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Oligomeric status of human cystathionine beta-synthase modulates AdoMet binding

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Cystathionine beta-synthase (CBS) plays a key role in the metabolism of sulfur-containing amino acids. CBS is a multidomain tetrameric enzyme allosterically activated by S-adenosylmethionine (AdoMet). Recent crystallographic analyses of engineered CBS lacking the loop made up of residues 516–525 revealed discrepancies in AdoMet binding compared to previous biophysical studies on a full-length CBS. Here, we show that removal of the loop 516–525 functionally eliminates the high affinity sites responsible for kinetic stabilization of the full-length enzyme and yields a dimeric AdoMet-inducible enzyme, in which kinetic stabilization is now exerted by AdoMet binding to the remaining low affinity sites.

Keywords: calorimetry; cystathionine beta-synthase; oligomerization; S-adenosylmethionine

Human cystathionine beta-synthase (CBS; EC 4.2.1.22), which plays a crucial role in conversion of essential amino acid methionine to cysteine, adopts a multidomain architecture containing the N-terminal heme binding region, central catalytic core and the C-terminal S-adenosylmethionine (AdoMet) binding regulatory domain [1]. The catalytic core harbors pyridoxal-5′-phosphate, where the condensation of homocysteine with serine occurs yielding cystathionine and water. Cystathionine is subsequently transformed into cysteine and alpha-ketobutyrate by cystathionine gamma-lyase. Deficiency in CBS activity chiefly due to the presence of missense pathogenic mutations in *cbs* gene is the most common inherited disorder of sulfur amino acid metabolism [2]. The CBS deficiency is characterized by accumulation of homocysteine and decreased levels of cysteine with clinical symptoms, such as skeletal and connective tissue defects, thromboembolism and mental retardation.

Recent advances in CBS crystallography yielded structures of full-length CBS with and without bound AdoMet and allowed for clarification of a complex regulatory mechanism of CBS activation upon AdoMet binding [3–5]. The structures revealed a tandem of the so-called CBS domains per monomer (CBS1 and CBS2) forming a regulatory domain where AdoMet binds. Although there are two potential clefts within the CBS regulatory domain, which could house

Abbreviations
AdoMet, S-adenosylmethionine; CBS, cystathionine beta-synthase; CBSΔ516-525, human CBS lacking residues 516–525; CD, catalytic domain; DLS, dynamic light scattering; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; RD, regulatory domain.

AdoMet, only one site was found occupied by AdoMet in AdoMet-bound CBS structures. It is important to note that crystallization of CBS was facilitated by an engineered construct lacking a flexible loop of residues 516–525 from CBS2 domain (CBSΔ516-525). While such deletion had no effect on activation of the construct by AdoMet, it was accompanied by a change in the enzyme's oligomeric status from a homotetrameric WT to a homodimeric CBSΔ516-525 [3].

Although crystal structures of CBSΔ516-525 extend our understanding about allosteric regulation of CBS by AdoMet, there is some controversy on AdoMet binding capacity of native full-length CBS WT. Previous microcalorimetric studies showed that CBS WT contains two distinct AdoMet binding sites with different stoichiometry and properties [6]. High affinity sites (K_d ~ 10 nM) bind two moles of AdoMet per tetramer and enhance the kinetic stability of the regulatory domain (i.e. binding slows down their irreversible denaturation), while low affinity sites (K_d ~ 400 nM) accommodate up to a further four AdoMet molecules per tetramer and are responsible for activation of the enzyme. While thermodynamic stability is defined by the equilibrium between the native functional protein and the low amount of unfolded and partially unfolded states, the kinetic stability relates to the time scale of irreversible denaturation, i.e. to the height of the free energy barrier separating the native state and all the non-functional states (e.g. irreversibly denaturated, aggregated, proteolyzed species) [7]. In some instances, kinetic and thermodynamic stabilities can be directly related when the irreversible step is rate-limiting [8]. It has been proposed that kinetic stabilization of the native state is therefore often needed for a protein to remain functional during a biologically-relevant time scale in addition to its thermodynamic stability [7]. However, it seems as if CBSΔ516-525 binds only one AdoMet molecule per monomer, thus four moles of AdoMet per tetramer rather than six moles of AdoMet per CBS WT tetramer. Since the addition of AdoMet to CBSΔ516-525 resulted in approximately fivefold increase of catalytic activity, the binding sites characterized crystallographically are likely those associated with the enzyme activation. In addition, both CBS WT and CBSΔ516-525 contain some AdoMet bound when recombinantly expressed and purified from *Escherichia coli* [5,9]. One reasonable explanation for this binding discrepancy is that CBS WT indeed contains up to two binding sites/monomer, one of them partially occupied when purified from *E. coli* (due to their higher affinity), while removal of the 516–525 loop abolishes these high affinity sites. We must note that based on our previous model for AdoMet binding to full-length CBS [6], the low affinity sites may also contribute to kinetic stability of the regulatory domain, but their effect must be much weaker (close to negligible) than that of the high affinity sites. Consequently, if the high affinity sites are removed in CBSΔ516-525, the low affinity sites might become the only source of kinetic stabilization in the presence of AdoMet, even though one might expect a lower stabilizing effect.

In an attempt to address this discrepancy in AdoMet binding capacity of CBS, we have biochemically characterized CBS WT along with several CBSΔ516-525 variants and described their AdoMet binding by isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC) and dynamic light scattering (DLS).

