Journal of Pharmaceutical and Biomedical Analysis 107 (2015) 311–317

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

A comprehensive platform to investigate protein–metal ion interactions by affinity capillary electrophoresis

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ARTICLE INFO

Article history:
Received 5 November 2014
Received in revised form 2 January 2015
Accepted 7 January 2015
Available online 14 January 2015

Keywords:
ACE
Protein
Metal ion
Interaction
Mobility shift

ABSTRACT

In this work, the behavior of several metal ions with different globular proteins was investigated by affinity capillary electrophoresis. Screening was conducted by applying a proper rinsing protocol developed by our group. The use of 0.1 M EDTA in the rinsing solution successfully desorbs metal ions from the capillary wall. The mobility ratio was used to evaluate the precision of the method. Excellent precision for repeated runs was achieved for different protein metal ion interactions (RSD% of 0.05–1.0%). Run times were less than 6 min for all of the investigated interactions. The method has been successfully applied for the interaction study of Li^{+}, Na^{+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Al^{3+}, Ga^{3+}, La^{3+}, Pd^{2+}, Ir^{3+}, Ru^{3+}, Rh^{3+}, Pt^{2+}, Pt^{4+}, Os^{3+}, Au^{3+}, Au^{+}, Ag^{+}, Cu^{1+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cr^{3+}, V^{3+}, MoO_4^{2-} and SeO_3^{2-} with bovine serum albumin, ovalbumin, β-lactoglobulin and myoglobin. Different interaction values were obtained for most of the tested metal ions even for that in the same metal group. Results were discussed and compared in view of metal and semimetal group's interaction behavior with the tested proteins. The calculated normalized difference of mobility ratios for each protein–metal ion interaction and its sign (positive and negative) has been successfully used to detect the interaction and estimate further coordination of the bound metal ion, respectively. The comprehensive platform summarizes all the obtained interaction results, and is valuable for any future protein–metal ion investigation.

1. Introduction

1.1. Protein–metal ion interaction

Approximately 30% of all proteins are metalloproteins, in which metals are important for their function [1]. Therefore, the selectivity of each protein for specific metal ions is of particular interest. The protein properties such as net charge, dipole moment, donating and accepting electrons and the number of potential ligands for metal ions inside a binding site are the major factors affecting the interaction with metal ions [2,3]. The metal ion's valency, atomic radius and charge accepting capacity contribute to the binding of a certain metal ion to its suitable binding sites [2,3]. The metal ion ligands react preferentially with the proteins of the similar overall hardness or softness [4–8]. Therefore, the interaction selectivity can be estimated according to the hard and soft acid and base theory (HSAB) [4–8] (Table 1).

1.2. Analytical techniques for studying protein–metal interactions

When comparing different techniques for investigating protein metal ion interactions, their challenges should be taken into account. The complexity of sample and their special preparation are the most challenging part for each technique. Most of the useful techniques require pure sample for analysis such as X-ray crystallography, nuclear magnetic resonance (NMR), Fourier transform infra-red spectroscopy (FTIR), circular dichroism (CD) spectroscopy, surface plasmon resonance (SPR) and atomic force microscopy (AFM) [9,10]. NMR technique performs with highly concentrated samples, and is restricted for small and soluble proteins (<40 kDa) due to often observed spectral overlaps for large proteins [11]. The preparation of biological crystal for X-ray crystallography is predominately difficult and in some cases impossible [9]. Since most of biological samples are impure, it makes sense to

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http://dx.doi.org/10.1016/j.jpba.2015.01.017
0731-7085/

Table 1
Prediction of the metal ions selectivity to various donor ligands using HSAB concept [4–8].

