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Elucidation of tRNA–cytochrome *c* interactions through hydrogen/deuterium exchange mass spectrometry

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ABSTRACT

Cytochrome *c* (cyt *c*) is a mitochondrial protein responsible for transferring electrons between electron transport chain complexes III and IV. The release of cyt *c* from the mitochondria has been considered as a commitment step in intrinsic apoptosis. Transfer RNA (tRNA) has recently been found to interact with the released cyt *c* to prevent the formation of the apoptosome complex, thus preventing cell apoptosis. To understand the molecular basis of tRNA–cyt *c* interactions, we applied hydrogen/deuterium exchange mass spectrometry (HDXMS) to analyze the interactions between tRNA and cyt *c*. $tRNA^{Phe}$ binding to cyt *c* reduced the deuteration level of cyt *c* in all analyzed regions, indicating that tRNA binding blocks the solvent-accessible regions and results in the formation of a more compact conformation. Substitution of the $tRNA^{Phe}$ with the total tRNA from brewer's yeast in the HDXMS experiment significantly reduced deuteration in the N-terminus and the region 18–32 residue of cyt *c*, where all tRNAs are bound. To clarify the cause of binding, we used synthesized single-stranded oligonucleotides of 12-mer dA and dT to form complexes with cyt *c*. The exchange of the nucleotide bases between adenine and thymine did not affect the deuteration level of cyt *c*. However, the regions 1–10 and 65–82 showed minor decreases after unstructured dA or dT DNA binding. Collectively, these results reveal that cyt *c* maintains its globular structure to interact with tRNA. The region 18–32 selectively interacts with tRNA, and N-terminal 1–10 interacts with oligonucleotides electrostatically.

1. Introduction

Cytochrome *c* (cyt *c*) is a multifunctional heme protein involved in adenosine triphosphate (ATP) production and programmed cell death. Although the mitochondria produce ATP, cyt *c* transfers electrons between electron transport chain (ETC) complexes III and IV in the mitochondria [1–3]. The ETC unbound cyt *c* located on the lipid membrane is associated with cardiolipin (CL) anions in the intermembrane space [4–6]. Activated cyt *c* exhibits peroxidase activity and thus peroxidizes CL in the presence of H_2O_2 [7–9], which can be converted to free oxide radicals by superoxide dismutase under stress [10]. The oxidized CL is flipped from the inner to outer mitochondrial membrane and subsequently causes high permeability of the outer membrane of mitochondria, leading to cyt *c* release [11,12]. The release of cyt *c* from the mitochondria is a commitment step in programmed cell death [13,14]. The released cyt *c* then binds to the Apaf-1 adapter protein to form the apoptosome complex [15], which is an irreversible step in apoptosis.

Transfer RNAs (tRNA) are molecules that transfer coded amino acids to ribosomes for translation. tRNAs also interact with the released cyt *c* to prevent the formation of the apoptosome complex before commitment to cell death [16,17]. The tRNA–cyt *c* interaction, which prevents entry into programmed cell death, implicates the additional role of tRNA in the regulation of apoptosis [18]. The conformational change and subsequent fragmentation of tRNA occur in the early stage of oxidative stress treatments such as ischemic reperfusion, toxic injury, and irradiation [19]. MicroRNAs derived from these tRNA fragments regulate cell proliferation and the DNA damage response [20]. Angiogenin-cleaved tRNA halves also interact with cyt *c* and rescue cells resulting from osmotic stress-induced apoptosis [21].

In addition to the function of transporting electrons and interacting with tRNA, cyt *c* exhibits peroxidase activity. The binding of cyt *c* to tRNA or ETC complexes results in conformational changes that markedly affect the peroxidase activity of cyt *c* as well as its interaction with CL [22,23]. The structural and conformational flexibility of cyt *c* enables rearrangement of the surface charge distribution and its affinity to the binding partner, thereby contributing to its versatile cellular functions [4].

The peptide amide H/D exchange technique has been employed to study the mechanisms of protein folding [24–26] and protein dynamics,

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domain structure, regional stability, and function [27,28]. The slowly exchanging peptide amide hydrogen is in equilibrium with the protons in the solvent [29,30]. The rate of exchange of the amide proton is affected by the solvent accessibility, the local stability and dynamics of that specific peptide bond. Measurements of the deuteration level in the peptide bonds have been used to characterize the changes in solvent accessibility caused by protein-binding interactions and conformational changes [31].

Formation of the tRNA–cyt *c* complex is a novel concept for the regulation of apoptosis. The interaction between tRNA and cyt *c* may be initiated by the electrostatic attraction force and subsequently result in conformational changes in cyt *c*. Although numerous cyt *c* crystal and nuclear magnetic resonance (NMR) structures have been reported, the details of the tRNA–cyt interactions have not been elucidated. In the present study, we applied hydrogen/deuterium exchange mass spectrometry (HDXMS) to analyze the conformation of cyt *c* after binding to tRNA and single-stranded DNA (ssDNA). Researchers have successfully used HDXMS to analyze conformational changes in proteins. Comparison of the single tRNA species of $tRNA^{Phe}$, natural total tRNA, and unstructured ssDNA will provide crucial information for predicting the tRNA-binding regions and to understand the structural changes in cyt *c* after tRNA binding.

2. Materials and methods

2.1. Material

Ferric-cyt *c* (oxidized form) from equine heart (≥95%) and transfer ribonucleic acid phenylalanine ($tRNA^{Phe}$) specific from brewer's yeast were purchased from Sigma Aldrich. Deuterium oxide (D_2O, 99.9%) was bought from Cambridge Isotope Laboratories. Immobilized pepsin on 6% agarose beads were purchased from Thermo Scientific. Single strand DNA 12-mer dA and 12-mer dT were synthesized by Protech Technology Enterprise Co, Taiwan. Yeast tRNA from brewer's yeast was purchased from Thermo Fisher Scientific Inc. 20X D_2O buffer contains 2.5 M Tris and 1 M NaCl at pH 7.5 in H_2O.