Materials and methods

Enzymes

The preparation of CBS WT, CBSΔ516-525 and its variants with permanent C-terminal 6xHis tag followed the procedure described previously [9,10] with a few modifications [4,11]. The CBSΔ516-525 variants were prepared by using a QuikChangeII XL mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's protocol with expression plasmid for CBSΔ516-525 as template and mutational oligonucleotides synthesized by Integrated DNA Technologies (Coralville, IA, USA). All constructs were verified by DNA sequencing before expression and purification.

Biochemical characterization of the purified constructs

Protein concentrations were determined by the Bradford method (Thermo Scientific, Waltham, MA, USA) using BSA as a standard according to the manufacturer's recommendations. For calorimetric studies, protein concentration was estimated spectrophotometrically by UV absorption using molar extinction coefficient of 103 800 $M^{-1} \cdot cm^{-1}$ determined previously for CBS WT [6]. Denatured proteins were separated by SDS/PAGE using 10% polyacrylamide precast gels (Mini-PROTEAN TGX; Bio-Rad Laboratories, Hercules, CA, USA). The native samples were separated in 4–15% polyacrylamide gradient precast gels (Mini-PROTEAN TGX; Bio-Rad Laboratories). For visualization, the denatured gels were stained with Simple Blue (Invitrogen, Carlsbad, CA, USA). Western blot analysis of crude cell lysates under denaturing or native conditions was performed as described previously [12]. The CBS catalytic activity was determined by a previously described radioisotope assay using [^{14}C(U)] L-serine as the labeled substrate, essentially as described elsewhere [10,12].

Isothermal titration calorimetry

Isothermal titration calorimetry experiments were performed in a ITC_{200} microcalorimeter (Malvern Instruments, Malvern, UK). CBS proteins were placed in the sample cell at 24 μM monomer and 400–500 μM AdoMet in the titrating syringe. Routinely, 1 × 0.5 μL followed with 42 × 0.9 μL injections (spaced 150 s) were performed. Experimental temperature was set at 25 °C. All samples were prepared in 20 mM HEPES pH 7.4. Thermodynamic binding parameters were obtained using models with one or two independent type of sites models available with the software provided by the manufacturer as described in [6,9].

Analyses of ITC titrations based on a binding polynomial formalism for the particular case of a macromolecule with two identical and independent binding sites can be found elsewhere [13]. Briefly, stepwise binding of AdoMet (L) to dimeric CBSΔ516-525 (M) can be described by these two equilibria:

$$M + L \leftrightarrow \mathrm{ML}$$

$$\mathrm{ML} + L \leftrightarrow \mathrm{ML_2}$$

which are described by the following macroscopic equilibrium constants:

$$K_1 = \frac{[\mathrm{ML}]}{[M][L]}$$

$$K_2 = \frac{[\mathrm{ML_2}]}{[\mathrm{ML}][L]}$$

The molar fraction of the different ligation states can also be designated as α_0 (M), α_1 (ML) and α_2 (ML_2). In the particular case of two identical and independent sites, the microscopic constant k (provided by the ITC fittings) is related to K_1 and K_2 as follows: $K_1 = 2k$ and $K_2 = k/2$. The dependence of the population of different ligation species on L concentration (i.e. [AdoMet]) can be calculated from the binding polynomial P ($P = 1 + 2k[\mathrm{AdoMet}] + k^2[\mathrm{AdoMet}]^2$) using the following expressions:

$$\alpha_0 = \frac{1}{P}$$

$$\alpha_1 = \frac{2k[\mathrm{AdoMet}]}{P}$$

$$\alpha_2 = \frac{k^2[\mathrm{AdoMet}]^2}{P}$$

Differential scanning calorimetry

Differential scanning calorimetry experiments were performed in a VP-DSC scanning microcalorimeter (Malvern Instruments). Protein samples were prepared at 4–5 μM monomer in 20 mM HEPES pH 7.4 with 0–50 μM AdoMet. Scans were performed using a rate 1.5 °C·min^{-1} in a temperature range 10–90 °C. Data analyses were performed using two-state irreversible kinetic models with first-order kinetics as described elsewhere [6].

Dynamic light scattering

Dynamic light scattering was carried out in a DynaPro MSX instrument (Wyatt Technology Corporation, Santa Barbara, CA, USA) using 1.5 mm path length cuvettes and 12 μM protein monomer at 25 °C in the absence or presence of 500 μM AdoMet in 20 mM HEPES pH 7.4. Data were analyzed as described previously [13].

Results

Initial characterization of the purified CBS constructs

We successfully purified truncated human CBS (45 kDa CBS), CBSΔ516-525, CBS WT and five CBSΔ516-525 variants carrying single site mutations (pathogenic R125Q, D198V, D444N and S466L and artificial E201S) to homogeneity (>98%) carrying the permanent 6xHis tag at the C-terminus and compared them side by side using SDS/PAGE and native-PAGE followed by Western blot and CBS activity assay (Fig. 1). All constructs yielded a single band on SDS/PAGE followed by Western blot using anti-CBS antibody indicating homogeneous preparations and no degradation of the purified enzymes (Fig. 1A). While the lack of the entire regulatory domain in 45 kDa CBS resulted in substantially faster migration in the gel, we were not able to differentiate CBS WT from CBSΔ516-525 variants lacking 10 amino acid residues from the CBS2 domain of the regulatory region. However, missing residues in CBSΔ516-525 variants resulted in a formation of homodimers on the contrary to the homotetrameric CBS WT when subjected to a native-PAGE followed by Western blot probed with anti-CBS antibody (Fig. 1B). The loss of the entire regulatory domain in 45 kDa CBS also resulted in a dimeric CBS enzyme, which in turn became permanently activated (Fig. 1C). Basal activity and AdoMet response of CBS WT and CBSΔ516-525 were essentially identical; however, the presence of a mutation in CBSΔ516-525 variants resulted in differential behavior of the constructs. While pathogenic mutations R125Q and D198V in the catalytic core seems to have no impact on CBS specific activity and AdoMet response, the artificial mutation E201S in the same region yielded permanently activated, the AdoMet

