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Kinetic characterization of recombinant human cystathionine β-synthase purified from *E. coli*

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ABSTRACT

Cystathionine β-synthase (CBS) catalyzes the pyridoxal-5′-phosphate-dependent condensation of L-serine and L-homocysteine to form L-cystathionine in the first step of the transsulfuration pathway. Although effective expression systems for recombinant human CBS (hCBS) have been developed, they require multiple chromatographic steps as well as proteolytic cleavage to remove the fusion partner. Therefore, a series of five expression constructs, each incorporating a 6-His tag, were developed to enable the efficient purification of hCBS *via* immobilized metal ion affinity chromatography. Two of the constructs express hCBS in fusion with a protein partner, while the others bear only the affinity tag. The addition of an amino-terminal, 6-His tag, in the absence of a protein fusion partner and in the absence or presence of a protease-cleavable linker, was found to be sufficient for the purification of soluble hCBS and resulted in enzyme with 86–91% heme saturation and with activity similar to that reported for other hCBS expression constructs. The continuous assay for L-Cth production, employing cystathionine β-lyase and L-lactate dehydrogenase as coupling enzymes, was employed here for the first time to determine the steady-state kinetic parameters of hCBS, *via* global analysis, and revealed previously unreported substrate inhibition by L-Hcys ($K_i^{L\text{-}HCYS} = 2.1 \pm 0.2$ mM). The kinetic parameters for the hCBS-catalyzed hydrolysis of L-Cth to L-Ser and L-Hcys were also determined and the $k_{cat}/K_m^{L\text{-}CTH}$ of this reaction is only ~2-fold lower than the $k_{cat}/K_m^{L\text{-}SER}$ of the physiological, condensation reaction.

Cystathionine β-synthase (CBS)[2] catalyzes the condensation of L-serine (L-Ser) and L-homocysteine (L-Hcys), a toxic metabolic intermediate of L-methionine (L-Met) metabolism, to produce L-cystathionine (L-Cth). Mammalian CBS is the only enzyme known to contain both pyridoxal 5′-phosphate (PLP), the catalytic cofactor, and heme [1], which is bound by the ~70-amino acid, N-terminal domain [2,3]. In contrast, CBS from yeast (*Saccharomyces cervisiae*) lacks the N-terminal domain and does not bind heme [4,5]. The gene encoding CBS is located on the 21st chromosome in humans and is linked to the genetic disorders of homocystinuria and Down syndrome [6–8]. Homocystinuria is an autosomal recessive disease, characterized by elevated plasma L-Hcys levels, with clinical manifestations including thromboembolism and connective tissue defects [7,9].

As the substrate of the transmethylation and transsulfuration pathways, L-Hcys is situated at a metabolic branch point. The flux of L-Hcys through these competing pathways is regulated, *via* allosteric regulation, by the ubiquitous methyl donor *S*-adenosylmethionine (SAM) [10,11]. Human CBS is activated 2–3-fold by SAM, thereby increasing the flux of L-Hcys through the transsulfuration pathway when the cellular methionine pool exceeds the level required to maintain homeostasis [11–13]. The ultimate product of the transsulfuration pathway, L-Cys, is the immediate precursor of glutathione, the major compound responsible for maintaining cellular redox homeostasis. It has been proposed that the heme cofactor of hCBS plays a redox-regulatory role, thereby providing a potential feedback link between glutathione and the transsulfuration pathway [14]. However, recent evidence suggests that a simple regulatory mechanism is unlikely and that heme-mediated regulation would be both temperature and pH-dependent [15–17].

Purification of hCBS from mammalian tissues is complicated by its susceptibility to proteolysis and tendency to aggregate [18,19]. Therefore, recombinant systems were developed for the expression of hCBS in *Escherichia coli*. For example, Bukovska et al. [20] developed a system, in which hCBS is expressed as a fusion protein with β-galactosidase (β-gal). Alternative *E. coli* expression systems, in which hCBS is expressed in conjunction with glutathione-*S*-trans-

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[2] *Abbreviations used:* ALA, δ-aminolevulinic acid; CBL, cystathionine β-lyase; CBS, cystathionine β-synthase; hCBS, human CBS; yCBS, yeast CBS; L-Cth, L-cystathionine; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); EDTA, ethylenediamine tetra acetic acid; L-Hcys, L-homocysteine; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria broth; LDH, L-lactate dehydrogenase; NADH, β-nicotinamide adenine dinucleotide (reduced form); Ni–NTA, Ni–nitrilo triacetic acid; PLP, pyridoxal 5′-phosphate.

