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Short report of Ph.D. thesis

Molecular Mechanisms of Homocystinuria: Spatial Arrangement of Human Cystathionine β-Synthase

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CONTENT

ABSTRAKT

Chybné sbalování mutantních enzymů je považováno za hlavní patogenetický mechanismus homocystinurie z deficitu cystathionin beta-synthasy (CBS). Cílem této práce bylo studium molekulových mechanismů, které vedou k chybnému sbalování mutantních forem CBS.

V první části práce jsme studovali prostorové uspořádání normální lidské CBS. Pomocí diferenčního kovalentního značení povrchově dostupných aminokyselinových zbytků jsme identifikovali kontaktní plochu mezi katalytickým jádrem a regulační doménou v lidské CBS a následně jsme navrhli strukturní model plnodélkového enzymu. V další části práce jsme studovali evoluční divergenci proteinových struktur CBS. Provedli jsme fylogenetickou analýzu, která odhalila unikátní uspořádání pro CBS z třídy nematod; doménová architektura CBS ze *Caenorhabditis elegans* byla podrobně studována experimentálně. Nakonec jsme studovali konformační vlastnosti vybraných mutantních forem lidské CBS, které měly do různé míry narušenou tvorbu tetrameru a sníženou enzymovou aktivitu. Pomocí proteolytických technik s využitím thermolysinu jsme analyzovali devět mutantních forem, které byly exprimovány v *E.coli*. Zjistili jsme, že rozbalení struktury je běžným jevem při chybném sbalování mutantních CBS. Důležitost rozbalení proteinů pro patogenesi deficitu CBS byla dále prokázána pomocí analýzy dalších devíti purifikovaných mutantních variant, které disponovaly nenarušenou tetramerní strukturou a normální enzymovou aktivitou. Tato data ukázala, že odlišná míra rozbalení proteinu je spolehlivým ukazatalem patogenity mutantních CBS a proteolýza thermolysinem za nativních podmínek může být důležitým nástrojem pro biochemické vyhodnocení patogenních variant.

Tato práce zvyšuje porozumění patogenních mechanismů deficitu CBS a poskytuje znalosti, které mohou být v budoucnosti využity pro vývoj chaperonové terapie a následně pro zlepšení péče o pacienty.

ABSTRACT

Protein misfolding is considered to be the major pathogenic mechanism in homocystinuria due to cystathionine beta-synthase (CBS) deficiency. The aim of this work was to study molecular mechanisms underlying protein misfolding of CBS mutants.

Firstly, we studied spatial arrangement of the normal human CBS protein. Using data from differential covalent labeling of solvent-exposed aminoacid residues, we identified interdomain contact area between the catalytic core and the regulatory domain in human CBS, and we subsequently generated the structural model of the full-length CBS. In the next step, we studied evolutionary divergence of CBS protein structures. We performed phylogenetic analysis that revealed unique spatial arrangement of CBS enzyme from nematodes and the domain architecture of CBS from *Caenorhabditis elegans* was studied experimentally in more detail. Finally, we determined conformational properties of a representative set of CBS mutants that exhibited in various extent affected formation of tetramers and decreased catalytic activity. Using thermolysin-based proteolytic techniques for analysis of nine mutants expressed in *E.coli*, we found that an unfolded structure is a common intermediate occurring in CBS misfolding. The importance of protein unfolding for the pathogenesis of CBS deficiency was further shown by analysis of additional nine purified mutants that were properly assembled into tetramers and possessed normal catalytic activity. These data demonstrated that altered protein unfolding is a reliable marker of pathogenicity of CBS mutants and proteolysis with thermolysin under native conditions may be an important tool for biochemical assessment of pathogenic variants.

This study advances our understanding of molecular pathology in CBS deficiency and provide knowledge useful for development of chaperone therapy and future improvement in patient care.

1. INTRODUCTION

Homocystinuria due to cystathionine β-synthase (CBS) deficiency is the most common enzymopathy affecting the metabolism of sulfur amino acids, with a worldwide prevalence of 1:344,000 (*1*). The CBS deficiency is a multisystemic disease that affects vasculature, connective tissues, and central nervous system (OMIM# 236200). More than 150 variant alleles have been described in CBS-deficient patients (annotated in CBS Mutation Database; http://cbs.lf1.cuni.cz/cbsdata/cbsmain.htm) and the missense mutations leading to aminoacid substitions in the CBS protein represent 86 % of all analysed patient allelles.

