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Structural and Biochemical Characterization of Human Adenylosuccinate Lyase (ADSL) and the R303C ADSL Deficiency Associated Mutation

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Abstract

Adenylosuccinate lyase (ADSL) deficiency is a rare autosomal recessive disorder, which causes a defect in purine metabolism resulting in neurological and physiological symptoms. ADSL executes two non-sequential steps in the *de novo* synthesis of AMP: the conversion of phosphoribosylsuccinyl-aminoimidazole carboxamide (SAICAR) to phosphoribosylaminoimidazole carboxamide (AICAR), which occurs in the *de novo* synthesis of IMP, and the conversion of adenylosuccinate (AMPS) to AMP, which occurs in the *de novo* synthesis of AMP and also in the purine nucleotide cycle, using the same active site. Mutation of ADSL's arginine 303 to a cysteine is known to lead to ADSL deficiency. Interestingly, unlike other mutations leading to ADSL deficiency, the R303C mutation has been suggested to more significantly affect the enzyme's ability to catalyze the conversion of SAMP than that of SAICAR to their respective products. To better understand the causation of disease due to the R303C mutation, as well as to gain insights as to why the R303C mutation potentially has a disproportional decrease in activity toward its substrates, the wild-type (WT) and the R303C mutation of ADSL were investigated enzymatically, and thermodynamically. Additionally, the X-ray structures of ADSL in its apo form as well as with the R303C mutation were elucidated, providing insight into ADSL's cooperativity. By utilizing this information a model for the interaction between ADSL and SAICAR is proposed.

Adenylosuccinate lyase (ADSL) deficiency is a rare autosomal recessive disorder characterized by serious neurological and physiological symptoms such as psychomotor retardation (PMR), expression of autistic features, structural brain abnormalities, axial hypotonia, seizures, peripheral hypotonicity, ataxia, muscle wasting, growth retardation and

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The atomic coordinates and structure factors have been deposited with the Brookhaven Protein Data Bank (PDB codes 4FLC, 4FFX).

SUPPORTING INFORMATION AVAILABLE

The supplementary figures S1, Specific activity vs [Substrate] plots of WT and R303C ADSL, S2, Calorimetric Titration of ADSL with AMP and AICAR, and S3, Missing electron density for R303 when mutated to cysteine in ADSL are provided in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

strabismus (1–3). More than 60 cases of ADSL deficiency have been reported worldwide, but it is widely considered to be underdiagnosed (4–6). ADSL deficiency is clinically diagnosed by elevated levels of succinylaminoimidazolecarboxamide riboside (SAICAR) and succinyladenosine (S-Ado) in urine, plasma, and cerebrospinal fluid (7). Three distinct phenotypic groups have been established: 1) Neonatal; 2) Type I; 3) Type II. The neonatal form results in fatal neonatal encephalopathy and has a S-Ado:SAICAR ratio of <1. Type I patients experience early onset, severe PMR, and have a ratio of ~1. Type II patients experience later onset with mild PMR, and have a ratio of 2–4 (8). There are three main hypotheses that describe the difference in ratio: 1) nonparallel reduction in the enzyme's activity on its substrates; 2) differential dephosphorylation or transport of the substrates out of the cells (9, 10), and 3) inability of ADSL mutant protein to form an active purinosome, which is formed when purine synthesis is required and is needed for appropriate channeling of SAICAR through the *de novo* purine synthesis pathway (11).

ADSL carries out two non-sequential steps of *de novo* AMP synthesis, the conversion of succinylaminoimidazolecarboxamide ribonucleotide (SAICAR) and succinyladenosine monophosphate (SAMP) into aminoimidazolecarboxamide ribotide (AICAR) and adenosine monophosphate (AMP), respectively, with the concomitant release of fumarate in each case (12). The conversion of SAMP to AMP is also part of the purine nucleotide cycle. So far, all mutations, with one exception, result in a proportional loss of enzyme activity with the two substrates. The ADSL carrying the R303C mutation has been observed as a homozygous mutation in two unrelated patients. It is a type II mutation and results in the mildest observed form of ADSL deficiency. Interestingly, this mutation shows a more severe loss in activity with SAMP than with SAICAR, although the extent of disproportionality varies from study to study, perhaps reflecting differences in how the enzyme assays were carried out. Some investigators were unable to detect activity with SAMP in extracts from fibroblasts (13), while others found 3% of normal activity (8). In other cases using different recombinant ADSL constructs, 7% and 18 % of wild-type (WT) activity was observed with SAMP and 44% with SAICAR (13, 14). The reason for the non-parallel loss of activity is not yet understood, nor is the reason why this mutation leads to such a mild phenotype. Hypotheses include the possibility that different amino acids in the active site bind the two substrates, that cysteine is not able to interact well with SAMP, or that there are alterations in the stability of the active site cleft (13, 14).

Crystal structures of the bacterial ADSL and other studies have provided insight into the active site and the catalytic mechanism; however these studies predominately use bacterial ADSL from organisms such as *Thermotoga maritima* or *Escherichia coli* (15, 16). *T. maritima* and *Homo sapiens* have a sequence identity of 25% and similarity of 57%. *E. coli* and *H. sapiens* have a sequence identity of 23% and a similarity of 57%. ADSL was found in these bacterial species to function in the pathway as a homotetramer (Fig 1). Interestingly, three monomers contribute to each of the four active sites in the tetramer (9, 17). The use of *Bacillus subtilis* ADSL in particular as a model to study properties of mutant enzymes in some cases involved modification of additional amino acid residues in the *B. subtilis* ADSL to more closely resemble human ADSL (18, 19). However, attempts to make use of a *B. subtilis* model system to replicate the R303C phenomenon have proven difficult because *B. subtilis* and *H. sapiens* only have a sequence identity of 30% and a similarity of 63%. Specifically, the sequence diversity of *B. subtilis* and *H. sapiens* results in multiple alterations within the active site including, but not limited to, an asparagine residue in place of the corresponding R303 in human ADSL, as well as an arginine residue in place of human corresponding T354. Not surprisingly, the removal of *B. subtilis*'s asparagines at the corresponding human ADSL R303 position for cysteine did not generate a disproportional reduction in catalytic ability (18, 19). This observation as well as others suggest that the study of the bacterial enzyme, while initially useful for understanding some of the basic

features of ADSL, such as its tetrameric nature and participation of three subunits in forming each active site, may not be as useful for understanding the effects of various disease causing mutations on the human enzyme. Only limited structural information exists of human ADSL active site in the unpublished SAMP bound and S-AMP/AMP-fumarate bound PDB deposited entries (2J91, 2VD6). Current studies on disease-associated mutations of human ADSL have focused on correlation of substrate activity with clinical phenotypes, thermal stability of ADSL, activity of hybrid WT ADSL and mutants, and global changes in structure (14, 20–23). They have not investigated local structural changes or binding properties to the products within the ADSL active site. In this study, we present structural and biochemical characterization data of WT and mutant R303C ADSL by enzyme kinetics, product binding by isothermal titration calorimetry (ITC), and X-ray crystallography to reveal the effects of the R303C mutation that results in nonparallel reduction in enzyme activity.