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ferase (GST), were subsequently developed by Shan and Kruger [21] and by Janosik et al. [22], although the hCBS produced retains an 11- or 23-residue extension at the amino-terminus, respectively, following proteolytic cleavage. Recently, a modified GST-hCBS expression construct was reported, which reduced the tag remaining at the amino-terminus of hCBS, following proteolytic cleavage, to a single glycine residue [23]. Expression of hCBS as a fusion protein with GST in *E. coli* has allowed yields of 2–11 mg/L and the variability in yield of purified hCBS reported is largely due to the aggregative tendency of the full-length enzyme [12,23,24].

Each of the hCBS expression systems currently employed rely upon GST-based affinity purification and share a requirement for protease cleavage to remove the fusion partner, followed by an additional purification step. In contrast, the addition of a 6-His affinity tag to the carboxy-terminus of truncated yeast CBS (ytCBS) enabled its purification *via* a single chromatographic step and, as the kinetic parameters of the enzyme were identical to wild-type ytCBS, subsequent removal of the 6-His tag is not required [25]. Therefore, to investigate the feasibility of employing Ni–nitrilo triacetic acid (Ni–NTA) affinity chromatography to purify hCBS, a 6-His tag was incorporated in a series of expression constructs: 6-His-GST/hCBS, 6-His-GFP/hCBS, 6-His-linker/hCBS, 6-His/hCBS and hCBS/6-His. The constructs bearing an amino-terminal 6-His tag were found to be optimal for the rapid purification of active, soluble hCBS with heme saturation of ~90%. Steady-state kinetic parameters for the physiological condensation of L-Ser and L-Hcys were determined *via* global fit analysis, enabling quantification of substrate inhibition by L-Hcys.

Materials and methods

Reagents

δ-Aminolevulinic acid (δ-ALA), L-lactate dehydrogenase (LDH), β-nicotinamide adenine dinucleotide (β-NADH, reduced form), Tris–HCl, thrombin, dithiothreitol (DTT), isopropyl β-D-1-thiogalactopyranoside (IPTG), DNase I, lysozyme, L-Cth, L-Ser and L-Hcys thiolactone were purchased from Sigma–Aldrich. L-Hcys was prepared from the thiolactone via the method described by Kashiwamata and Greenberg [26]. Protease inhibitor tablets were a Roche product and nickel–nitrilotriacetic acid (Ni–NTA) resin was from Qiagen. Potassium phosphate (monobasic and dibasic), 5, 5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and imidazole were Fisher Scientific products. The 6-His-tagged cystathionine β-lyase (CBL) coupling enzyme, employed in the CBS assay, was expressed and purified as described previously [25]. Oligonucleotide primers were synthesized by Integrated DNA Technologies Inc. All restriction endonucleases and T4 DNA ligase were obtained from New England BioLabs. The pGEX4T-hCBS expression construct was the generous gift of Dr. Warren Kruger (Fox Chase Cancer Center, Philadelphia).

Preparation of the 6-His-GST/hCBS expression construct

The sequence spanning both the GST and hCBS coding regions was amplified from the pGEX4T-hCBS vector with the GSTf-His-NcoI (CAT GCC ATG GCC CAC CAC CAT CAC CAC CAT TCC CCT ATA CTA GGT TAT TGG) and hfCBSr-XbaI primers (GCT CTA GAT CAC TTC TGG TCC CGC TCC TGG GCG GCC ACG), which comprise a NcoI restriction endonuclease site and 6-His tag and a XbaI site, respectively, and introduced at the corresponding sites of the pTrc-99a expression vector, under control of the IPTG-inducible *trc* promoter, to produce the 6-His-GST/hCBS expression construct [21].

