Available online at www.sciencedirect.com

ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 399–406

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

www.elsevier.com/locate/jpba

Review

Conformational mobility of immobilized proteins

Ruin Moaddel*, Irving W. Wainer

Gerontology Research Center, National Institutes in Aging, National Institutes of Health,
5600 Nathan Shock Drive, Baltimore, MD 21224-6825, United States

Received 17 August 2006; accepted 20 August 2006

Abstract

Cellular membrane fragments have been immobilized on the surface of a silica-based liquid chromatographic support and on the surface of glass capillaries to create immobilized receptor and drug transporter columns. These columns have included phases containing one subtype of the nicotinic receptor (α3β2, α3β4, α4β2, α4β4) and the P-glycoprotein transporter. A key question in the application of these columns to drug discovery and development is the ability of the immobilized receptor or transporter to undergo ligand and/or co-factor induced conformational changes. Using frontal affinity chromatographic techniques and non-linear chromatographic techniques it has been demonstrated that the immobilized nicotinic receptors undergo agonist-induced conformational shifts from the resting to desensitized states with corresponding changes in binding affinities and enantioselectivities. Ligand-induced allosteric interactions and ATP-driven conformational changes have also been demonstrated with the immobilized Pgp stationary phase. The results demonstrate that the immobilized proteins retained their ability to undergo conformational mobility and that this is an attractive alternative to allow for the full characterization of multiple protein conformations.

Keywords: Nicotinic receptor; P-glycoprotein transporter; Conformational activity; Affinity chromatography; Displacement chromatography

Contents

1. Introduction 399
2. Methodologies 400
   2.1. Immobilization of solubilized components of the cellular membranes on the IAM stationary phase 400
   2.2. Immobilization of solubilized components of the cellular membranes on the surface of open tubular columns 400
   2.3. Characterization of the immobilized protein stationary phases by frontal chromatography 401
   2.4. Characterization of the immobilized protein stationary phases by non-linear chromatography 401
3. Nicotinic receptor 402
4. P-glycoprotein transporter (Pgp) 404
5. Conclusion 406
References 406

1. Introduction

In recent years, there has been increased interest in the conformational mobility of proteins as part of drug discovery programs. In the past, little importance was placed on this aspect of binding, but it is now known, that proteins can exist in multiple conformations. Two models dominate, the ligand-induced model and conformational selection model [1]. The ligand-induced model states that the ligand binds to the lowest energy conformation of the protein inducing a conformational change to accommodate the ligand. The second model, the conformational selection model, states that proteins exist in multiple conformations at all times. The ligand then selectively binds to one of the conformations increasing the population of that conformation over the others and subsequently eliciting its response. In any case it has been shown that upon binding of the ligand, the protein-ligand

---

* Corresponding author. Tel.: +1 410 558 8294; fax: +1 410 558 8409.
E-mail addresses: moaddelru@grc.nia.nih.gov, rmoaddel@comcast.net (R. Moaddel).

0731-7085/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jpba.2006.08.021

complex assumes a new conformation that has lower energy than the protein alone.

Conformational mobility appears to be an essential requirement for proper protein function. For example, the binding of a substrate to an enzyme produces conformational changes which lead to the transitional state, and subsequently formation and release of the product [1]. For transmembrane proteins, such as ligand gated ion channels, LGIC, agonist binding produces conformation changes that open an ion channel and facilitate the transfer of ions from one side of the cellular membrane to the other.

The structural requirements for the binding of agonists to target proteins, both on the molecular and protein levels have been extensively studied [1]. The structural requirements for competitive inhibitors, compounds which bind at the agonist binding site but do not activate the receptor, have also been extensively investigated. A third class of ligands, allosteric modifiers, contains compounds that bind at a site or sites on the receptor other than the active site [2,3]. The binding of these compounds produces conformational changes in the protein, which either increase (cooperative allosteric) or decrease (anti-cooperative allosteric) the activity of the agonist. Allosteric modifiers represent a new class of therapeutic agents which have not been extensively studied [4]. A key aspect to the successful exploitation of allosteric modifiers is the understanding and measurement of the conformational changes they produce in the target receptor.