EXPERIMENTAL PROCEDURES

Materials

Chemicals, biochemicals, buffers, and solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Pittsburgh, PA), Fluka Chemical Corp. (Milwaukee, WI), or EM Science (Cincinnati, OH). The Centricon and Ultrafree centrifugal filter devices were obtained from Millipore Co. (Billerica, MA). Nickel-nitrilotriacetic acid-agarose, a QIAspin kit, and high throughput crystal condition screens were purchased from QIAGEN. Additive HT Screen was purchased from Hampton Research. QuikChange site directed mutagenesis kit was purchased from Stratagene. SAICAR was prepared enzymatically from AICAR purchased from Sigma- Aldrich Chemical Co. as described by Zikanova, *et al* (24). Enzymes and reagents used for molecular biology procedures were obtained from New England Biolabs, Inc. (Ipswich, MA). The sources for the components of Luria-Bertani (LB) media are reported elsewhere (15).

Site-directed mutagenesis, Enzyme Expression, and Purification

The initial WT ADSL construct was obtained from Dr. Roberta F. Colman, Department of Chemistry and Biochemistry, University of Delaware, Newark, DE. The full description of the initial WT ADSL construct is described in Lee and Colman (20). In short, the full length human ADSL gene (1-484 residues) was constructed in pET-14b vector containing a 5' end *NdeI* restriction site and a 3'-end *BlnI* restriction site and a thrombin cleavable N-terminal histidine tag. In order to overexpress the human enzyme in *E. coli*, the vector was transformed into *E. coli* Rosetta 2(DE3)pLysS. The WT and R303C ADSL were purified to homogeneity using a Qiagen Ni-NTA column. Purity was assessed by SDS-PAGE gel electrophoresis (data not shown). After purification, protein was stored in Enzyme Storage Buffer (50 mM potassium phosphate buffer, pH 7.0, containing 150 mM KCl, 1 mM DTT, 1 mM EDTA, and 10% (v/v) glycerol) at –80°C. Introduction of point mutations in the human ADSL plasmid was done using QuikChange site-directed mutagenesis. The QIAspin kit was used for cDNA extraction and purification. DNA sequencing was performed at the CU Cancer Center DNA Sequencing & Analysis Core to confirm mutations. Slight modifications were made to the purification for ITC and X-ray Crystallography. Following the purification using the Qiagen Ni-NTA column, thrombin was added to the eluted fraction and it was dialyzed overnight at 4°C in ADSL Running Buffer (5 mM HEPES pH 7.0, 150 mM KCl, 2 mM DTT) to cleave the His-tag. The protein was purified by size exclusion chromatography using S200 sepharose resin and concentrated to 10 mg/mL for crystallography, and to 200–400 μ M for ITC. Concentrations were measured by the absorbance at 280 nm using an experimentally determined extinction coefficient of 0.782 L

$\text{g}^{-1}\text{cm}^{-1}$ (43,150 $\text{M}^{-1}\text{cm}^{-1}$) for thrombin cleaved ADSL and 0.770 $\text{L g}^{-1}\text{cm}^{-1}$ for His-tagged ADSL following previously established procedures (25).

Static Light Scattering

Size Exclusion Chromatography-Multi-Angle Light Scattering measurements were performed on a Wyatt miniDAWN TREOS connected to a Shimadzu UFLC and a Wyatt WTC-030S5 size exclusion column. Samples of 350 μg of His-tagged ADSL diluted in Enzyme Storage buffer were run in duplicate. PBS was used as the mobile phase and flowed at 0.5 mL/min. Calibration was checked using 2 mg/mL bovine serum albumin. Data were analyzed with ASTRA software, and 0.185 was used as the dn/dc value.

Enzyme Assays and Kinetic Studies

Enzyme kinetic experiments were performed on a UV-Vis Spectrophotometer Evolution 3000 from Thermo Scientific using 1 mL quartz cuvettes at 25°C. ADSL with the His-tag intact was used for enzyme assays, as it has been shown that the His-tag does not affect ADSL activity for SAMP (26). Experiments were run with concentrations of 0.11 mg/mL and 0.19 mg/mL for WT and R303C ADSL, respectively. Frozen samples were incubated for ~2 hours at 25°C before measurements were taken to assure restoration of full activity (26). SAMP enzyme assays of ADSL were measured in triplicate at 25°C in 40 mM Tris-HCl (pH 7.4) with varying concentrations of SAMP (1 – 60 μM). Specific activity was measured from the decrease in absorbance of SAMP at 282 nm as it was converted to AMP and fumarate. The assay was monitored over 30 seconds in a 1 mL volume. The difference in extinction coefficient of 10,000 $\text{M}^{-1}\text{cm}^{-1}$ between SAMP and AMP was used to calculate the specific activity. SAICAR enzyme assays of ADSL were measured in triplicate at 25°C in 40 mM Tris-HCl (pH 7.4) with varying concentrations of SAICAR (1 – 100 μM). Specific activity was measured from the decrease in absorbance of SAICAR at 269 nm as it was converted to AICAR and fumarate. The assay was monitored over 30 seconds in a 1 mL volume. The difference in extinction coefficient of 700 $\text{M}^{-1}\text{cm}^{-1}$ between SAICAR and AICAR was used to calculate the specific activity. To determine the kinetic constants, the initial velocity data were fitted to the Hill equation, $v = V_{\text{max}} * [\text{S}]^n / (K_{0.5}^n + [\text{S}]^n)$ for WT and R303C ADSL and $v = V_{\text{max}} * [\text{S}] / (K_M + [\text{S}])$ for R303C ADSL, using the Enzyme Kinetics Module 1.3 of Sigma Plot version 10 (SPSS Inc.). The k_{cat} value was calculated from V_{max} and the enzyme concentration $[\text{E}]$ via the equation $k_{\text{cat}} = V_{\text{max}} / [\text{E}]$.

