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Quartz crystal microbalance with dissipation and microscale thermophoresis as tools for investigation of protein complex formation between thymidylate synthesis cycle enzymes

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ABSTRACT

Thymidylate synthase (TS) and dihydrofolate reductase (DHFR) play essential role in DNA synthesis, repair and cell division by catalyzing two subsequent reactions in thymidylate biosynthesis cycle. The lack of either enzyme leads to thymineless death of the cell, therefore inhibition of the enzyme activity is a common and successful tool in cancer chemotherapy and treatment of other diseases. However, the detailed mechanism of thymidylate synthesis cycle, especially the interactions between cycle enzymes and its role remain unknown.

In this paper we are the first to show that human TS and DHFR enzymes form a strong complex which might be essential for DNA synthesis. Using two unique biosensor techniques, both highly sensitive to biomolecular interactions, namely quartz crystal microbalance with dissipation monitoring (QCM-D) and microscale thermophoresis (MST) we have been able to determine DHFR–TS binding kinetic parameters such as the K_d value being below 10 μM (both methods), $k_{on}=0.46\times10^4\ M^{-1}\ s^{-1}$ and $k_{off}=0.024\ s^{-1}$ (QCM-D). We also calculated Gibbs free energy as in the order of −30 kJ/mol and DHFR/TS molar ratio pointing to binding of 6 DHFR monomers per 1 TS dimer (both methods). Moreover, our data from MST analysis have pointed to positive binding cooperativity in TS–DHFR complex formation. The results obtained with both methods are comparable and complementary.

1. Introduction

Thymidylate synthase (TS, EC 2.1.1.45), dihydrofolate reductase (DHFR, EC 1.5.1.3) and serine hydroxymethyltransferase (SHMT, EC 2.1.2.1) are enzymes constituting the thymidylate biosynthesis cycle and providing *de novo* synthesized thymidylate that is crucial for DNA synthesis and repair (Carreras and Santi, 1995; Liu et al., 2002; Kisliuk, 2006). Previous studies have shown that lack of TS and DHFR enzymes or inhibition of their activity leads to the thymineless death of a cell, as DNA cannot be synthesized due to the deficiency of thymidylate derivative - deoxythymidine triphosphate (dTTP). Therefore, the enzymes are highly important drug targets in many different disorders including cancer (Wilson et al., 2014).

In most organisms, including human, these enzymes function as distinct proteins, however in protozoa and plants they are encoded by a single gene and expressed as a bifunctional enzyme DHFR-TS with autonomous binding sites, similar to those of monofunctional enzymes. Taking this under consideration, we have suspected that human monofunctional TS and DHFR form an active complex while involved in thymidylate synthesis cycle.

Years of meticulous studies devoted to this cycle highlight its importance and have indicated the possibility that the protein–protein interactions and formation of metabolic complex may be necessary for cells to proliferate. McGaughey et al. (1996), using affinity chromatography, have demonstrated the specific binding between immobilized T4 bacteriophage-encoded dCMP deaminase (the enzyme providing dUMP for TS reaction) and TS, as well as DHFR and several other proteins involved in DNA metabolism and repair. The results obtained by Reddy and Pardee (1980) on mammalian cells were supporting the hypothesis that six enzymes involved in DNA metabolism (TS and DHFR among them) were translocated into nuclei of cells that are about to replicate DNA, and form the complex that was named by the authors “replitase”. The authors suggested that the assembly of the complex possibly signaled the initiation of the S phase of the cell cycle. Finally, recent studies presented by Stover group have shown that in mammalian cells SHMT serves as a scaffold protein translocating

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DHFR and TS to nucleus to assemble the complex required for DNA replication and repair. This translocation was essential to prevent uracil accumulation in nuclear DNA. Moreover, nuclear *de novo* thymidylate synthesis appeared to be critical for maintaining DNA integrity (Anderson et al., 2012; MacFarlane et al., 2011; Woeller et al., 2007). However, in spite of some evidence supporting formation of the complex between TS and DHFR, the detailed mechanism of thymidylate synthesis cycle functioning, especially the nature of protein–protein interactions between particular enzymes remains unknown.

The aim of our studies is to prove the existence of such interactions and fully analyze the human TS–DHFR complex formation, as it most likely is necessary for the DNA synthesis to occur. The obtained data might be of great importance especially in anticancer drug development, where the rising problem is the increase of target enzyme resistance to inhibitor (Banerjee et al., 2002; Volpato and Pelletier, 2009). The elucidation of interactions between thymidylate synthesis cycle enzymes may lead to development of such new category of drugs that could target the DHFR–TS complex formation (instead of either enzyme catalytic activity) and, consequently, cause inhibition of DNA synthesis.

In this paper we present evidence for *in vitro* human DHFR–TS complex formation using unique biomolecular interaction analysis tools. The protein–protein binding has been detected and characterized with the use of two sensitive techniques: quartz crystal microbalance with dissipation monitoring (QCM-D) and microscale thermophoresis (MST).