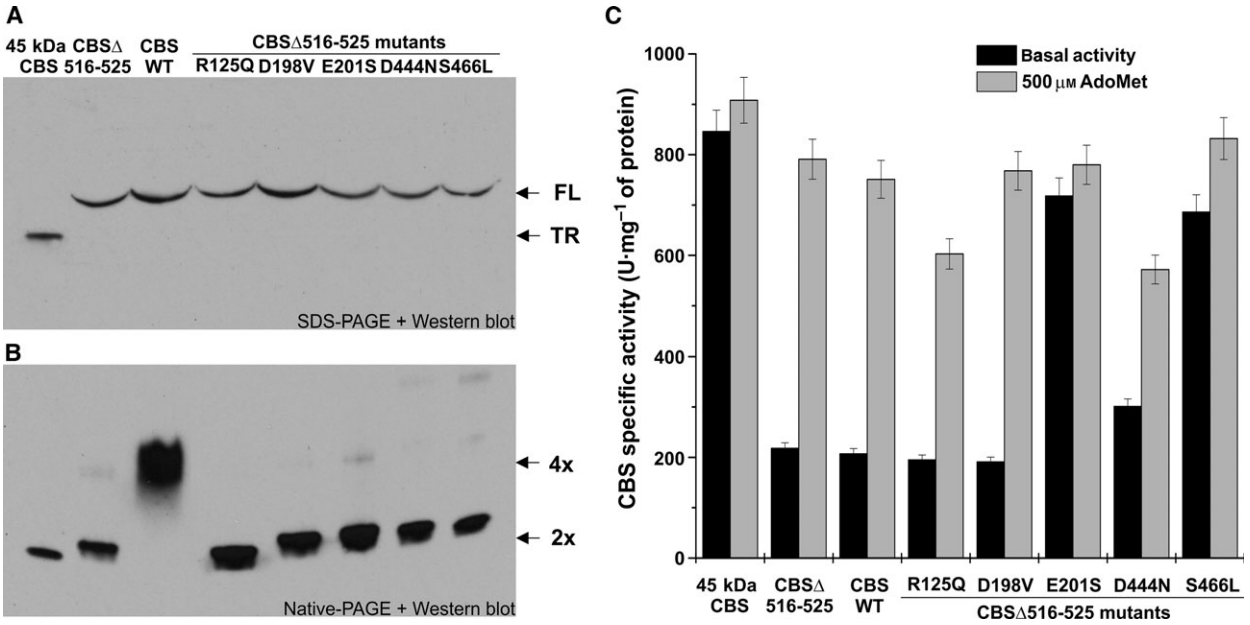

Fig. 1. Initial characterization of the purified CBS constructs. SDS/PAGE (A) and native-PAGE (B) followed by Western blot analysis. The 100 ng of each construct were resolved in 10% (A) or gradient 4–15% (B) MiniProtean TGX gel, blotted on PVDF membrane and probed with anti-CBS antibody. Truncated (TR) or full length (FL) constructs yielded homodimers (2×) or tetrameric species (4×) on a native PAGE. (C) The purified enzymes were tested for their specific activity in the canonical condensation of serine and homocysteine in the absence (black bars) or presence of 500 μM CBS allosteric activator AdoMet (grey bars). Error bars indicate standard errors of the means of triplicates.

unresponsive enzyme. Pathogenic mutations D444N and S466L in the C-terminal regulatory region resulted in increased basal activity compared to both CBS WT and CBSΔ516-525, which in case of the CBSΔ516-525 S466L construct was comparable to activities obtained for AdoMet-stimulated CBS enzymes.

Binding of AdoMet

We performed titrations with AdoMet at conditions designed to distinguish between different binding capacities of CBS WT and CBSΔ516-525 variants. Figure 2 shows representative raw titrations and binding isotherms, while Table 1 summarizes thermodynamic parameters for AdoMet binding as determined by ITC. As previously reported [6,9,14], AdoMet binding to CBS WT is consistent with a binding capacity of about 1.5 moles of AdoMet/monomer into two types of independent binding sites. Attempts to analyze these binding isotherms using a sequential binding model with a total binding capacity of four binding sites per tetramer did not provide convergent fittings (data not shown), supporting the notion that these isotherms cannot be satisfactorily explained by four dependent (i.e. cooperative) binding sites per tetramer.