Isothermal Titration Calorimetry (ITC)

ITC experiments were conducted using a NANO-ITC system (TA Instruments, Utah, USA). Thrombin cleaved WT ADSL was dialyzed overnight against a solution of 25 mM HEPES pH 7.0, 150 mM KCl, and 2 mM DTT. AMP was brought to a concentration of 2.5 mM using the buffer in which WT ADSL was dialyzed. ITC runs of WT ADSL with AMP were performed in duplicate and comprised of one 1 μL injection followed by 24, 2 μL injections for a total of 25 injections of 2.5 mM AMP into 0.250 mM WT ADSL. Each injection was spaced 250 seconds apart. ITC experiments were performed in an identical method, injecting AICAR instead of AMP into 0.225 mM WT ADSL. In addition, AMP and AICAR were titrated in an equal manner into 0.280 mM R303C ADSL. Data sets were analyzed with NanoAnalyze software and fit to an independent model concurrently with NanoAnalyze blank constant model to adjust for heat of dilution. The blank constant model was derived using heats from the last injections deemed by the blank function to be occurring during saturated conditions.

Crystallization of WT and R303C ADSL

Initial crystal conditions for both WT and R303C ADSL were determined from high-throughput screening of Qiagen Nextel screens, Classics and PEG I, in a 96 sitting drop format using an Art Robbins Phoenix robot. The drops consisted of 0.4 μ L protein solutions and 0.4 μ L precipitate in a 100 μ L reservoir. Initial screening exhibited multiple hits; however, a solution containing 0.1 M Tris pH 8.0, and 20% (w/v) PEG 6000 produced the most viable crystals for WT ADSL, and 0.1 M Tris pH 8.5 and 20% (w/v) PEG 8000 for the ADSL mutant R303C. These crystals were then optimized using Additive HT Screen from Hampton Research. Final WT and R303C ADSL crystals were obtained through vapor diffusion using a 500 μ L reservoir with 4 μ L drops mixed 1:1 with protein solution and, for native ADSL, a precipitant gradient of 18–28% (w/v) PEG 6000, 0.1 M Tris pH 8.0, and 12.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and for R303C, a precipitant gradient of 18–28% (w/v) PEG 8000, 0.1 M Tris pH 8.5, and 12.5 mM spermine tetrahydrochloride.

X-ray structural determination of ADSL Structures

All X-ray native datasets were collected using crystals mounted on nylon loops and submerged in a 5 μ L cryo solution of 26% (w/v) PEG 6000 and 0.1 M Tris pH 8.0 for WT ADSL, and 26% (w/v) PEG 8000, 0.1 M Tris pH 8.5 for R303C ADSL. In order to preserve the crystals they were submerged and flash frozen in liquid nitrogen. Frozen crystals were mounted under a stream of dry N_2 at 100 K. A WT ADSL dataset with resolution to 2.70 $\text{\AA}$ and ADSL mutant R303C dataset with resolution to 2.60 $\text{\AA}$ were collected at the 21-ID-D Life Science-Collaborative Access Team beamline at the Advanced Photon Source Synchrotron. Both datasets were collected at 0.9789 $\text{\AA}$ with a MarMosaic300 CCD detector (Rayonix). All data was subsequently processed and scaled using Scalepack (27) and subsequent phases were determined and refined using Phaser (28). Initial models for both WT and R303C ADSL were elucidated by molecular replacement using ADSL structure 2J91 as a search model for Phaser (28). Both structures were refined using iterative cycles of model building and structure refinement using WinCOOT (29) and REFMAC (30), respectively. Water molecules were added to $2Fo-Fc$ density peaks that were greater than 1σ using the “Find Water” winCoot program function. The final model was checked for structural quality using the CCP4 suite programs: Procheck and Sfccheck. Data processing and refinement statistics are shown in Table 1.

Modeling of AMP/AICAR and Fumarate Binding in the Active Site

Apo structures of WT ADSL with either AICAR or AMP were neutralized with the TLEAP module from the Amber10 simulation package (31, 32) and solvated using TIP3P water molecules (33). The final system was then energy minimized using the Amber 10 simulation package to obtain the final structure. Initially, each system's protein was restrained, and the solvent was minimized for 5,000 steps to remove any unfavorable contacts that occurred within the water. Then, each system (solvent and molecule) was allowed to energy minimize for 20,000 steps, starting with 10,000 steps of steepest descent and switching to 10,000 more steps of conjugate gradient minimization. The force field used was ff99SB (34). We removed residues outside of 30 $\text{\AA}$ from AMP/AICAR active site. This was done to reduce computational demand by ignoring distant parts of the protein not relevant for the present problem. However, 30 $\text{\AA}$ was long enough to focus on a sufficiently large region around the active site to avoid any artifact due to small system size. All the calculations were performed at University of Denver's High Performance Computing facility, which is a Linux cluster with 22 twin nodes, each node having eight cores.

RESULTS

WT and R303C ADSL Enzyme Kinetics

To ensure that the previously observed reduction in activity of the ADSL possessing the R303C mutation was not due to aberrant tetramer formation or outright global instability of the mutated protein in solution, static light scattering was employed to determine the polymeric distribution of the R303C and WT ADSL. As expected, WT ADSL is predominantly found to be a tetramer, $92.7 \pm 2.6\%$, with aggregates contributing the final percentage. Similarly, R303C ADSL is predominantly a tetramer, $94.3 \pm 0.1\%$, with aggregates contributing the final percentage. Observed weights of the WT and R303C ADSL tetramers were 225.0 ± 2.9 kDa and 214.5 ± 12.2 kDa, which is in close agreement with the His-tagged WT and R303C ADSL predicted tetrameric weights, suggesting that the R303C mutation results in no degradation of the ADSL tetramer.