Quartz crystal microbalance is a thickness shear mode acoustic wave resonator that has become widely popular for studying various biological processes due to its satisfactory performance (Cooper and Singleton, 2007). In this method mass uptake on sensor's surface can be registered as changes of quartz crystal oscillator resonance frequency due to piezoelectric phenomenon (Okahata et al., 2000; Tan et al., 2009). The main advantages of this method include high sensitivity, rapid and facile operation and label-free monitoring (Tan et al., 2009). In this work, a QCM-D was used as a tool not only to detect biomolecular recognition in aqueous solution but also to characterize the binding kinetics of the studied interactions. The viscoelastic layers of water rich protein adsorbents have been characterized using the dissipation mode which allows for monitoring of the energy damping due to frictional movement and conformational changes of the molecules immobilized on the quartz oscillator (Limson et al., 2004; Nicolini et al., 2012).

One of the most common application areas of QCM-D includes the investigation of protein–protein interactions (Cooper and Singleton, 2007; Spera et al., 2013). This technique enables determination of analyzed interactions specificity, kinetics and proteins affinities. Moreover, it is also possible to evaluate the kinetic profile of protein adsorption and associated conformational change on various surfaces (Shen et al., 2001). There have also been reports on using QCM-D for the investigation of adsorption/desorption cycles (Si et al., 2002), protein subunits and peptides screening (Melles et al., 2005), density analysis of proteins (Voros, 2004) as well as solvent and ions influence on protein–protein interactions (Sadik and Cheung, 2001).

Microscale thermophoresis is a free solution technique used for biomolecular interactions studies, that can be carried out in wide range of buffers and biological liquids (Duhr and Braun, 2006; Wienken et al., 2010). It allows a quantitative analysis of protein interactions by exploiting a unique phenomenon of thermo diffusion describing the variable movement of different particles along temperature gradient. The movement of molecules in the generated heat-wave differs according to various molecular properties and additionally further diverge while binding to the second particle. Therefore, unlike the most of biophysical techniques, MST is highly sensitive to alterations of wide variety of molecule parameters occurring during complex formations, from changes in particle mass and charge to modifications in molecule conformation and hydration shell (Seidel et al., 2013). Moreover, as a technique conducted in buffer solution, it enables *in vitro* studies on molecules binding without the need of immobilizing one of the particles, regardless of binding partner size or physical properties.

The MST technique is a highly adequate method for protein–protein interactions studies, since it enables monitoring of the protein complex formation, gives an insight into mechanism of binding and allows determination of the process kinetics. MST can be utilized as a tool for evaluation of protein complexes dissociation coefficient in experiments involving either incubation of purified recombinant proteins (Hallström et al., 2013; Latz et al., 2013), additional incubation of proteins with their cofactors (Jerabek-Willemsen et al., 2011), or cell lysate probed with protein in search of the binding partner (Derler et al., 2013). The method allows for monitoring of the formation of protein dimers, tetramers and higher oligomer structures (Liu et al., 2013). In addition, MST enables measurements of the antibody affinity towards target protein as well as affinity changes upon protein modifications that further lead to elucidation of the exact epitope recognized by a given antibody (Miles et al., 2013).

In this work we combine these two ultra-sensitive and novel techniques as tools for characterizing the protein–protein interactions of the enzymes involved in thymidylate synthesis cycle. To our knowledge, to date there are no reports on complying these two methods together which is a new approach in characterizing the protein–protein complex formation. We show that these tools are complementary for biomolecular analysis and allow characterization of the kinetic parameters of the complex formation.

2. Materials and methods

2.1. Enzymes preparation

Methods for preparation of the recombinant human thymidylate biosynthesis cycle enzymes, as well as the source of recombinant human CK2α protein and lipase Amano AY30 utilized in this paper studies are to be found in Supporting information, Appendices A.

2.2. Quartz crystal microbalance with dissipation monitoring (QCM-D) measurements

QCM-D measurements were carried out using commercially available HisTag capturing sensors (5 MHz, QSX 340, Q-Sense) that consist of chelated Cu^{2+} ions coupled to a passivating PEG background coating. Prior to the experiments the sensor's surface was cleaned according to the manufacturer protocol. The frequency and dissipation variations were monitored using Q-Sense E1 QCM-D (Q-Sense, Sweden) device equipped with a liquid flow cell setup. All experiments were carried out in the following protein buffer solution: 20 mM Tris–HCl (pH 7.6) containing 100 mM NaCl, 10 mM $MgCl_2$ and 0.05% Tween 20 at 200 μl/min flow rate and 25 °C.

2.2.1. In-situ quartz crystal functionalization with HisTag labeled TS

In a typical QCM-D experiment, the receptor molecules are immobilized on the surface of the quartz crystal, while the ligand molecules are freely dissolved in the surrounding solution. In the present work, TS with HisTag label served as a receptor, therefore it was immobilized on the surface of the sensor according to the following procedure.