In contrast to CBS WT, the binding of AdoMet to CBSΔ516-525 variants is consistent with the existence of a single type of binding sites with a much lower binding capacity for AdoMet: on average 0.5 mole of AdoMet/monomer (Fig. 2, Table 1). With exception of CBSΔ516-525 D444N mutant, the K_d of these sites is about 1 μM, a value close to that of the low affinity sites in CBS WT. The D444N mutant has a binding affinity about fivefold lower than the remaining CBSΔ516-525 variants, which correlates with the finding that a significantly higher concentration of AdoMet is needed for a full activation of CBS D444N compared to the WT enzyme [15,16]. It should be noted that for all CBSΔ516-525 variants studied here, the ITC titrations provided robust estimations of all binding parameters. Accordingly, the values of the dimensionless c parameter (c = [protein monomer]/K_d) ranged from 4 (CBSΔ516-525 D444N) to 30 (CBSΔ516-525 R125Q), and thus they are in the range of optimal values for an accurate and simultaneous determination of the binding parameters [17]. Therefore, the higher binding capacity of the CBSΔ516-525 D444N cannot be explained by an inaccurate estimation of the total binding capacity, even though the origin of such capacity is currently unknown. The higher binding capacity of the D444N mutant may plausibly imply that the sites are essentially AdoMet free as purified due to their lower affinity for the cofactor. Indeed, the crystal structure of AdoMet-bound

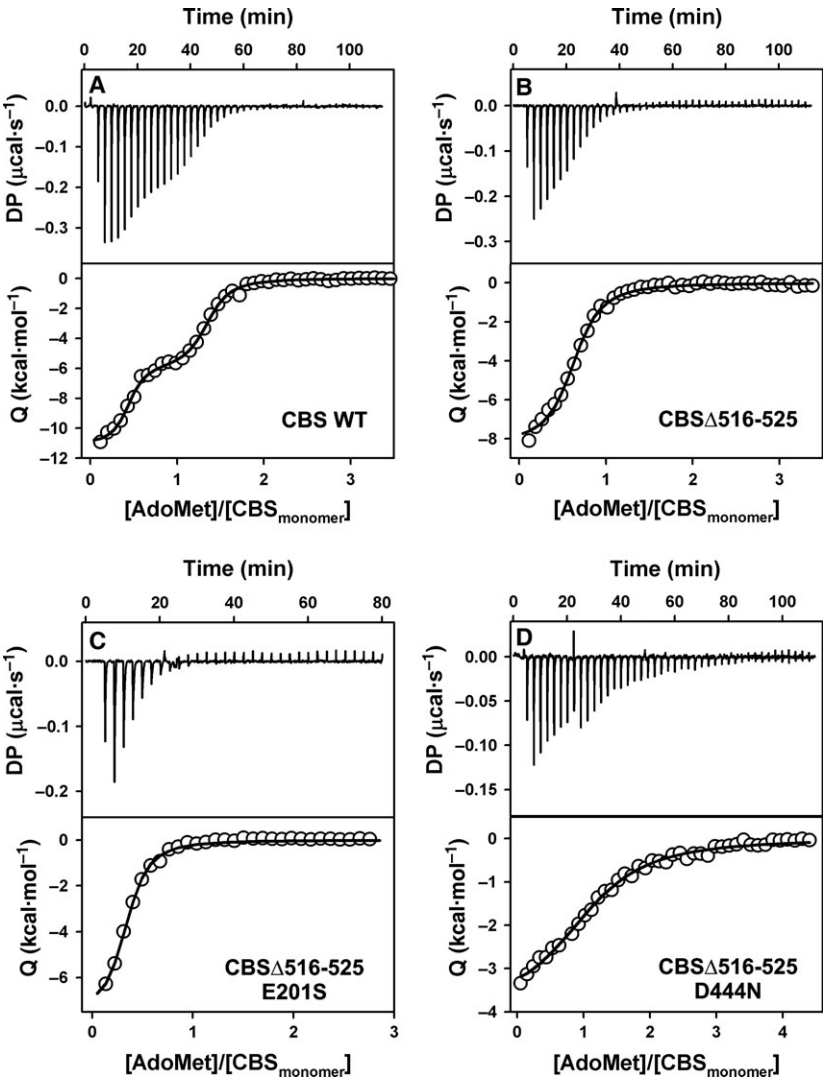

Fig. 2. Binding of AdoMet to various CBS constructs as examined by ITC. Representative titrations of CBS WT and CBSΔ516-525 variants with AdoMet.

Table 1. Thermodynamic parameters for AdoMet binding as determined by ITC. Values for AdoMet binding sites (*N*) are expressed per CBS monomer. The N/A stands for not applicable.

	Site 1				Site 2			
	N	K_d (μM)	ΔH (kcal·mol^{-1})	ΔS (cal·mol^{-1}·K^{-1})	*N*	K_d (μM)	ΔH (kcal·mol^{-1})	ΔS (cal·mol^{-1}·K^{-1})
CBS WT	0.41 ± 0.01	0.012 ± 0.003	−11.1 ± 0.2	−1.1	0.92 ± 0.01	0.45 ± 0.05	−6.0 ± 0.2	8.9
CBSΔ516-525	0.61 ± 0.01	1.14 ± 0.09	−8.3 ± 0.1	−0.7	N/A	N/A	N/A	N/A
CBSΔ516-525 R125Q	0.62 ± 0.01	0.83 ± 0.07	−8.8 ± 0.1	−1.6	N/A	N/A	N/A	N/A
CBSΔ516-525 D198V	0.40 ± 0.01	1.33 ± 0.18	−9.9 ± 0.4	−6.4	N/A	N/A	N/A	N/A
CBSΔ516-525 E201S	0.33 ± 0.01	1.06 ± 0.10	−7.9 ± 0.2	1.0	N/A	N/A	N/A	N/A
CBSΔ516-525 D444N	1.13 ± 0.03	6.25 ± 0.40	−4.0 ± 0.1	10.5	N/A	N/A	N/A	N/A
CBSΔ516-525 S466L	0.20 ± 0.01	1.64 ± 0.19	−12.9 ± 0.8	−17.0	N/A	N/A	N/A	N/A

CBSΔ516-525 E201S mutant explains well this lower affinity, since residue D444 is required for the stabilization and the positioning of AdoMet within the binding cavity of the C-terminal regulatory domain [4]. Alternatively, the mutation introduced additional binding sites in this variant, a hypothesis that would be

confirmed upon determination of the high-resolution structure of this mutant in the presence of AdoMet.