Previously, a report noted that the ADSL carrying the R303C mutation displayed non-parallel decrease in activity at a single concentration (14). Additionally, the kinetic parameters for human ADSL utilization of SAICAR have never been fully determined. Interestingly, Stone et. al. 1993, details a K_M and k_{cat} of ADSL for SAICAR (32). Unfortunately, the boundaries of ADSL were not well established at the time of that study, resulting in their use of a 25 amino acid N-truncated version of ADSL and not accounting for cooperativity (33). Unfortunately, a subsequent study by Kmoch et al. (2000) also did not account for ADSL cooperativity, nor did they report on the R303C mutant. This has left ambiguity to the kinetic parameters surrounding ADSL ability to use SAICAR as a substrate. To further investigate the R303C phenomenon and determine the K_M and k_{cat} of ADSL for SAICAR, kinetic assays were performed on both WT and R303C ADSL using SAICAR and SAMP as substrates (14). Both WT and R303C ADSL had measurable activity that could be evaluated by monitoring the UV absorbance of either SAMP or SAICAR. As WT ADSL was previously reported not to follow simple Michaelis-Menten kinetics for SAMP and perhaps be cooperative, the Hill equation was initially employed for calculating WT ADSL's kinetic parameters (Table 2; Fig. S1) (20). The resulting Hill coefficients of WT ADSL for SAMP and SAICAR are both significantly above one, indicating cooperativity for the substrates. The resulting k_{cat} and $K_{0.5}$ of WT ADSL for SAMP was in agreement with a prior study that utilized the non-truncated form of ADSL (20). For WT ADSL's utilization of SAICAR, its $K_{0.5}$ was 1.8 ± 0.1 μ M, which is only slightly higher than SAMP's. However, WT ADSL's k_{cat} for SAICAR was 1.6 fold higher than that for SAMP suggesting that ADSL is more kinetically efficient for the catalysis of SAICAR over SAMP. This is confirmed by WT ADSL's $k_{cat}/K_{0.5}$ for SAICAR being 2 fold higher than SAMP's. Additionally, the k_{cat} for both substrates was found not to be diffusion limited.

Introduction of the R303C mutation into ADSL has multiple effects. Initially expecting similar cooperativity observed in WT ADSL, the Hill equation was utilized (Table 2). Unlike WT ADSL, the R303C ADSL demonstrates no cooperativity, with a Hill coefficient for both substrates of one. As a result, the simple Michaelis-Menten equation was employed for R303C. The R303C ADSL mutation reduces k_{cat} to 4.5% and 30% of that relative to WT ADSL for SAMP and SAICAR, respectively. Interestingly, this 7-fold difference in residual *in vitro* activity is in line with cell free extract studies of a Type II patient's fibroblasts carrying the R303C mutation, which had 3% of normal SAMP activity and 30% of normal SAICAR activity (22, 35). As a result, the ratio of activity of WT ADSL for SAICAR:SAMP is 1.7, which is in agreement with a previously published ratio of 1.6, but the ratio of activity for the R303C mutant ADSL shifts to 11.5 (21). Interestingly, K_M values of R303C ADSL increased for SAICAR compared to that of WT ADSL. The change observed in K_M of R303C ADSL for SAMP is almost negligible, with the K_M for SAICAR increasing more than 4-fold. This may initially suggest the mutation affects the ability of

R303C ADSL to bind SAICAR more than SAMP resulting in the disparity in their cleavage. However, the rate of SAICAR cleavage by R303C ADSL is 5 times more rapid than that for SAMP at R303C ADSL's SAMP K_M . Therefore, the effect of the R303C mutation on ADSL may not solely be reflective in its divergent ability to bind SAICAR and SAMP. It suggests that R303C may affect the catalytic mechanism of SAMP and SAICAR in addition to binding of the substrates.

Isothermal Titration Calorimetry (ITC)

To explore the possible thermodynamic factors involved in the non-parallel reduction in activity resulting from the R303C mutation and in general ADSL-substrate interactions, ITC was performed on WT and R303C ADSL with their products AMP and AICAR. By measuring the amount of heat liberated per injection as a function of the molar ratio of the substrate and protein, thermodynamic parameters for R303C and WT ADSL's interaction with their products were calculated (Table 3, Fig. S2). Intriguingly, product binding does not show any evidence of cooperativity, as the data fit to an independent model rather than a multiple binding site model. This does not necessarily rule out the possibility that the substrate binding is cooperative, but suggests that cooperativity may require inclusion of interactions between the fumarate component of the substrates and ADSL, which is lacking in the products.

Beyond the absence of cooperativity observed, the K_d values for WT ADSL were 54 and 34 μM for AMP and AICAR, respectively (Table 2, Fig S1a,b). The thermodynamic properties that are responsible for AICAR and AMP binding to WT ADSL globally are similar with the enthalpic component, ΔH , being the predominant driving force and the entropic component, ΔS , being unfavorable. Although similar, the thermodynamic properties are not identical. AICAR binding liberates an additional 18 kJ/mol, suggesting an additional hydrogen bond may be formed between AICAR and WT ADSL that is absent in a AMP and WT ADSL complex. The lack of this additional enthalpic contribution is partially offset in the AMP and WT ADSL complex by the reduced unfavorable entropic component compared to the AICAR and WT ADSL complex. Overall, this leads to a difference in ΔG of AMP and AICAR to WT ADSL to be only 1.2 kJ/mol in AICAR's favor.

Intriguingly, the R303C mutation impacts the ability of ADSL to form a complex with AMP and AICAR in two distinct manners. The enthalpic component for both products decreases by a similar degree of ~30 kJ/mol suggesting the loss of one to two hydrogen bonds, whose identity could be common between the two products and ADSL. Additionally, the R303C mutation shifts the ADSL complex formation with AMP and AICAR from an unfavorable to favorable entropic event. Taken together, these thermodynamic factors result in a similar decrease of ΔG and increases of 76 and 88 μM in the K_d of AMP and AICAR respectively. As a result, the R303C mutation appears to negatively affect ADSL's interaction with AMP and AICAR almost indistinguishably.

