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Purification and characterization of the wild type and truncated human cystathionine β-synthase enzymes expressed in *E. coli*

Nina Frank [1], Jana O. Kent [1], Markus Meier, Jan P. Kraus *

Department of Pediatrics, University of Colorado School of Medicine, UCHSC, RC1 North, Rm. 4128, 12800, Mail Stop 8313, P.O. Box 6511, Aurora, CO 80045-0511, USA

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Abstract

In this paper, we describe the expression and characterization of recombinant human cystathionine β-synthase (CBS) in *Escherichia coli*. We have used a glutathione-*S*-transferase (GST) fusion protein vector and incorporated a cleavage site with a long hinge region which allows for the independent folding of CBS and its fusion partner. In addition, our construct has the added benefit of yielding a purified CBS which only contains one extra glycine amino acid residue at the N-terminus. In our two-step purification procedure we are able to obtain a highly pure enzyme in sufficient quantities for crystallography and other physical chemical methods. We have investigated the biochemical and catalytic properties of purified full-length human CBS and of two truncation mutants lacking the C-terminal domain or both the N-terminal heme-binding and the C-terminal regulatory regions. Specifically, we have determined the pH optima of the different CBS forms and their kinetic and spectral properties. The full-length and the C-terminally truncated enzyme had a broad pH 8.5 optimum while the pH optimum of the N- and C- terminally truncated enzyme was sharp and shifted to pH 9. Furthermore, we have shown unequivocally that CBS binds one mole of heme per subunit by determining both the heme and the iron content of the enzyme. The activity of the enzyme was unaffected by the redox status of the heme iron. Finally, we show that CBS is stimulated by *S*-adenosyl- L-methionine but not its analogs.
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Keywords: Homocystinuria; *S*-adenosylmethionine; Pyridoxal-5′ phosphate; Heme

Cystathionine β-synthase (CBS)[2] (L-Serine hydrolyase (adding homocysteine), EC 4.2.1.22) is central to sulfur amino acid metabolism in eukaryotes, and complete CBS deficiency is the principal cause of homocystinuria in humans. The CBS-catalyzed condensation of serine and homocysteine generates cystathionine which is then cleaved with cystathionine γ-lyase (CGL) to yield cysteine [1].

Cysteine synthase (*O*-acetylserine sulfhydrase, OASS; CS), the CBS counterpart in prokaryotes, plants and enteric protozoa, catalyzes the formation of cysteine from *O*-acetylserine and hydrogen sulfide (H_2S) with the concomitant release of acetic acid. Unlike mammals, these organisms are able to assimilate inorganic sulfur into organic sulfur and are therefore essential players in the sulfur cycle in nature. CS and CBS are affiliated with the large β-family of pyridoxal-5′-phosphate (PLP) dependent enzymes and have a high degree of sequence and structural conservation within the catalytic core [2,3], (see also www.uchsc.edu/sm/cbs/cbsdata/cbsprotein.htm). An evolutionary relationship between these enzymes is also illustrated by the fact that some CBS enzymes have retained CS activity [4]. CS and CBS, together with other enzymes such as β-cyanoalanine synthase, the β-subunit of trypto-

* Corresponding author. Fax: +1 303 724 3838.
E-mail address: Jan.Kraus@UCHSC.edu (J.P. Kraus).
[1] These authors contributed equally to this work.
[2] *Abbreviations used:* CBS, Cystathionine β-synthase; GST, glutathione S-transferase; PLP, pyridoxal 5′-phosphate; AdoMet, S-adenosyl-L-methionine; CS, cysteine synthase; OASS, *O*-acetylserine sulfhydrase; β-gal, β-galactosidase; OAS, *O*-acetyl serine; AdoHcy, S-adenosylhomocysteine.

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phan synthase and cysteine lyase, catalyze primarily β-replacement reactions [5,6]. Despite catalyzing mostly one reaction type, CS and CBS can utilize a wide range of substrates [7].

CBS is the only PLP dependent enzyme which contains heme [7]. The heme is co-ordinated by Cys 52 and His 65 residues located in the N-terminal region of the enzyme. The function of this ligand is yet to be determined; however, several laboratories have shown that it is not directly involved in the catalytic process [8–11], thus contradicting previous reports that have shown catalytic involvement [12,13]. CBS from lower eukaryotes such as *Saccharomyces cerevisiae* do not contain heme [8,9]. Thus the absence of this cofactor in CBS from lower eukaryotes suggests that the function of heme in this enzyme is unique to higher organisms.

Human CBS activity is regulated by *S*-adenosyl-L-methionine (AdoMet) [14]. Interestingly, all CS and some CBS enzymes from lower eukaryotes are not responsive to AdoMet suggesting that the requirement by CBS of a heme moiety and regulation by AdoMet may have emerged at the same time evolutionarily and may be interrelated.