Previous studies showed that protein misfolding plays an important role in the pathogenesis of CBS deficiency (*2, 3*). This was demonstrated by observing large inactive aggregates devoid of heme on native electrophoresis, increased abundance of mutant proteins in the insoluble fraction and by their rescue after the expression at a lower temperature that facilitated correct folding.

Abberant folding of mutants may be corrected by the presence of chaperones during protein expression and chaperone therapy represents an interesting option for treatment of conformational disorders (*4*). This strategy may be also useful for therapy of CBS deficiency as was demonstrated by successful rescue of a number of mutants by treatment with chemical chaperones, CBS ligands and inhibitors of proteasome (*5-8*). However, clinically relevant compounds possessing chaperone-activity towards CBS mutants have not yet been found. The efficacy and the specificity of the search for pharmacological chaperones may be increased if the structure-function mechanisms of protein folding/misfolding are known.

Human CBS is a pyridoxal-5´-phosphate (PLP) dependent modular protein (551 amino acids; 61 kDa) composed of the N-terminal heme-binding domain (1-69), an evolutionary conserved catalytic core (70-413) and the C-terminal regulatory domain (414-551) (*9*). Allosteric cross-talk between the catalytic core and the regulatory domain modulates CBS activity. The 3-D structure of human CBS has been solved by X-ray crystalography for the C-terminally truncated protein missing regulatory domain (*10, 11*) and the heme-binding pocket along with the catalytic site containing PLP were described at an atomic resolution. However, the spatial arrangement of the full-length CBS has not yet been determined, possibly due to interdomain flexible motions that prevent successful crystalization of the protein. Using H/D exchange followed by mass spectrometric detection, the polypeptide region 359-385 was identified to form interdomain contact area, and structural model of the full-length CBS was proposed (*12*). However, this structural model has not been supported and/or refined by additional structural approaches.

In addition to study spatial arrangement of wild-type CBS, structural characterization of pathogenic variants may provide an insight into molecular mechanisms of CBS deficiency. However, only a few CBS mutants have been purified into homogeneity in yields sufficient for conformational study, including the C-terminal mutants (namely I435T, D444N and S466L) (*12, 13*), a double linked mutant P78R/K102N (*14*) and the R266K mutant (*15*). Thus, the majority of clinically relevant CBS variants have not been structurally characterized and knowledge about structure-function mechanisms underlying misfolding of CBS mutants clearly needs to be expanded.

2. AIMS OF THE STUDY

The main goal of this PhD. study was the structural characterization of wild-type and mutant CBS proteins. The specific aims were as follows:

1. To study spatial arrangement of the full-length CBS using covalent labeling of solvent-exposed aminoacid residues and to build a structural model of the enzyme.

2. To study the conservation of CBS structure by *in silico* analysis and to characterize unusual domain architecture of CBS in *Caenorhabditis elegans*.

3. To describe conformational properties of representative CBS mutants and to study structural basis of their dysfunction.

4. To determine whether human plasma contains CBS enzyme and to test the utility of this phenomenon for diagnosing CBS deficiency.

3. METHODS

3.1 Surface mapping of human CBS

3.1.1 Purification of CBS proteins

The CBS proteins were expressed in *E.coli* as fusion proteins with the N-terminal GST tag. The C-terminally truncated (45CBS; aminoacid residues 1-413) CBS was purified by affinity chromatography using Glutathionine-Sepharose

(Amersham Biosciences) and by subsequent on-column cleavage of the fusion protein by Pre-Scission protease. The full-length CBS was purified by two step procedure using affinity chromatography with glutathione Sepharose followed by hydrophobic interaction chromatography using Phenyl Sepharose (Amersham Biosciences). The CBS proteins were purified with yields of ≈ 5 mg per liter of bacterial culture. The procedures are thoroughly described in (*16, 17*).

3.1.2 Covalent labeling

CBS proteins were modified by diethylpyrocarbonate, N-bromosuccinimide, N-ethylmaleinimide, N-acetylimidazole, sulfo-N-hydroxysuccinimido acetate and 4-hydroxyphenylglyoxal. The reactions were quenched by buffer exchange using Zeba Desalt spin columns (ThermoFischer Scientific) with elution by 50mM NH_4HCO_3.

The structural integrity of modified CBS proteins was assessed by native gel electrophoresis and by determination of catalytic activity by radiometric assay using [^{14}C]-L-serine (Moravek Biochemicals) as a substrate followed by thin-layer chromatography and subsequent quantification of labeled cystathionine by PhosphorImager System (Molecular Dynamics).