X-ray structural elucidation of R303C and WT ADSL

Recently, a study proposed that the R303C mutation may distort the active site cleft (14). To investigate the structural effects of the R303C mutation on ADSL and how these effects might be linked to the lower activity and unique specificity of ADSL with the R303C mutation, the crystal structure of R303C ADSL (R303C-ADSL) was elucidated to 2.60 Å. A homo-tetramer in the asymmetrical unit was observed, with each monomer composed of 16 α -helices and 3 small β -sheets, which can be further divided into three domains (Fig. 1,2). Electron density was observed for the majority of all 5–476 ADSL residues in all monomers, with residues 5–112 forming domain 1, residues 113–364 as domain 2, and 364–476 as domain 3. A notable exception was the residue range 286–291 in all monomers. These

residues comprise the $\beta 3$ - $\alpha 10$ loop (also known as the C3 loop), which is located near ADSL's active site and expected to contain an important catalytic serine, S289. The lack of density for this region is not fully unexpected, as it has been unobserved in all but one bacterial ADSL X-ray structure. As a result, the loop is suggested to be highly flexible (Fig. 1). Apart from the expected differences in the positions of the residues just prior to and after the missing density of the $\beta 3$ - $\alpha 10$ loop, the only other notable difference observed between the four monomers of R303C-ADSL resides in domain 3 of the structure. This domain is on the periphery of the tetrameric complex likely resulting in the small alterations and higher B-factors between the four monomers in that region (Fig. 2).

Despite these differences on the periphery of the protein, from a distance the R303C-ADSL globally resembles that of the PDB entry 2VD6 ADSL with its four active sites a mixture of active site bound SAMP, or AMP with fumarate (ADSL-SAMP) as well as AMP bound ADSL PDB entry 2J91 (ADSL-AMP). Unlike ADSL-AMP where all of the active sites are indistinguishably bound to AMP, ADSL-SAMP has its active sites 1 and 2 filled with AMP and fumarate, while the SAMP was observed in active sites 2 and 4 (Fig. 3a,b). Both of these ligand bound structures previously have alluded to ADSL active sites being formed by three contributing monomers. For example, in the active site 1, the monophosphate group of AMP hydrogen bonds to three residues from chain A. The fumarate interacts with 3 residues from chain A bridging across to interactions with two residues from chain B, and one from chain D (Fig. 3a). The ADSL-SAMP active sites with SAMP bound have similar intermonomeric interactions (Fig. 3b). Not surprisingly, upon inspection of the corresponding active site of R303C ADSL, density for the R303 side chain is largely absent with only enough remaining to represent a mutation to cysteine at that position (Fig. 3c, Fig. S3). Based on the ligand bound structures, this mutation would appear to simply eliminate the hydrogen bonds formed between the monophosphate in the AMP, or SAMP, and ADSL. This loss of the phosphate-R303 interaction, which also likely exists with AICAR, could be reflected in the enthalpy change detected by ITC for AMP and AICAR. However, upon closer examination by superimposing the active sites of R303C-ADSL with the corresponding sites in ADSL-SAMP revealed that the two structures' corresponding active sites are not entirely identical, giving possible credence to an earlier study's suggestion that the R303C mutation may disrupt the active site cleft (14). Specifically, comparison of R303C-ADSL's active sites with ADSL-SAMP's corresponding fumarate and AMP bound active sites shows a 2.5 Å shift of α -helices 2, 3 and 4 (Fig. 4a). This shift is also observed between R303-ADSL and ADSL-AMP. Interestingly similar, but not exact, shifts are also observed between R303C-ADSL's active sites and the SAMP filled active sites of ADSL-SAMP. To determine if the shift in α -helices 2 – 4 was related to the R303C mutation or a conformational change related to substrate binding, the structure of WT ADSL in its apo form (WT-ADSL-apo) was elucidated to 2.70 Å (Fig. 4b). Similar to the ADSL-R303C and ADSL-SAMP, electron density for residues 283–293 ($\beta 3$ - $\alpha 10$ loop) was not observed. Similarly to R303C-ADSL, WT-ADSL's monomers were largely indistinguishable outside of the domain 3. Intriguingly, comparison of the WT-ADSL-apo with ADSL-SAMP revealed a 2.7 Å shift of α -helices 2 – 4 in line to those observed previously in the R303C-ADSL and ADSL-SAMP comparison, indicating that substrate binding is likely the major cause of the shifts observed between the ADSL-R303C and the ADSL-SAMP (Fig. 4c), while comparison of WT-ADSL-apo to R303C-ADSL revealed nearly identical structures (Fig. 4d). In addition, WT-ADSL-apo reveals an additional apo form of the ADSL active site that is divergent from product and substrate bound active sites previously observed.

Modeling of AMP/AICAR and Fumarate Binding in the Active Site

Currently, no crystal structure for WT ADSL with AICAR bound has been resolved. Utilizing the elucidated WT-ADSL-apo and ADSL-SAMP structures along with the

understanding of local active site shifts within the active site upon substrate binding; AICAR and fumarate were modeled into the active site. Specifically, the AMP and fumarate bound active site of 2VD6 was employed as a template for the initial location of AICAR and fumarate within an active site of the WT-ADSL-*apo*. Using the Amber10 simulation package, by energy minimization the ADSL-AICAR-fumarate model structure was determined (Fig. 5a). To validate the modeling parameters used, we also energy minimized the structure with AMP and fumarate instead in the WT-ADSL-*apo* active site. The resulting AMP and fumarate bound ADSL model was nearly identical to that of AMP and fumarate bound in ADSL-SAMP.

As is evident from the figure, the placement of the fumarate, phosphate and ribose groups of AICAR and SAMP within the ADSL active site is similar (Fig. 5b). However, the remaining structure of the two substrates forms divergent interactions within the active site. This divergence could be a result of a rotatable bond between AICAR's imidazole ring and carbonyl carbon, whereas the presence of the pyrimidine in AMP eliminates the ability of the corresponding bond to rotate (36). As a result, this flexibility in AICAR and an amine group substituent of its imidazole ring potentially allows the amine to stick out in a space previously occupied by a water molecule potentially facilitating hydrogen bond with the hydroxyl group of the conserved S334 (Fig 1). Additionally, the rotation of the AICAR amide causes an alteration in the R329 side chain to form a hydrogen bond with AICAR's amide group.