Thermal stability in the absence and presence of AdoMet

We have analyzed the thermal stability of CBS WT and CBSΔ516-525 variants by DSC. Figure 3 shows representative thermal denaturation profiles, while Table 2 summarizes key denaturation parameters for the regulatory (RD) and the catalytic domains (CD) obtained for CBS WT and CBSΔ516-525 variants using a two-state irreversible denaturation model [6]. As previously reported [6,9,14], thermal denaturation profile of CBS WT showed two main transitions with T_m values of 51.2 °C and 67.2 °C corresponding to

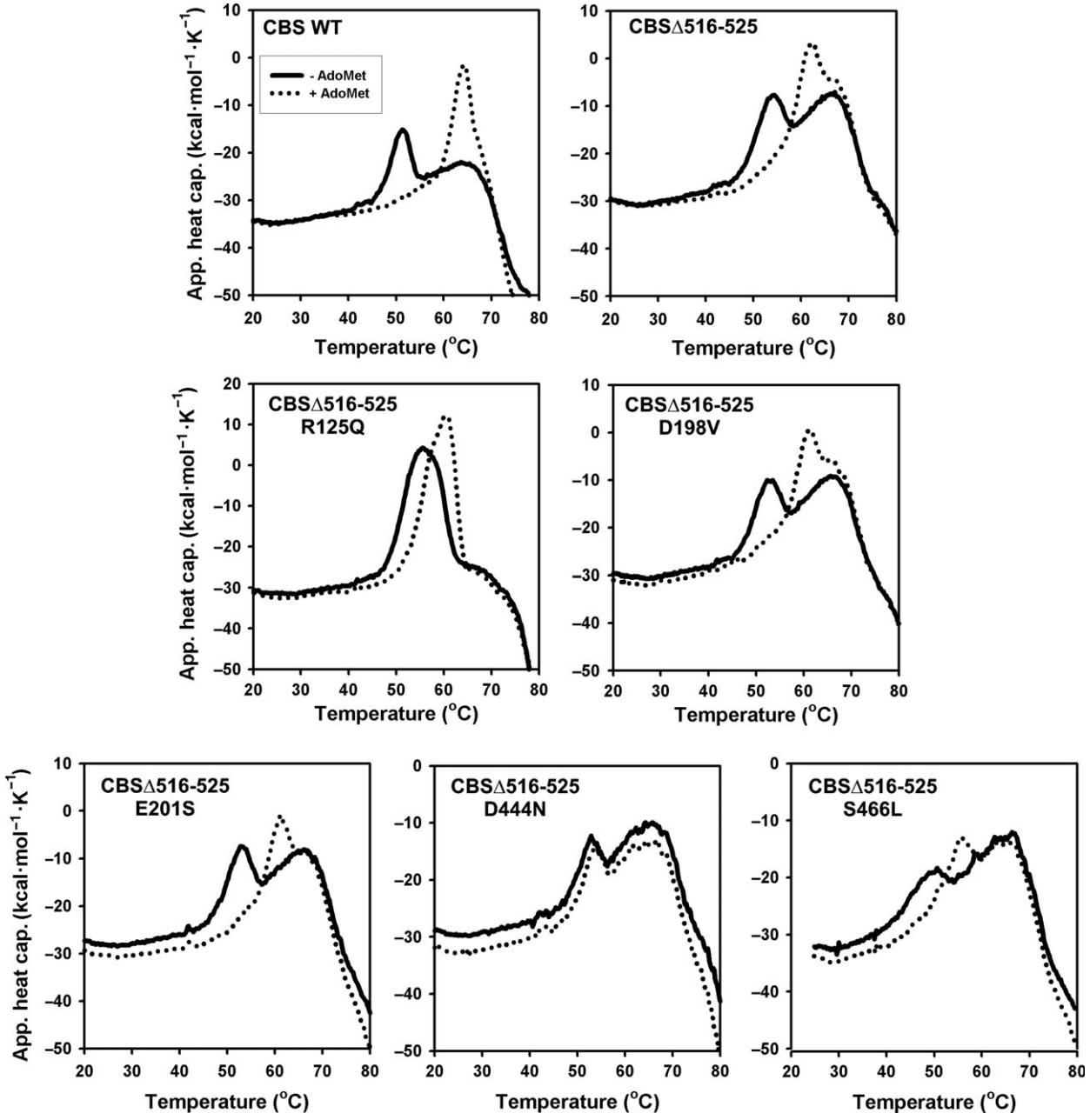

Fig. 3. Thermal denaturation profiles of CBS WT and CBSΔ516-525 variants analyzed by DSC. Solid and dotted lines show experimental traces in the absence and presence of 50 μM AdoMet, respectively. The scan rate was 1.5 °C·min^{-1} and the protein concentration was 5 μM in protein subunit.