2.2. MS/MS of pepsin digested cyt c

The prepared 50 μg of cyt *c* was digested by immobilized pepsin for 5 min on ice. The pepsin digested peptides was identified by MS/MS function of both Quadruple Ion Trap MS (Esquire 6000, Bruker) and quadrupole time-of-flight (Q-TOF) MS (micrOTOF-Q III, Bruker). The pepsin digested peptides were injected into a peptide trap (Optimize, 3 mm cartridge) linked to HPLC (L2130, Hitachi) with reverse phase C18 column (Biobasic 5 μm 50 mm × 1 mm, Thermo scientific) for separation. Gradient was set to 100%–0 Solvent A and 0–100% Solvent B in 40 min. Solvent A contains 0.1% TFA and Solvent B contains 80% Acetonitrile, 20% H_2O and 0.01% TFA. Most peptides were eluted between 20–40 min. Tandem mass was applied to these peptides by the methods of Auto MS/MS of Ion Trap MS. The experiments were done in triplicates. Parallel MS/MS experiments were done on the Q-TOF instrument and UHPLC (UltiMate 3000, DIONEX) with the same C18 column.

2.3. Peptide map of cyt c

Peptide map lists all identified fragments of the target protein, which determines the resolution of HDXMS. The sequence of the peptides was initially identified by MS/MS analysis on Quadruple Ion Trap MS in triplicate. The MS/MS profiles with intensity above 5000 were exported as 3 individual MGF files. These MGF files were imported into X!Tandem parser 1.7.7 to perform peptide identification [32]. The product ion pattern of each peptide was then further examined manually on X!Tandem viewer. The peptides that do not have the good matched product ions with theoretical product ions will be deleted. Any peptide that appeared in two of the three replicates was selected into the initial peptide pool. Through the MS/MS identification by Quadruple Ion Trap, there were 72 peptides in the initial peptide pool. There were 46 peptides in this 72-peptide pool further confirmed by Q-TOF MS/MS analysis, which utilizes similar protocol as Quadruple Ion Trap MS. These 46 peptides becomes our final peptide pool, which will be imported into HDexaminer 1.3 (Sierra Analytics). The *m/z* shifts that resulted in the best fit between the theoretical and actual isotope clusters of 41 peptides are below 0.1 Da in HDexaminer. The *m/z* shifts of the rest of the five peptides are within 0.1–0.2 Da.

2.4. Preparation of deuterated samples

D_2O buffer containing 95% D_2O was prepared by dilute 20X D_2O buffer in D_2O. Before hydrogen/deuterium exchange experiments, 5 μl of ferric cyt *c* (1 mM) was incubated with15 μl of tRNA (1.5 mM), $tRNA^{phe}$ (1.2 mM), 12-mer dA (1.2 mM) or 12-mer dT (1.2 mM) at equal volume in each binding experiment at 25 °C for 30 min. Hydrogen/deuterium exchange experiments were initiated by mixing 20 μl of prepared cyt *c*-tRNA or ssDNA complex with 60 μl of 95% D_2O buffer (125 mM Tris, 50 mM NaCl, pH 7.5) to a final concentration of 71.25% D_2O at pH 7.5. The samples were incubated at 25 °C for an additional 10 s, 30 s, 100 s, 300 s, 1000 s, 3000 s or 10000 s. The deuterium exchange was quenched by adding 120 μl of ice-cold quench solution (0.1% TFA), which acidified the sample to a final pH 2.5. The samples were then immediately digested by the pre-activated pepsin on agarose beads. H/D exchange peptides were subsequently separated by HPLC C18 column (Thermo scientific) and then sequentially analyzed by ESI-MS/MS. All steps were performed at 0 °C as previously described [33–35]. Back exchange levels of peptides were calculated by the same peptide after 24-hr H/D exchange as previously reported [33–35]. All of our H/D exchange experiments at each time point is one individual experiment, the correct trend of accumulation of deuteration indicating the technique repeatability of our HDXMS experiments in six experiments. The HDXMS experiments for $tRNA^{phe}$ and ssDNA contain only one set with 6 time points. The H/D exchange data set of $tRNA^{total}$ and control are triplicates to validate our technique.

2.5. Data analysis

Intensity threshold 5000 counts of the peptides were first filtered by Data Analysis 3.4 (Bruker Corporation). Each peptide was identified by the MS/MS analysis. HDexaminer 1.3 (Sierra Analytics) was used for the mass spectra analysis, which is similar to a previous version described [33–35]. Briefly, the results were incorporated into HDexaminer, which retrieved the charges, sequences and retention times from the mass data. The software evaluated the match between the experimental data and the theoretical mass envelopes, and gave the result a score for each peptide fragment. Every mass envelope was further manually examined to make sure the mass envelope identified correctly. Different time points at 0 s, 10 s, 30 s, 100 s, 300 s, 1000 s and 3000 s were calculated individually. The deuteration level of each peptide was obtained by the ratio of the incorporated deuteron number to the maximum possible deuteration number. Because of the fast off-exchange rate of the two N-terminal residues, those residues should not retain any deuterons after liquid chromatography and therefore were not included in calculation.

3. Results

3.1. Peptide map of cyt c

Construction of the peptide map of the pepsin-digested cyt *c* was based on the tandem MS of a quadruple ion trap and Q-TOF. A total of 72 peptides were identified by ion trap MS/MS. Further, Q-TOF