DISCUSSION

Origins of ADSL cooperativity

The elucidation of the ADSL-SAMP structure has previously been suggested to support cooperativity in human ADSL, as AMP and fumarate are found in two active sites and with the other two occupied by SAMP. However, previously limited biochemical data existed to support the origins or form of the cooperativity found in ADSL. Interestingly, the kinetic and thermodynamic properties of the WT ADSL and the R303C mutant suggest that the involvement of all three monomers that comprise an ADSL active site is necessary to achieve ADSL's positive cooperativity. Specifically, no cooperativity was observed when binding AICAR, or AMP, to WT ADSL. Binding of these products form the majority of their interactions with only two of the monomers. For example, in active site 1, AMP interacts with six residues from monomer A, and one from monomer B. Additionally, the phosphate group of AMP is anchored with two additional residues from a relatively immobile portion of monomer D. (Fig. 3a). This leaves the fumarate product to span interactions between monomer A's mobile α -helices 2 – 4, monomer B, and the near β 3- α 10 catalytic loop region of monomer D, suggesting that it is necessary for cooperativity to be observed. In line with this hypothesis, mutation of R303 to cysteine, which removes two hydrogen bonds, thus removing the majority of the interactions between monomer D and SAMP, results in no cooperativity (Fig. 3b).

Beyond the necessity for the substrate, or products, to involve all three monomers when binding to achieve an allosteric response, comparison of the WT-ADSL-*apo* and ADSL-SAMP active sites provided a glimpse into the model of allosteric regulation that ADSL undergoes. Specifically, the WT-ADSL-*apo* active sites are considerably more open to the bulk solvent than those of the ADSL-SAMP and ADSL-AMP structures (Fig. 6a). The 2.7 Å shift of α -helices 2, 3 and 4 observed between the WT-ADSL-*apo* active sites and those bound with AMP, or AMP and fumarate, illustrates a clamping down of the active site over SAMP, or AMP. With AICAR and SAICAR having homologous phosphate, fumarate, and ribose moieties to that of AMP, the same shift is likely to occur upon the binding of these ligands as well. This closure of the active site around SAMP suggests that a concerted model

of allosteric regulation would not be possible (Fig. 6c). In other words, if this shift occurred in a neighboring active site as a result of binding SAMP and SAICAR for the reaction running in the forward direction, SAMP attempting to bind to the neighboring active site would be sterically impeded. This would not be reflective of the positive cooperativity observed. Applying sequential allosteric regulation to the reverse reaction is less certain. The binding of AMP and AICAR would also be sterically impeded from binding to an active site that already underwent a shift of α -helices 2, 3 and 4. However, as illustrated by the thermodynamic information, the binding of AMP and AICAR does not illustrate cooperativity. With fumarate binding at the top of the active site, which is exposed to the bulk solvent, a concerted model cannot be ruled out in the reverse direction (Fig. 6b).

Beyond the sequential model of allosteric modulation of ADSL, in what order, or the extent to which the active sites fill with substrates remains an open question. The ADSL-SAMP structure has its two active sites on the same end of ADSL occupied by SAMP and other distal active sites bound with fumarate and AMP. The absence of SAMP in all of the active sites could be due to restraints of the enzyme imposed by the crystal lattice, but alternatively might propose that substrates bind to adjacent active sites first, or only can bind to them. The latter would infer that the active sites at one end of the ADSL tetramer communicate to its distal neighbors. Although the mild loss of cooperativity observed by Ariyananda et al. (20) by an ADSL mutation L311V at the center of the tetramer would support this possibility, additional structurally guided mutagenesis along monomer's interface and molecular dynamics simulations will be required to completely tease out the global allosteric nature of ADSL.

Catalytic Effects of R303C mutation

The loss of cooperativity because of the R303C mutation removal of hydrogen bonds with one of the three monomers comprising the active site as reflected in the structures of ADSL-SAMP, or ADSL-AMP, would not itself explain the divergent catalytic properties of ADSL observed towards SAICAR and SAMP. However, the combination of the kinetic and thermodynamic data coupled with the structural evidence suggests that the R303C mutation illuminates SAICAR as a better substrate for ADSL. At first glance, the unparallel reduction in k_{cat} between SAMP and SAICAR upon the R303C mutation could suggest that the divergence in catalytic ability of ADSL between SAMP and SAICAR is due to a lack of substrate binding that favors SAICAR, or AICAR binding over that of SAMP. However, the K_M for SAICAR is slightly elevated relative to that of SAMP indicating that binding of SAICAR might be slightly weaker (Table 2). Moreover, the ADSL ligand bound structures have R303 forming hydrogen bonds to the phosphate group that is conserved between SAICAR and AICAR (Fig. 3). Also, the thermodynamic data reflects that the mutation causes a loss of enthalpy at almost equal magnitude for both AICAR and AMP, reflective of a loss of the same ADSL-phosphate group interactions. Despite the loss of the bond to the phosphate group, the overall ΔG_{bind} for both products remains similar to WT ADSL. This is accomplished by shifting the product binding from an unfavorable to favorable entropic event. The resulting K_d s for both substrates are nearly equally decreased by approximately 2.5–3 fold. This near equal drop in K_d but not k_{cat} suggests that the R303C mutation is indirectly affecting the catalytic ability of ADSL divergently for its two substrates. In other words, this thermodynamic, kinetic, and structural evidence suggests that the substrate can bind to the active site at a significant affinity despite the R303C mutation. As a result, unlike WT ADSL, the R303C mutant is likely operating at rapid equilibrium state, where if the substrate binds to ADSL, most substrate will dissociate, and only a small amount will be converted to product.

This would suggest that ADSL is more efficient at cleaving the fumarate-AICAR/AMP bond of SAICAR than SAMP. The inequality of catalysis might suggest that SAICAR and

SAMP do not follow the same mechanistic pathway. The reaction mechanism for conversion of SAMP and SAICAR by ADSL has been previously described as a general acid-base mechanism resulting in β -elimination of fumarate (Fig. 7)(16). Although, the conversion of SAICAR to AICAR and fumarate could follow the same steps as the SAMP to AMP mechanism, due to the presence of an additional carbonyl in SAICAR, there is another possibility. In this SAICAR selective pathway, the first two steps are the same: the C $^{\beta}$ -proton is removed, and the negative charge is stabilized by the δ -carboxyl group. However, in the third step, as the electron density from the double-negatively charged carboxyl group is shifted down and the C $^{\alpha}$ -N6 bond is broken, a double bond is formed between N6 and the carbonyl carbon, and electron density is pushed up onto the carbonyl oxygen. Then, in the last step, this negative charge moves back down to form a C-O double bond as N6 becomes protonated by H159. This negatively charged oxygen may be stabilized by nearby positively charged residue, R235. This additional stabilization of the intermediate may be a factor in the faster reaction rate of SAICAR compared to SAMP.