Covalently modified proteins were reduced by dithiotreitol and cysteine residues were acetamidated with iodoacetamide. Subsequently, they were digested by trypsin, chymotrypsin and endoprotease Glu-C, and by their double combinations. The peptide mixture was fractionated by ZipTip (Promega), each fraction was mixed with the matrix solution (α-cyano-4-hydroxycinnamic acid) and measured using Autoflex II (Bruker Daltonics) in reflector positive mode (m/z range 500 to 4500). The mass spectrometer was externally calibrated using peptide calibration standard II (Bruker Daltonics). Mass spectra were proccessed by Flex Analysis, Biotools 3.0 and mMmass 3.0.

3.1.3 Computational modeling

The model of the CBS C-terminal domain was built by Modeller 9v3 using Bateman domain-containing protein MJ0100 from *Methanocladococcus jannaschii* (Protein Data Bank ID 3KPB) (*18*). The sequences were aligned using web service of PHYRE and PSI-BLAST. The generated model was evaluated by Prosa and statistical coupling/protein sector analysis (*19*). Docking of the C-terminal domain to the active core (Protein Data Bank ID 1JBQ) (*10*) was carried out using ZDOCK.

3.2 Characterization of CBS in *Caenorharbditis elegans* (CBS-1)

3.2.1 Purification of CBS-1

The CBS-1 was expressed in *E.coli* at 18 °C as a fusion protein with N-terminal cleavable GST and C-terminal uncleavable hexahistidine tag, respectively. The protein was purified using affinity column filled with Glutathionine Sepharose and subsequent on-column cleavage by Pre-Scission protease. In the next step, CBS-1 was purified by IMAC. Using elution buffer containing 75mM imidazole, CBS-1 was collected with the yield of approximately 1mg per 1 liter of bacterial culture.

3.2.2 Native size of CBS-1

The CBS-1 protein was analysed by size exclusion chromatography (Bio-Sil SEC HPLC Column, Biorad), native gel electrophoresis, Blue-native gel electrophoresis and cross-linking procedure using bis[sulfosuccinimidyl] suberate.

3.2.3 Catalytic properties of CBS-1

Substrate specificity and kinetic parameters was determined using HPLC and LC-MS/MS as described in detail previously (*20, 21*).

3.2.4 Analysis of truncated and active-site mutants

We further purified and characterized three CBS-1 mutants: Δ377-704, E62K and K421A. Catalytic activity of mutants was determined as decribed above. Moreover, proteins were analysed by native gel electrophoresis and by UV-VIS absorption (Shimadzu UV-2550), fluorescence (Perkin Elmer LS55) and circular dichroism (Jasco J-810) spectroscopy.

3.3 Conformational analysis of CBS mutants

3.3.1 Study of crude cell extracts – sample preparation

The wild-type and mutant human CBS proteins were expressed at 37 °C in *E.coli* transformed with pHCS3 plasmids that produce untagged full-length enzymes.

3.3.2 Proteolysis with thermolysin under native conditions

Bacterial lysates (final protein concentration 1 mg/ml) containing 10 mM $CaCl_2$ were cleaved by thermolysin from *Bacillus thermoproteolyticus* for the time course of 20 min. The concentration of thermolysin ranged from 0.4 to 200 μg/ml. At the selected time point, proteolysis was quenched in 20 mM EDTA.

3.3.3 Pulse proteolysis in a urea gradient

Cell lysates (final protein concentration 1 mg/ml) containing 10 mM $CaCl_2$ and urea (0-7M) were incubated overnight at 4 °C. The samples were digested by thermolysin (0.2 mg/ml) at 25°C for 60 s. The proteolytic pulse was quenched in 20 mM EDTA.

3.3.4 Evaluation of the proteolytic courses

The amount of the remained uncleaved proteins was assessed by SDS-PAGE followed by Western Blotting using purified chicken anti-hCBS serum H19 (HenA) as a primary antibody and rabbit anti-chicken horseradish-peroxidase-conjugated secondary antibody (ThermoFisher Scientific).

The signal was quantified using Gene Tools software and fitted into exponential (native proteolysis) or sigmoidal (pulse proteolysis) equations by Origin 8.0 (Originlab).

3.3.5 Preparation of purified CBS mutant proteins

Purification of CBS mutants was performed in the laboratory of our collaborator Dr. Kraus at University of Colorado and was reported previously (*8, 15*).