Preparation of the 6-His/hCBS and 6-His-GFP/hCBS expression constructs

The *eGFP-1* gene was amplified from the pEGFP-1 plasmid (Clontech) using the GFP-Fc-NcoI-His (5′-TAG ACC ATG GCG CAT CAT CAC CAT CAC CAT ATG GTG AGC AAG GGC GAG-3′) and GFP-Rc-KpnI (5′-GGC ATG GAC GAG CTG TAC AAG TAA-3′) primers and inserted between the NcoI and KpnI sites of pTrc-99a. A 6-His tag was incorporated immediately following the start codon of GFP, in the GFP-Fc-NcoI-His primer. The pTrc99a-GFP construct was subsequently modified to introduce a linker sequence, encoding a thrombin proteolytic cleavage site at the KpnI site at the 3′ end of the eGFP-1 gene. The linker was designed to incorporate a unique SpeI restriction site, not present in the vector, such that digestion with SpeI endonuclease, followed by re-ligation of the vector, would enable selection of a construct containing a single copy of the linker. The KpnI site, at the 3′ end of the linker, was subsequently converted to a NdeI site *via* whole-plasmid, *Dpn*I-mediated mutagenesis [27,28]. The pTrc99a vector contains a NdeI site, outside of the multiple cloning site, which was subsequently converted to a BglII site. The hCBS gene was amplified from the pGEX4T-hCBS vector with the hCBS-Fc-NdeI (5′-GAC AAA CAT ATG CCT TCT GAG ACC CCC C-3′) and hCBS-Rc-SalI (5′-GGA TCC GAT CGA CTT CAC TTC TGG TCC CGC-3′) primers. The pTrc99a/GFP-linker vector contains a pair of NdeI sites, one situated at the junction of the 6-His tag and the GFP gene and the second at the 3′ end of linker sequence. Therefore, insertion of the amplified hCBS sequence between the NdeI and SalI sites of this plasmid substituted the GFP-linker sequence with the hCBS amplicon to produce the 6-His/hCBS expression construct. The NdeI site between 6-His tag and GFP of the pTrc99a/GFP-linker plasmid was subsequently changed to a BmtI site *via* whole-plasmid, *Dpn*I-mediated mutagenesis and insertion of the amplified hCBS sequence at the NdeI and SalI sites of this plasmid produced the 6-His-GFP/hCBS expression construct.

Preparation of the hCBS/6-His and 6-His-linker/hCBS expression constructs

Whole-plasmid, *Dpn*I-mediated mutagenesis was employed to replace the NcoI site of the pTrc-99a multiple cloning site with a NdeI site and to remove the existing NdeI site to produce a modified pTrc-99a vector. The hCBS sequence was amplified from the pGEX4T-hCBS vector with the hCBS-Fc-NdeI (GGG AAT TCC ATA TGC CTT CT GAGA CCC CC) and hCBS-Rc-His-SalI (TAT ATG TCG ACA TGA TGA TGA TGA TGA TGT CAC TTC TGG TCC CGC TCC TGG GCG GC) primers, the latter encoding a 6-His tag, and inserted at the NdeI and SalI sites of the modified pTrc-99a vector to produce the hCBS/6-His expression construct. The modified pTrc-99a expression vector was also altered to incorporate a sequence encoding a 6-His tag and a Factor Xa protease site at the 5′ end of the poly-linker. A mixture of 1 nmol of each of the linker-top (5′-TAG CCA TCA TCA TCA TCA TCA TAG CAG CGG CCA TAT CGA AGG TCG TCA) and linker-bottom (5′-TAT GAC GAC CTT CGA TAT GGC CGC TGC TAT GAT GAT GAT GAT GAT GGC) oligonucleotide primers were incubated at 70 °C for 5 min, annealing buffer was added (to a final concentration of 100 mM Tris–HCl, pH 7.5, 70 mM $MgCl_2$) and the mixture was incubated at 70 °C for 5 min prior to gradual cooling to 25 °C, over a period of 1 h. The annealed linker was inserted at the introduced NdeI site of the modified pTrc-99a plasmid, the translation initiation site was subsequently reintroduced *via* whole-plasmid, *Dpn*I-mediated mutagenesis and the hCBS sequence, amplified from the hCBS/6-His construct with the pSECseq0 (GGC GTC AGG CAG CCA TCG GAA GCT G) and hCBS-Rc-SalI vector and gene-specific primers, respectively, was