An alternative possibility, or potential additional factor, for the inequality of bond cleavage between SAMP and SAICAR is the ability of SAICAR to be positioned with the active site in a more catalytically effective orientation than SAMP. The loss of the hydrogen bonds that R303 contributed to substrate binding potentially affected the ability to orient SAMP and SAICAR ideally for cleavage. Unlike SAMP, which possess a pyrimidine ring, SAICAR has a single rotatable bond that has been previously suggested to infer greater flexibility to adopt different conformations (36). This also might explain the lack of unparallel reduction in catalytic ability of *B. subtilis* ADSL. In that case, *B. subtilis* ADSL has an arginine in place of human ADSL T354 that may provide a similar influence. Unfortunately in the Palenchar *et al.* (2003) study that looked at *B. subtilis* ADSL corresponding human ADSL R303C mutation (*B. subtilis* ADSL N276C), only performed a double mutation to mimic the human active site configuration and did not include a single mutation for the corresponding arginine at human ADSL's T354. This prevented observing whether elimination of anchoring ADSL's substrates by a third arginine resulted in unparallel catalytic ability.

Additionally, the thermodynamic data suggests that AICAR forms an additional hydrogen bond not present in the ADSL-AMP complex. The enthalpic divergence between the two substrates remains after the introduction of the R303C mutation. The ability of SAICAR to possess an additional point of contact within the ADSL active site could ensure that SAICAR properly oriented within the active site more often than SAMP, contributing to the unparallel k_{cat} between SAMP and SAICAR observed. Based on the ADSL-AICAR-fumarate model, the additional hydrogen bond could be reflective of an interaction between AICAR and the highly conserved S334 (Fig 5a). Curiously, even in human ADSL's distant homologue of *B. subtilis*, this serine is conserved. This serine has not previously been implicated in substrate binding or catalysis, as it does not interact directly with SAMP or AMP within the ADSL-SAMP and ADSL-AMP structures. Naturally, additional site-directed mutagenesis efforts within the active site including S334, or an X-ray structure of ADSL with SAICAR, or AICAR, will be necessary to fully identify the ADSL residue acceptor that forms the additional hydrogen bond with AICAR and bring final clarity to the unparallel catalytic activity observed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADSL	Adenylosuccinate lyase
WT	Wild Type
SAICAR	succinylaminoimidazolecarboxamide ribonucleotide
AICAR	aminoimidazolecarboxamide ribotide
SAMP	succinyladenosine monophosphate
AMP	adenosine monophosphate
PMR	psychomotor retardation
SAICar	succinylaminoimidazolecarboxamide riboside
S-Ado	succinyladenosine
DTT	DL-Dithio-threitol
EDTA	Ethylenediaminetetraacetic acid tetrasodium salt dihydrate
Ap	ampicillin
ITC	Isothermal Titration Calorimetry
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate
IPTG	isopropyl-β-D-thiogalactoside
LB	Luria-Bertani
NCBI	National Center for Biotechnology Information
PEG	polyethylene glycol
PPT	phenylpyruvate tautomerase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tris	tris(hydroxymethyl)aminomethane

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Figure 1.

Sequence alignment of ADSL from various species. ADSLs are from *H. sapiens* (GenBank AAC83935.1), *M. musculus* (GenBank AAB60684.1), *B. subtilis* (NCBI Reference Sequence: YP_003865018.1), and *C. griseus* (NCBI Reference Sequence: NP_001230974.1). Secondary structure of hADSL according to Defined Secondary Structure of Proteins (DSSP) is represented by green cylinders (helical regions), orange arrows (β-sheet regions), black lines (loops), and black dashes (unstructured regions). Mutation site 303 is marked with an asterisk. The location of helices α3 and α4 is highlighted with a red box. The C3 loop is enclosed in black brackets. Catalytic residues H159 and S289 are marked with blue asterisks. Domains 1, 2, and 3 are indicated with bars in shades of brown.

Figure 2. Side and Top view of WT homo-tetramer. $P2_12_12_1$ asymmetric unit of WT crystal. Monomers A, B, C and D are rendered red, green, blue, and yellow, respectively. Domains 1–3 are indicated for Monomer C. Active sites are indicated with arrows.

Figure 3.

Comparison of ADSL active sites for WT, WT with substrate, and R303C mutant. (a) Wall-eyed stereo view of an ADSL-SAMP active site with AMP and fumarate occupying the active site. AMP is rendered pink and fumarate is rendered tan. Heteroatoms are colored according to their element. Water molecules (grey) are depicted as spheres and are scaled to 50% for clarity. The monomer each residue belongs to is given in parentheses after the residue number. (b) Wall-eyed stereo view of an ADSL-SAMP active site with SAMP occupying the active site. SAMP is rendered purple, and heteroatoms are colored according to their element. Water molecules (grey) are depicted as spheres and are scaled to 50% for clarity. The monomer each residue belongs to is given in parentheses after the residue number. (c) Wall-eyed stereo view of R303C ADSL active site. Monomers A, B and D are rendered red, green, and yellow, respectively. Heteroatoms are colored according to their element. Water molecules (cyan) are depicted as spheres and are scaled to 50%, and the side chain of R85 is hidden for clarity. The monomer each residue belongs to is given in parentheses after the residue number.

Figure 4.

Substrate induced α -helices 2, 3 and 4 shift. (a) Wall eyed stereo view comparing the mobile loop $\alpha 2$ - $\alpha 4$ between ADSL-SAMP (grey) and R303C-ADSL. (b) Wall-eyed stereo view of WT active site colored salmon, light yellow and pale green, with waters colored aqua. Heteroatoms are colored according to their element. Water molecules are depicted as spheres and are scaled to 50%, and the side chain of R85 is hidden for clarity. The monomer each residue belongs to is given in parentheses after the residue number. (c) Wall eyed stereo view comparing the mobile loop $\alpha 2$ - $\alpha 4$ between ADSL-SAMP and WT-ADSL-apo. Coloring is as in (a) and (b). (d) Wall eyed stereo view comparing the mobile loop $\alpha 2$ - $\alpha 4$ between R303C-ADSL and WT-ADSL-apo. Coloring is as in (a) and (c).