The purification of CBS from mammalian tissues is complicated by its tendency for aggregation and its susceptibility to proteolysis [15]. Previously, an *Escherichia coli* expression system using β-galactosidase (β-gal) was used for the purification of CBS [16]. This system had several disadvantages. There was variable undesirable proteolytic cleavage within *E. coli* between β-gal and CBS. The proteolytic cleavage produced free β-gal and CBS prematurely making it impossible to use an affinity chromatography step to obtain purified CBS. Because of these complications, we decided to purify CBS using an alternate expression system. The expression of CBS with a Glutathione-*S*-Transferase (GST) affinity tag was first described by Warren Kruger's laboratory [17]. The disadvantage of that system was that the cleaved purified CBS still retained 11 non-CBS residues at its N-terminus [18]. We have previously expressed human CBS with a GST tag, which upon purification and removal of the fusion partner yielded CBS with 23 extra residues at the N-terminus [19]. Subsequently, we demonstrated that N-terminal elongation altered the affinity of the enzyme for Hcy. This enzyme had ~9-fold lower K_m for Hcy [20] compared to the WT enzyme [21] and was used to obtain the first CBS structure [22]. Here we describe a method to purify CBS using a GST fusion protein which yields CBS as close to normal sequence as possible. Using our construct we are able to obtain a purified enzyme with only one extra small amino acid residue, glycine, at the N-terminus of the purified CBS protein.

The new method gives us several other benefits. First, the vector we chose has a long recognition sequence for the precision protease cleavage site which provides a convenient long hinge region between GST and CBS allowing independent folding of the two enzymes. Second, this system gives the added benefit of a shorter fusion partner which enables us to rapidly purify large amounts of CBS in an easy, two-step procedure. In this paper we describe the expression and purification of the full-length wild type CBS as well as CBS which does not contain the C-terminal regulatory region (1–413) and CBS lacking both the N- and C-terminal regions (71–400). The 71–400 CBS does not contain heme and is highly conserved with the CS family of enzymes. We have further characterized all three forms of CBS with respect to their biochemical and physical properties.

Materials and methods

Chemicals

Unless stated otherwise, all materials were purchased from Sigma. L-[U-^{14}C] serine was obtained from Perkin Elmer Life Sciences.

Cloning of CBS wild type, CBS (1–413) and CBS (71–400)

In order to express the human CBS wild type protein, a construct pGEX-6P-1 HCBS WT was prepared. This construct enabled us to express CBS as a fusion protein with a glutathione-*S*-transferase (GST) tag, which could be later cleaved off with PreScission protease (Amersham Biosciences, Piscataway, NJ). First, the commercially available pGEX-6P-1 vector (Amersham Biosciences, Piscataway, NJ) was modified by destroying the internal Apa I site located at the nucleic acid position 3890 of the pGEX-6P-1 vector as described previously [11] and cut with ApaI/SalI restriction enzymes to create an ApaI–SalI cassette. Then, two primers (sense: 5′-ctctgagaccccccaggcagaagtggggcc-3′ and antisense: 5′-ccacttctgcctggggggtctcagagggcc-3′) encoding the last two C-terminal amino acids of the PreScission protease site and the first ten amino acids of the CBS enzyme were designed. Each primer (1.5 nmol) in 50 μl total volume was phosphorylated in a separate tube using 10 U of polynucleotide kinase (New England Biolabs Ipswich, MA). Following the phosphorylation, the primers were mixed and boiled in a water bath for 2 min. 4 M sodium chloride was added immediately to a final concentration of 10 mM, and the mixture was allowed to slowly cool at room temperature. Under these conditions, the two primers hybridize while forming a short double-stranded insert with ApaI–ApaI overhangs.

The cDNA portion of the previously constructed pAX5$^-$ HCBS WT vector [16] encoding CBS amino acids 1–551 was cut out using ApaI and SalI restriction endonucleases. Finally, the pGEX-6P-1 vector cassette, the two hybridized primers and the fragment cut out of the pAX5$^-$ HCBS WT vector were ligated.

The pGEX-6P-1 HCBS 1–413 construct, yielding human CBS protein truncated at the C-terminus by 138 amino acid residues was prepared from the pGEX-6P-1 HCBS WT clone. The latter was digested with SphI and SalI, which released the portion coding for the C-terminal region spanning amino acids 337–551. Subsequently, we replaced the missing region with the corresponding cDNA portion cut out from a previously constructed pAX5$^-$ CBS 1–413 clone.

The construction of the pGEX-6P-1 HCBS 71–400 expression plasmid has been described previously [11].

All constructs were transformed into *E. coli* BL21 cells (Stratagene) and their authenticity was confirmed by DNA sequencing using a Thermo Sequenase Cy5.5 sequencing kit (Amersham Biosciences) and the Visible Genetics Long-Read Tower System-V3.1 DNA Sequencer according to the manufacturer's instructions.

Expression and purification of wild type, 1–413 and 71–400 CBS

The purification of wild type human CBS (1–551) and two truncated forms, 1–413 CBS and 71–400 CBS, was performed as described previously [11,19] with some modifications. Protein expression was carried out at 30 °C, as the slower expression appears to have beneficial influence on

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