Table 2. Thermal denaturation parameters for the regulatory and catalytic domains as determined by DSC using a two-state irreversible denaturation model. Temperature in parenthesis for the regulatory domain represents the T_m in the presence of 50 μM AdoMet. The N/A stands for not applicable.

	Regulatory domains		Catalytic domains	
	T_m (°C)	E_a (kcal·mol^{-1})	T_m (°C)	E_a (kcal·mol^{-1})
CBS WT	51.2 ± 0.1 (~ 64.2)	58.5 ± 0.7	67.2 ± 0.1	45.0 ± 0.5
CBSΔ516-525	53.5 ± 0.1 (~ 62.2)	79.9 ± 0.8	69.2 ± 0.1	86.4 ± 3.3
CBSΔ516-525 R125Q	N/A			
CBSΔ516-525 D198V	52.4 ± 0.1 (~ 61.1)	81.9 ± 0.8	68.3 ± 0.1	72.3 ± 1.7
CBSΔ516-525 E201S	52.6 ± 0.1 (~ 61.3)	84.7 ± 1.1	69.5 ± 0.7	75.2 ± 8.1
CBSΔ516-525 D444N	52.6 ± 0.1 (~ 53.1)	116.2 ± 4.2	67.0 ± 0.2	54.0 ± 3.7
CBSΔ516-525 S466L	48.0 ± 0.1 (~ 55.5)	49.2 ± 1.3	68.7 ± 0.2	62.2 ± 2.2

denaturation of RD and CD, respectively. The CBSΔ516-525 variants presented similarly well resolved two thermal transitions to CBS WT, which T_m for both RD and CD denaturation were generally up-shifted by 2 °C. The CBSΔ516-525 R125Q mutant yielded a single asymmetric peak with a thermal transition at about 55 °C suggesting a strong destabilization of the CD due to the presence of the missense mutation. Additionally, a significant destabilization of the RD in the CBSΔ516-525 S466L mutant was observed.

Addition of 50 μM AdoMet increased thermal stability of the RDs of CBSΔ516-525 variants generally by 8–9 °C, which was lower than for CBS WT (~ 13 °C). The results suggest a lower AdoMet-mediated kinetic stabilization in CBSΔ516-525 variants. In the case of CBSΔ516-525 D444N mutant, the stabilization was essentially non-existent at the tested concentration of AdoMet most likely due to enzyme's lower binding affinity as found by ITC.

We have previously proposed that denaturation of the CBS WT RD in the presence of high AdoMet concentrations follows this simple mechanism [6]:

$$R_4L_{[2,4]} \leftrightarrow R_4L_{[0,4]} + 2L \rightarrow F_4$$

in which the fully AdoMet-saturated CBS tetramer $R_4L_{[2,4]}$ exists in an equilibrium with a less kinetically stable state $R_4L_{[0,4]}$. This kinetically unstable state undergoes irreversible denaturation to a final state F_4, which is formed upon "release" of two AdoMet molecules ($2L$) from the high-affinity sites. We want to note that this mechanism was proposed considering the number of AdoMet molecules and the affinity of the sites involved in kinetic stabilization as derived from the T_m and denaturation rate dependences on AdoMet concentrations upon analyses using a phenomenological model as well as a more elaborated kinetic mechanism (see [6] for a detailed description). To build up this mechanism, we also considered that in the ligand binding equilibrium there are multiple ligation species (most of them at very low populations at the AdoMet concentrations used), but importantly, even though the population of $R_4L_{[0,4]}$ is low at high AdoMet concentrations, it is critical to determine kinetic stability since it is the most populated state among those kinetically sensitive to denaturation (i.e. those states with lower occupancy of the low affinity sites, $R_4L_{[0,3]}$ to $R_4L_{[0,0]}$; please refer to our previous work [6], particularly to Figure 7, for more detailed explanation).

Taking into account the AdoMet binding capacity of CBSΔ516-525 variants as well as the significant kinetic stabilization of their RDs by this ligand, the number of AdoMet molecules released prior to the transition state of the rate-limiting step (ν) as determined from the AdoMet concentration dependency of the T_m values on AdoMet concentration can be calculated from the following equation (Fig. 4) [6]:

$$\ln[\text{AdoMet}] = \text{constant} - \left(\frac{E_a}{\nu R T_m}\right)$$

where E_a is the activation energy, T_m is the melting temperature (i.e. the temperature of maximum of the transition) and R is the ideal gas constant. The value of ν determined from the slope of a ln[AdoMet] versus $1/T_m$ plot using this equation is 1.6 ± 0.1, consistent with a scenario in which the AdoMet free dimer is likely the kinetically sensitive state. Using experimental binding affinities at 25 °C and a binding polynomial formalism (see Methods), the fraction of different ligation states can be readily calculated (Fig. 5). At the AdoMet concentrations used in DSC experiments, the mole fraction of α_0 (no AdoMet bound per CBSΔ516-525 dimer) is about 0.01–0.001 at 25 °C. This result agrees well with the ~ 25-fold increase in kinetic stability derived from DSC analyses at 50 μM AdoMet at higher temperatures (around the T_m, 53–62 °C), considering that the fraction of α_0 would be higher at this