3.3.6 Analysis of CBS mutants by spectroscopic techniques

The conformation of purified mutants was assessed using circular dichroism (Jasco J-810), fluorescence (Perkin Elmer LS55) and second-derivative UV (Agilent diode array model 8453) spectroscopy.

3.3.7 Analysis of purified mutants by proteolytic techniques

The analysis was performed as described above (Sections 3.3.2-3.3.4) with modifications as follows: purified proteins (0.5 mg/ml) were cleaved with

thermolysin (0.1 mg/ml) and the amount of the uncleaved protein was assessed by SDS-PAGE followed by staining with Coomassie Blue.

3.4 Analysis of human plasma

The CBS activity was measured using stable isotope substrate 2,3,3-^{2}H serine and labeled cystathionine was detected by LC-MS/MS (Agilent 1100 LC System coupled with API 3200 triple quadrupole mass spectrometer).

4. RESULTS AND DISCUSSION

4.1 Surface mapping of normal human CBS enzyme

Initially, we developed labeling procedures for nine modifiers (diethylpyrocarbonate, N-bromosuccinimide, N-ethylmaleinimide, N-acetylimidazole, sulfo-N-hydroxysuccinimido acetate, 4-hydroxyphenylglyoxal, tetranitromethane, iodine and 2-hydroxy-5-nitrobenzyl bromide) with model proteins such as chicken lysozyme, horse cytochrom c and human serum albumin. All modifiers used, with the exception of diethylpyrocarbonate, reacted with specific aminoacid residues as was described in the literature (reviewed in (*22*)). Interestingly, our study revealed that diethylpyrocarbonate reacts not only with the solvent-exposed histidine but also with lysine residues. This finding enabled more effective and accurate analysis of this labeling reaction in further studies.

The feasibility of covalent labeling for structural analysis of CBS was tested using the 45CBS. Six of nine modifiers tested provided relevant information about aminoacid residues in native proteins since the modification reactions did not affect quarternary structure nor the catalytic activity of labeled proteins (feasible modification agents are specified in Section 3.1.2).

Comparing reactivity of side-chains in the 45CBS and the full-length CBS, we identified 46 identically reactive residues and 4 diferentially modified sites that were reactive only in the 45CBS but not in the full-length CBS, namely the K172/K177, R336, K384 and W408/W409/W410 aminoacid residues.

Using computational modeling exercise, we found that differentialy reactive residues may form interdomain contact area that is responsible for modulation of CBS activity. Based on these data, we generated structural model of the normal human full-length CBS enzyme.

Our model has been strongly supported by a recent X-ray crystalographic study (*23*) that described 3-D structure of CBS from *Drosophila melanogaster*. Taken

together, our study expanded significantly knowledge about spatial arrangement of human CBS.

4.2 Characterization of CBS-1 in *Caenorharbditis elegans*

Using *in-silico* searches together with experimental confirmation (RT-PCR and the expression analysis of GFP-tagged proteins) we found that ZC373.1 is the only transcriptionally active gene encoding CBS in *C.elegans* (CBS-1). The CBS-1 protein does not form oligomers and possesses conserved tandemly arranged catalytic domains in one polypeptide. Interestingly, only the C-terminal domain binds the PLP cofactor and catalyses the β-replacement reactions as was demonstrated by analysis of truncated (Δ377-704) and active site (E62K and K421A) mutant proteins. The funtion of the noncatalytic N-terminal conserved domain is unclear. Our data indicate that this module is important for stability and/or proper folding of CBS-1 biomolecule. Taken together, the nematode CBS-1 possesses a unique spatial arrangement that is specific only for nematodes as was revealed by the phylogenetic analysis. This study shows that domain architecture of CBS is not conserved in all metazoan species and demonstrates evolutionary divergence of the CBS protein structure.

4.3 Conformational analysis of human CBS mutants

4.3.1 Study in crude cell extracts

Since the majority of CBS mutant proteins are not amenable to purification, we used an approach permitting a conformational study directly in crude cell extracts. The approach uses thermolysin and comprises two proteolytic techniques, namely proteolysis under native conditions, and pulse proteolysis in a urea gradient.

Using this methodology we analysed three most common mutations (T191M, I278T and G307S) together with representative mutations in each functional domain of the CBS molecule (H65R, A114V, E302K, R369C, R439Q and D444N).

Our study showed that proteolysis under native conditions is a robust technique useful even for highly unstable CBS mutants, whereas pulse proteolysis in a urea gradient has limited value due to instability of mutant proteins.