Figure 5.

ADSL-AICAR-fumarate model. (a) Wall-eyed stereo view of model of WT active site with AICAR and fumarate occupying the active site. AICAR and fumarate are rendered yellow. Heteroatoms are colored according to their element. The monomer to which each residue belongs is given in parentheses after the residue number. (b) View of model of WT active site with AMP and fumarate overlaying model of WT active site with AICAR and fumarate. WT with AMP is rendered grey with AMP in pink and fumarate in tan, while WT with AICAR residues are teal with AICAR and fumarate in yellow.

Figure 6.

Constriction of the ADSL active site upon substrate binding. (a) Surface rendering of WT-ADSL-apo active site 1. WT active site depicted in salmon, light yellow and pale green corresponding to monomer A, B, and D respectively. R85 side chain is shown with transparency to reflect the lack of electron density for the side chain in all monomers. AMP and fumarate from active site 1 of ADSL-SAMP was placed in the apo active site for scaling purposes. (b) Surface rendering of SAMP and fumarate bound active site 1 of ADSL-SAMP. Coloring of light grey, medium grey, and dark grey correspond to monomers A, B, and D respectively. (c) Surface rendering of SAMP and fumarate bound active site of ADSL-SAMP. Coloring of light grey, medium grey, and dark grey correspond to monomers A, D, and C respectively. All waters were removed from surface renderings in (a-c).

Figure 7.
Proposed mechanism for SAICAR to AICAR plus fumarate and for SAMP to AMP plus fumarate.

Table 1**Data Collection and Refinement Statistics ADSL**

	WT APO	R303C ADSL
<i>Data Collection</i>		
Space Group	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁
Unit Cell Dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	85.9, 105.2, 215.0	85.8, 105.6, 217.2
α=β=γ (degrees)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	50.0-2.70	50.0-2.60
No. Reflections Observed	241,786	263,513
No. Unique Reflections	48,725	58,820
Rmerge (%)	10.3 (63.1) *	12.4(35.0) *
I/σI	14.2(4.3) *	18.9(4.8) *
% Completeness	89.6 (90.2) *	95.4 (97.1)
<i>Refinement</i>		
Resolution Range	50.0-2.70	50.0-2.60
No. Reflections in Working Set	44,972	55,609
No. Reflections in Test Set	2,450	2,979
Rwork (%)	24.3	23.0
Rfree (%)	29.2	29.8
Mean B-factor (Å ²)	27.5	55.1
Protein B-factor (Å ²)	27.6	54.9
Water B-factor (Å ²)	19.2	50.1
RMS deviation:		
Bond Lengths (Å)	0.01	0.01
Bond Angles (degrees)	0.90	1.01
Protein/Water Atoms	14,605/214	14,764/144
Monomers in Asymmetric Unit	4	4

* The last resolution shell is shown in parentheses.

^a $R_{\text{merge}} = \sum h \sum i |I_i(h) - \langle I(h) \rangle| / \sum h \sum i I_i(h)$, where $I_i(h)$ is the *i*th measurement and $\langle I(h) \rangle$ is the weighted mean of all measurements of $I(h)$.

^b R_{work} and $R_{\text{free}} = \sum (|F(h)o| - |F(h)c|) / \sum |F(h)o|$ for reflections in the working and test sets.

Table 2

Enzymatic Activities of ADSL with SAMP and SAICAR at 25 °C

	SAMP		SAICAR	
	WT	R303C	WT	R303C
$k_{cat,H}$ (1/sec)	52.2 ± 1.8	2.33 ± 0.07	90.2 ± 1.9	27.8 ± 0.8
$k_{cat,H}$ (%)	100 ± 3	4.5 ± 0.1	100 ± 2	31 ± 1
$K_{0.5}$ (μ M)	2.1 ± 0.2	2.3 ± 0.12	1.8 ± 0.1	8.4 ± 0.8
Hill Coeff	1.5 ± 0.2	1.03 ± 0.08	1.22 ± 0.09	0.94 ± 0.06
$k_{cat,H}/K_{0.5}$ ($\text{sec}^{-1}\mu\text{M}^{-1}$)	25.0	0.99	49.6	3.37
$k_{cat,M}$ (1/sec)	N/A	2.36 ± 0.04	N/A	27.2 ± 0.4
$k_{cat,M}$ (%)	N/A	4.5 ± 0.1	N/A	30 ± 1
K_M (μ M)	N/A	2.4 ± 0.2	N/A	7.8 ± 0.4
$k_{cat,M}/K_M$ ($\text{sec}^{-1}\mu\text{M}^{-1}$)	N/A	0.99	N/A	3.45

The k_{cat} , $K_{0.5}$, K_M , and Hill coefficient were determined by varying substrate concentration and fitting data to the Hill Equation (H) or the Michaelis-Menten Equation (M) in Sigma Plot. Protein was reconstituted for 2 hours at 25 °C prior to measurements. R303C activity is relative to % WT. The values are shown along with their standard errors. N/A stands for not applicable.

Table 3

ITC Thermodynamic Parameters of ADSL

	AMP		AICAR	
	WT	R303C	WT	R303C
K _d (μM)	54 ± 3	130 ± 8	34 ± 2	121.9 ± 0.9
ΔH (kJ*mol ⁻¹)	-35 ± 2	-5.9 ± 0.2	-53 ± 1	-16.7 ± 0.5
TΔS (kJ*mol ⁻¹)	-12 ± 1	15.92 ± 0.06	-28 ± 1	5.3 ± 0.5
ΔG (kJ*mol ⁻¹)	-23.9 ± 0.1	-21.8 ± 0.2	-25.1 ± 0.1	-21.95 ± 0.02
n	0.99 ± 0.01	1.01 ± 0.04	1.00 ± 0.02	1.00 ± 0.02

Data sets were collected in duplicate and analyzed with NanoAnalyze software and fit to an independent model concurrently with a blank constant model to adjust for heat of dilution. All measurements were from 25 injections of 2.5 mM AMP into 170 μL protein in 25 mM HEPES, pH 7.0, 150 mM KCl, 2 mM DTT at 20°C.