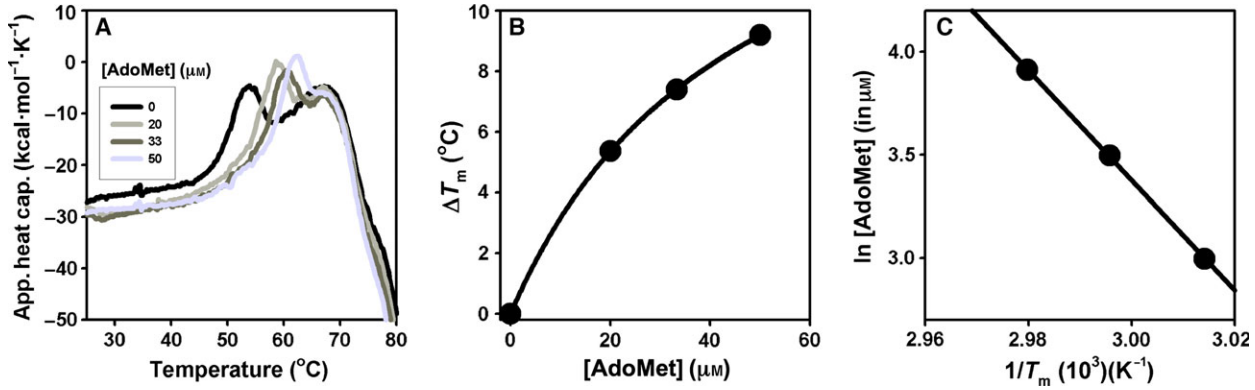

Fig. 4. Stabilization of CBSΔ516-525 regulatory domain upon AdoMet binding. (A) DSC traces for CBSΔ516-525 at the indicated AdoMet concentration. (B) Dependence of ΔT_m on AdoMet concentration for the denaturation of the CBSΔ516-525 regulatory domain. (C) Dependence of $1/T_m$ on the natural logarithm of AdoMet concentration. The slope equals to $(-E_a/\nu \cdot R)$ and yields the moles of AdoMet (ν) released prior to the transition state of the denaturation rate-limiting step ($\nu = 1.6 \pm 0.1$).

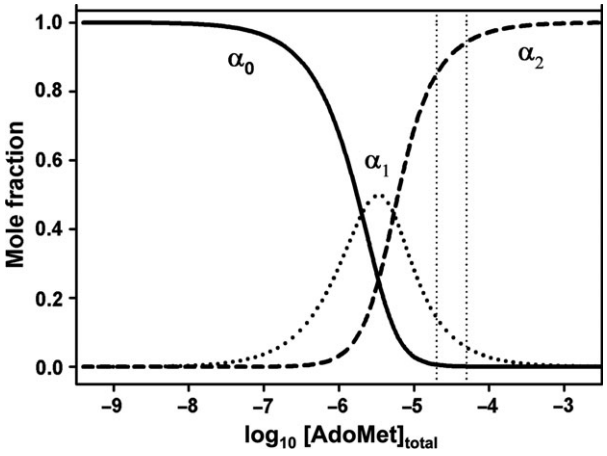

Fig. 5. Dependence of ligation species for CBSΔ516-525 on AdoMet concentration using a binding polynomial formalism. Thick solid, dashed and dotted lines indicate the mole fraction of designated ligation species at certain AdoMet concentration, while vertical thin dotted lines represent the AdoMet concentrations used in DSC experiments. Total CBSΔ516-525 concentration was 2 μM.

ligand concentration as temperature is increased (due to the negative binding enthalpy; see Table 1). Therefore, our data support that α_0 is the kinetically sensitive state overall.

AdoMet binding does not affect the oligomeric status of CBS

Different AdoMet binding capacity of CBS WT and CBSΔ516-525 allows for an hypothesis that AdoMet binding may affect the oligomeric equilibrium of human CBS. As native electrophoresis could not be used due to the charged nature of AdoMet and size exclusion HPLC chromatography did not yield conclusive results (data not shown), such effect could be evaluated in solution using DLS with results shown in Fig. 6. Samples analyzed in the absence and presence of 50 μM AdoMet confirmed the presence of a main component (at equilibrium) representing over 95% of the sample (Fig. 6A), which correlates well with the data from native electrophoresis (Fig. 1B). Assuming spherical shape of studied CBS constructs, DLS was able to detect a different oligomeric status of CBS WT (radius of ~ 5 nm, estimated molecular weight about 200 kDa) and CBSΔ516-525 variants (radius of ~ 4.2 nm, estimated molecular weight about 100 kDa) again endorsing the data from native electrophoresis (Fig. 6B). Lastly, polydispersities of studied CBS variants were mostly in 10–20% range suggesting that one oligomeric species predominates: tetramer in CBS WT and dimer in CBSΔ516-525 variants (Fig. 6C). More importantly, the presence of AdoMet had negligible effect on hydrodynamic behavior of studied CBS constructs thus discouraging the hypothesis that AdoMet mediates the shift of the CBS oligomeric equilibrium.

