NMR STUDIES OF HUMAN CYSTATIN C – STABLE ISOTOPE LABELING OF HUMAN CYSTATIN C

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Abstract: The objective of this study was to obtain a double and triple labeled human cystatin C (hCC). Another objective was to record sets of 2D and 3D NMR spectra of the hCC dimer (in a solution) using a 700 MHz spectrometer. The data obtained during attempts to determine the NMR structure should provide useful information about chemical shifts of amino acid residues. They will certainly accelerate solving the human cystatin C NMR structure.

In this paper the main focus is put on triple isotopic labeling, protein overproduction and NMR analysis of hCC. The first two processes lead to obtaining hCC labeled with stable isotopes of carbon (^{13}C), nitrogen (^{15}N) and hydrogen (^{2}H) (double labeled hCC was obtained with a similar method). The obtained protein was later used for the purpose of NMR spectra.

Keywords: NMR, overproduction, stable isotopes, proteins, labeling

1. Introduction

Human cystatin C (hCC) is the most widespread kind of cystatin in mammalian organisms. This small protein (13 kDa, 120 amino acid residues) is a member of a superfamily of natural, papain-like, cystein protease inhibitors. It is involved in many different types of mechanisms which lead to various diseases such as ascerebral amyloid angiopathy, dementia [1]. Human cystatin C is one of the most important extra- and transcellular cystatin protease inhibitors. Its monomeric form is present in all human body fluids such as cerebrospinal fluid, seminal plasma, milk, synovial fluid, saliva, tears, urine and blood plasma. The concentration of cystatine C in those fluids is generally a constant value (*e.g.* 0.8–1.2 mg/l for plasma). Therefore, a change in the plasma concentration of hCC has been proposed as an kidney function index. This proposition has been possible owing to the fact that increased plasma levels are almost exclusively associated with a reduction in the GFR (glomerular filtration rate) [2]. Even though hCC

is a monomer (under physiological conditions), attempts of its crystallization have resulted in obtaining a dimeric form only [3]. This phenomenon results from a three-dimensional exchange of domains (3D domain swapping) [4]. The NMR structure of the monomeric form of human cystatin C has not been found to date. In 1997 Ekiel *et al.* [5] were very close to the discovery of this structure but failed due to technical limitations (many sequential NOE signals missing). Compared with 1997, the parameters of NMR techniques such as resolution have increased. This opens new opportunities for scientists engaged with the protein structure determination.

2. Labeling and overproduction of human cystatin C

The human cystatin C dimer was obtained by amplification of the hCC coding gene. The gene was incorporated into plasmid DNA and then overexpressed in a properly selected strain of bacteria (*E.coli*). The triple labeled hCC wild type protein and its natural mutant V57G were overexpressed in special microbiological media containing isotopically labeled ingredients. Such ingredients as glucose labeled with the ^{13}C isotope, ammonium chloride labeled with the ^{15}N isotope and deuterium oxide were used for the purpose of overproduction of isotopically labeled hCC. The process of overproduction required a transfer of bacteria between two media containing different ingredients (Figure 1). The obtained protein was later purified by chromatographic techniques.

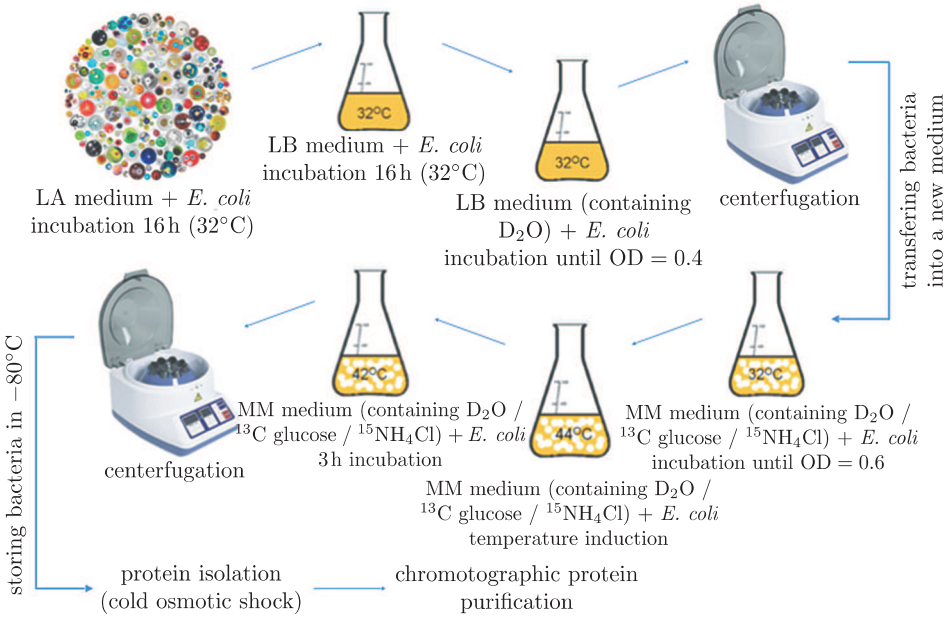

Figure 1. Method of overproduction of isotopically labeled proteins

3. NMR studies

The sequence-specific assignment of ^{1}H, ^{13}C and ^{15}N backbone resonances was performed on the hCC dimer, synthesized and purified in the ^{13}C and ^{15}N double labeled form. NMR spectra of hCC, synthesised and purified in the ^{2}H, ^{13}C and ^{15}N triple labeled form were also obtained. All the NMR spectra were obtained in a water solution using a VARIAN VNMRS 800 MHz spectrometer and analyzed using the CARA program [6].

NMR data were used as input for CS23D2.0 – a web server for rapid generation of accurate 3D protein structures using the assigned NMR chemical shifts only as input [7].

4. Results

After the analysis of ^{1}H, ^{15}N, $^{13}C_{n-1}$, $^{13}C_{\alpha n-1}$, $^{13}C_{\alpha}$ and $^{13}C_{\beta n-1}$ chemical shifts it is easy to see that it is only 97 out of 240 amino acid residues that are defined. Many sequential signals are missing. It was possible to perform a sequential assignment using $^{13}C_{\alpha}$ and $^{13}C_{\alpha n-1}$ chemical shifts for some fragments of the hCC dimer. The NMR structure of the hCC dimer is shown in Figure 2. The NMR dimer structure is different from the X-ray one. However, the comparison of the NMR monomer with the X-ray monomer structure clearly shows many similarities (RMSD 1.755). The differences between X-ray and NMR structures are observed mainly in the (1–10) N-terminal fragment and the AS loop (Figure 2).

The NMR spectrum of triple labeled in comparison with double labeled hCC should show more chemical shifts and give a higher resolution picture of the 3D cystatin C structure.

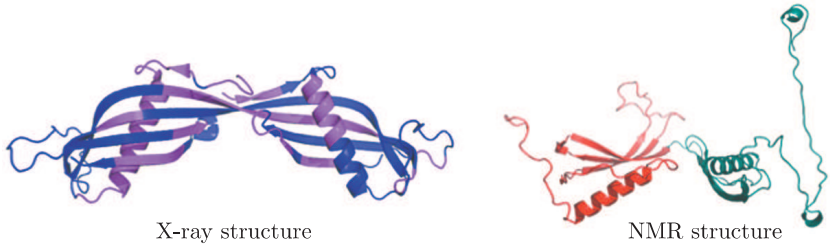

Figure 2. Comparison of hCC X-ray and NMR structures; fragments obtained with NMR marked pink on the X-ray structure

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