV-Visible spectrophotometer (Perkin-Elmer) equipped with a thermoelectric cuvette holder. Measurements were made in quartz cuvettes with a 1 cm path length. Initial rates were corrected for the background reaction. Kinetic parameters k_{cat} and K_m were calculated from the initial rates as previously described,[13] using ideally a minimum of five substrate concentrations ranging from at least 4-fold below K_m to at least 4-fold above K_m (Fig. 6).

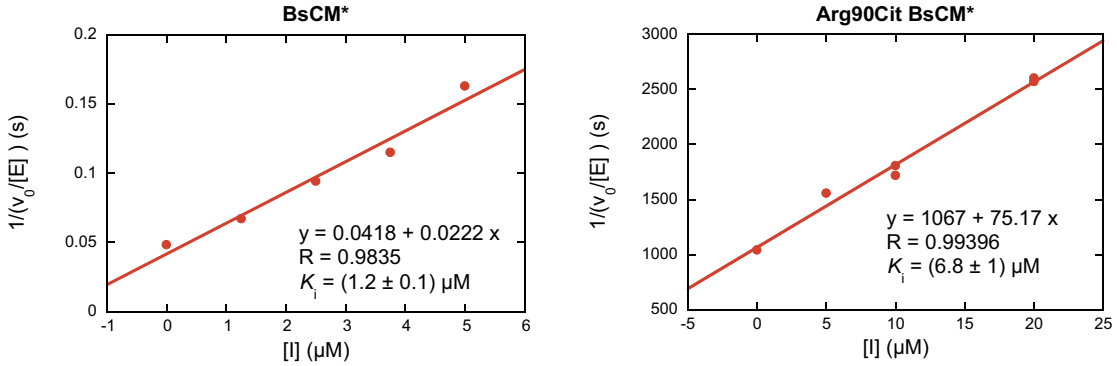
Figure 6. Kinetic assays of semisynthetic BsCM* and Arg90Cit BsCM*



Enzyme inhibition studies. The chorismate mutase-catalyzed conversion of chorismate to prephenate was performed in the presence of 0 to 20 μM of inhibitor **4**, an oxabicyclic dicarboxylic acid that was designed as a transition state analog,[14] at 30 °C in 50 mM potassium phosphate buffer, pH 7.5, at a substrate concentration close to K_m. The ratio of [E]/v_0 was plotted against the inhibitor concentration [I] (Fig. 7), and K_i was calculated using equation (2) and the independently determined values for k_{cat} and K_m:[15]

$$K_i = \frac{K_m}{k_{cat} \cdot [S] \cdot slope} \qquad (2)$$

Figure 7. Inhibition assays of semisynthetic BsCM* and Arg90Cit BsCM*



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