Discussion

Data shown in this study represents a missing piece of supporting evidence, which will address the discrepancy between the biochemical data on AdoMet binding by a full-length human CBS WT [6] and the crystallographic data on activated AdoMet-bound engineered human CBSΔ516-525 construct [4,5]. We showed much lower AdoMet binding capacity of the CBSΔ516-525 variants, about 0.5–1 mole of AdoMet/monomer, compared to the native full-length enzyme

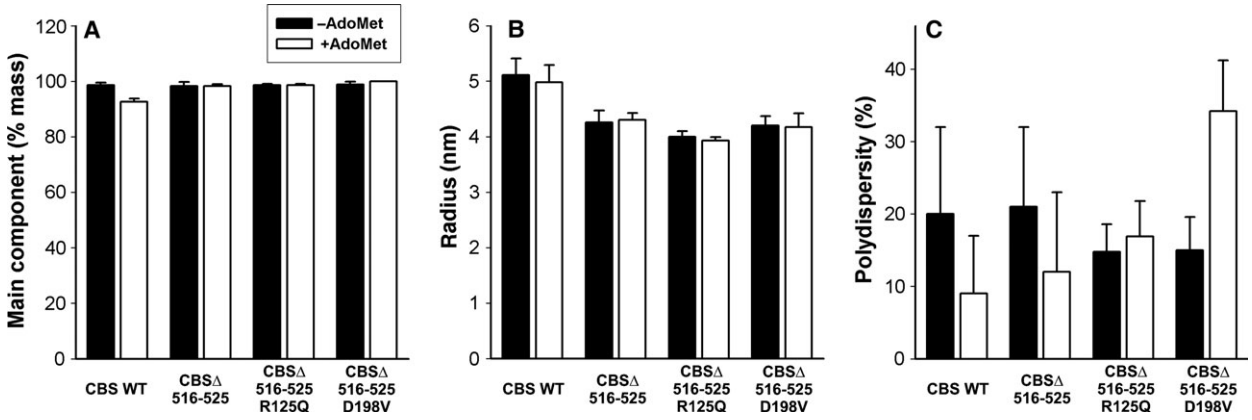

Fig. 6. Dynamic light scattering of CBS WT and CBSΔ516-525 variants. Data represent averages ± SEMs from three to six independent measurements.

of about 1.5 mole of AdoMet/monomer, which is in agreement with the occupancy of binding sites in crystal structures of the dimeric CBSΔ516-525 constructs. More importantly, ITC titrations indicate that all the sites are of a single type in contrast to CBS WT, where two types of AdoMet binding sites were clearly identified [6]. Binding sites found in CBSΔ516-525 variants are similar to the low affinity sites found in CBS WT suggesting that these variants lack the high affinity sites of CBS WT. Therefore, the AdoMet-bound binding sites characterized crystallographically (identified as site S2) are likely those leading to enzyme activation. Sites S1 present in the regulatory domain were found sterically occluded by the structural elements from the catalytic core of a complementary subunit in the crystallized CBSΔ516-525 dimer [3]. Despite being solvent accessible in the activated conformation, sites S1 remained empty due to the presence of large hydrophobic residues within the cleft that impede a proper accommodation of the adenine ring of AdoMet [4]. Additionally, residue N463, located at the first turn of helix α22 (a position usually occupied by a conserved aspartate in CBS domains [18]), weakens the potential interaction with the hydrosyl groups of a second molecule of AdoMet. These data suggest that site S1 most likely does not function as AdoMet binding site under basal or activated conditions. Since CBS WT contains two types of sites and has higher binding capacity compared to CBSΔ516-525, it has been proposed that the additional AdoMet binding sites are present or formed when the enzyme is assembled into higher oligomeric species (i.e. the tetrameric CBS WT) [5]. Our DLS data show that the main species existing in solution is a tetramer (CBS WT) and a dimer (CBSΔ516-525), while AdoMet addition has no large effect on the conformational equilibrium. Thus our data suggest that no binding sites in CBS WT arise from the assembly into higher oligomeric species upon AdoMet binding and that AdoMet does not modulate oligomerization of CBS.

On the other hand, the available data argue that there might be an additional non-canonical (i.e. outside of CBS domain cleft) binding site formed in the tetrameric CBS WT, which is not present in the dimeric CBSΔ516-525. Binding of AdoMet analogs, such as S-adenosylhomocysteine or S-adenosylornithine, yielded stoichiometry of two ligands per tetrameric CBS WT resulting in stabilization of the enzyme's regulatory domain without its activation [19]. Consistently, DSC experiments reveal that CBSΔ516-525 variants are kinetically stabilized (RD) by AdoMet, but this stabilization is weaker than for CBS WT. Our results also support that in the case of CBSΔ516-525, the kinetically sensitive species at high AdoMet concentrations is the CBSΔ516-525 dimer with no AdoMet bound.

Overall, our results allow reconciliation of the crystallographic analyses and previous biophysical models for AdoMet binding to CBS, as well as the stoichiometry and kinetic stabilizing effect of structural analogs of AdoMet [19]. CBS WT contains two different types of binding sites for AdoMet, a high affinity type that is operationally responsible for the kinetic stabilization of the RDs, and a low affinity type that leads to the activation of the enzyme. Consistently, binding of AdoMet analogs with no activating properties to the first type of sites lead to a modest stabilization (due to their lower binding affinity). Regarding the overall binding capacity of CBS WT, it is plausible that the tetramer binds up to two AdoMet molecules per monomer, even though the high affinity sites could be partially occupied as purified, which is a common

feature of metabolically available high affinity and slowly dissociating ligands bound to human proteins expressed in *E. coli* [9,13,20,21]. When the loop 516–525 is removed, the high affinity sites are no longer functional, complying with the crystallographic picture of AdoMet binding (associated to activation) recently described, and to a scenario in which, in the absence of the high affinity sites, the activating sites become the source of kinetic stabilization of RDs upon AdoMet binding.

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Author contributions

ALP and TM performed the research. ALP and TM designed the research study. ALP, JPK and TM contributed essential reagents or tools. ALP, LAMC and TM analyzed the data. ALP and TM wrote the initial draft. ALP, LAMC, JPK and TM revised the manuscript and approved the final version.

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