Proteolysis under native conditions revealed that the majority of examined CBS mutants is more susceptible towards cleavage indicating their propensity to unfolding. The extent of unfolding correlated well with with previously determined

catalytic activity and degree of tetrameric assembly of CBS mutants (*3*). Our study showed that an unfolded structure is a common intermediate occuring in misfolding of CBS proteins, and that proteolytic susceptibility under native conditions may be an important marker of pathogenicity of CBS mutations.

4.3.2 Study of purified CBS mutants

Previous study by Majtan et al. (*8*) demonstrated that CBS mutants could be purified if the culture media contained chemical chaperone. However, the purified CBS mutants retained normal catalytic activity, tetramer assembly and full heme saturation; the structural abnormalities responsible for pathogenicity of these mutations remained unknown.

In our study, we analyzed CBS mutants purified in previous Majtan´s studies (*8,15*), namely P49L, P78R, A114V, R125Q, E176K, R266K, P422L, I435T and S466L, using spectroscopic and proteolytic techniques. Using far UV-circular dichroism, fluorescence and second-derivative UV spectroscopy we found that global structure of CBS mutants is similar to that of the wild-type but the microenvironment of the chromophores of the studied mutants may be altered. In the next step, proteolysis with thermolysin under native conditions revealed that six of nine mutants were more susceptible towards digestion than the wild-type. Pulse proteolysis revealed that the R125Q and E176K mutants were less stable than wild-type whereas other mutant proteins possessed unaffected global protein stability. In conclusion, this study demonstrated that CBS mutants are prone to unfolding in spite of normal catalytic activity and unaffected formation of tetramers.

4.4 Analysis of CBS enzyme in human plasma

Using sensitive assay with deuterium-labeled substrate 2,3,3-^{2}H serine followed by LC-MS/MS detection of the labeled product cystathionine, we demonstrated that CBS protein is present in human plasma. However, the amount of CBS is low, approximately in the range of nanograms per milliliter, which does not permit application of the available immunological techniques for detecting the enzyme.

In the next step, we tested a utility of LC-MS/MS-based assay for diagnosis of the CBS deficiency. Our study showed that the catalytic activity in pyridoxine-nonresposive patient was significantly decreased whereas the activity of pyridoxine-responders overlapped with the activity of healthy controls. These data indicate that determination of CBS activity in human plasma may be used for non-invasive diagnosis of pyridoxine-nonresponsive CBS deficiency.

5. CONCLUSIONS

1) Using surface mapping we identified residues forming a contact area between the catalytic core and the C-terminal regulatory domain of normal human CBS. These data were used for generation of structural model of the full-length enzyme. The modeled structure is consistent with recently solved spatial arrangement of CBS from *Drosophila melanogaster* indicating that regulatory interface is conserved in Bateman domain-containing CBS proteins from different species.

2) We identified and characterized CBS in *Caenorhabditis elegans*. We found that this CBS protein possesses unique domain architecture and does not assemble into oligomers. This study provides novel data on evolutionary divergence of CBS protein structures.

3A) Using proteolysis with thermolysin under native conditions for analysis of CBS mutants in bacterial lysates, we found that unfolded structure is a common intermediate occuring in protein misfolding of pathogenic variants. The extent of unfolding of the studied CBS mutants correlated inversely with their catalytic activity and with the degree of tetrameric assembly.

3B) Furthermore, the majority of purified CBS mutants was more susceptible against proteolysis in spite of normal catalytic activity, heme saturation and tetramer formation. Both thermolysin-based studies show that proteolytic sensitivity of CBS mutants may represent an important marker of pathogenicity of the CBS mutations.

4) Our study demonstrated that CBS protein is present in human plasma in low amounts that are insufficient for detection by immunological techniques. The only feasible approach for study of CBS in plasma is determination of catalytic activity by LC-MS/MS; we showed that this technique may be useful for non-invasive diagnosis of pyridoxine-unresponsive CBS deficiency.

6. REFERENCES

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7. ABBREVIATIONS

45CBS	C-terminally truncated cystathionine beta-synthase
CBS	cystathionine beta-synthase
GFP	green fluorescent protein
GST	glutathionine S-transferase
HPLC	high performance liquid chromatography
IMAC	immobilized metal ion affinity chromatography
LC-MS/MS	liquid chromatography – tandem mass spectrometry
PLP	pyridoxal-5´-phosphate
RT-PCR	reverse transcription - polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis

8. LIST OF AUTHOR´S PUBLICATIONS

8.1 Publications cited in PhD. thesis

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