Ligand-induced conformational changes in isolated proteins have been studied using multiple techniques, including the steady state and time-resolved fluorescence, far-UV circular dichroism and NMR relaxation studies [5,6]. Although these methods provide detailed information about the secondary structure and can provide very important functional data, they do not provide extensive binding data for a set of ligands to the different conformations. The spectroscopic techniques, in particular CD have been used to monitor the formation of the drug/protein complex, thus, providing direct data on the binding interaction between the protein and a drug [7]. In these cases, they are looking at gross changes in the molecule. However, if the binding site is small relative to the overall size of the protein, these techniques are unable to detect conformational changes associated with ligand binding, cf. [8].

Currently, quantitative analysis of the immobilized conformation can be carried out, but it often requires immunoprecipitation analysis, SDS-PAGE and Western blotting for conformational analysis, which is time consuming and laborious [9]. Another approach to the study of affect of protein conformation on ligand binding utilizes surface plasmon resonance (SPR). An example of this method is the characterization of the binding properties of the active conformation of the Bax protein, a protein involved in the mitochondrial pathway for apoptosis [9]. In this study, the Bax protein was immobilized using a monoclonal antibody that recognizes only its active conformation and then studied using SPR. However, only the active conformation could be studied in these experiments and the immobilized protein was not conformationally mobile.

An alternative technique for the measurement of ligand-induced conformational changes is bio-affinity chromatography, which utilizes liquid chromatographic stationary phases containing immobilized proteins in a flow system. Initial studies with immobilized human serum albumin (HSA) demonstrated that the chromatographic retention of a compound on the HSA stationary phase correlated with the compound's binding affinity to the immobilized protein [10]. It was also demonstrated that the HSA column could also be used to identify positive allosteric effects on binding affinity, such as the S-warfarin induced increase of the binding affinity of *S*-oxazepam hemmisuccinate [11] and the ibuprofen induced increase in the affinity of lorazepam as well as the negative allosteric effects of *S*-oxazepam on the binding of *R*-oxazepam [12].

A key advantage of bio-affinity chromatography is the ability to use this technique with both cytosolic and transmembrane proteins. Since transmembrane proteins constitute the largest family of therapeutic drug targets, this is a key advantage. In addition, it has been demonstrated that this approach can also be used to investigate induced conformational changes in the immobilized target. This aspect of bio-affinity chromatography will be explored in this review using data from the studies of the nicotinic acetylcholine receptor (a LGIC) and P-glycoprotein (a transmembrane drug transporter).

2. Methodologies

2.1. Immobilization of solubilized components of the cellular membranes on the IAM stationary phase

The amount of cellular membranes used to produce the membrane-column from transfected cells is dependent on the level of expression of the target protein and can range between 10^6 and 10^7 cells [13]. The necessary amount of cells is initially homogenized to lyse the cells. The solution is subsequently centrifuged and the pellet is solubilized overnight in the presence of protease inhibitors, peptidase inhibitors, salts and detergent and in some case in the presence of polyhydroxy compounds for protein stability. The resulting solution is centrifuged to remove insoluble proteins and the solubilized solution is then mixed with immobilized artificial membrane (IAM) particles, which contain phosphatidyl choline head groups. After mixing for 1 h, the suspended particles are dialyzed for a period of 1–3 days, depending on the protein stability in the environment. After dialysis, the particles are collected and washed by centrifugation. The protein-rich stationary phase is then packed into a HR 5/2 column (Amersham Pharmacia Biotech, Uppsala, Sweden) to yield a 150 mm × 5 mm (i.d.) chromatographic bed and washed for a period of at least 6 h before starting the frontal analysis experiments.

2.2. Immobilization of solubilized components of the cellular membranes on the surface of open tubular columns

Cellular membranes can also be directly immobilized on the surface of silica capillaries to create open tubular chromatography columns [14]. This approach utilizes a significantly smaller amount of cells, 10^4 to 10^5 cells, which are homogenized and solubilized in the same manner, with the exception that a much