	Hard[a]	Borderline[a]	Soft[a]
Acids	Class A cations e.g. Li^+, Na^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Al^{3+}, Ga^{3+} High oxidation state of class B cations e.g. V^{3+}, Cr^{3+}, Co^{3+}, Fe^{3+} Lanthanide and actinide cations	Medium oxidation state of Class B cations (almost divalent) e.g. Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pd^{2+}	Low oxidation state of Class B cations (monovalent, heaviest) e.g. Cu^+, Ag^+, Cd^{2+}, Au^+, Hg^+
Base	Carboxylates (glutamate and aspartate), hydroxyl group (serine, theronine, tyrosine), guanidinium (arginine), carbonyl, alcohols, amines, ether, water, nitrate, sulphate, phosphate, carbonate, etc.	Imidazole (histidine), amides (asparagine, glutamine), nitrogen of the peptide bond, indole (tryptophan), pyrrole (porphyrin), nitrite, azides, nitrogen gas. Pyridine, aniline, chloride, etc.	Thiols (cysteine), thioethers (methionine), phenyl (phenylalanine), ethylene, cyanide, etc.

[a] Acids with the same charge become softer (less hard) as a radius increases. Acids become harder (or less soft) as a charge (oxidation state) increases.

discuss and compare the two most powerful techniques with high separation efficiency for this purpose, namely affinity chromatography [12] and affinity capillary electrophoresis (ACE) [13–15]. The affinity chromatography and their sub-techniques suffer from the disadvantages that a large amount of sample and materials are needed and the high cost of special affinity columns [12]. Recently, ACE was successfully used to investigate the interaction of ovalbumin isoforms and other proteins with different metal ions [13–15], the ability of this technique to investigate each isoform and separating a complex sample components make it more attractive than other techniques. Mass spectrometer provides binding information through m/z of target biomolecule before and after binding with ligand [16,17]. Nonetheless, direct infusion mass spectrometry (DIMS) could provide unreliable interaction information due to four main problems, (1) different electrospray ionization efficiency of each compound presents in a biological mixture, (2) lack of separation increase the competition between components to bind with a ligand, (3) low stability of some protein–metal complexes in the gas phase, (4) irrelevant binding properties in the gas phase [16,18]. Therefore, MS might sometimes show different binding behavior compared to other techniques. Therefore, ACE–UV can be considered as the first choice to investigate the protein–metal interactions also due to its moderate costs for reagents and instrumentation.

1.3. ACE

ACE can be classified into three main modes, dynamic equilibrium, pre-equilibrated and kinetic method. In the dynamic equilibrium mode, the relaxation time of the equilibrium is short with respect to the separation time. In the pre-equilibrated mode, solute and ligand are mixed outside the capillary to allow for equilibration and then they are introduced for separation. This mode is only suitable if the separation time is shorter than the relaxation time of the equilibrium. In between these two modes is the kinetic method [19].

In this study, one of the dynamic equilibrium ACE methods, namely the mobility shift-ACE, was used since most of the protein–metal ion interactions have short equilibrium relaxation time [13,14]. In-capillary protein–metal interaction will take place after injecting a sample containing protein into a capillary containing running buffer with metal ions.

The mobility ratio can be determined by the following equation [20]:

$$R = \frac{t_{EOF}}{t_{prot}} \tag{1}$$

where t_{EOF} is the migration time of the EOF marker and t_{prot} is the migration time of a protein. Please note that R is the ratio of the mobilities μ_{prot}/μ_{EOF} at the same time, since mobility μ is defined as lL/Ut, where l and L are the effective and the total length, respectively, and U is the applied voltage. All these parameters apart from the migration time t are the same for the protein and the EOF and hence can be canceled.

The mobility shift-ACE method offers fast screening of interaction depending on the electrophoretic mobility ratio (with respect to EOF marker) of a solute (protein) interacting with and without a ligand (free of metal ion) in the running buffer, R_i and R_f, respectively.

Changes in charge, mass and size of a protein after interaction with a metal ion can be determined by using the normalized difference of mobility ratios ($\Delta R/R_f$), where $\Delta R = R_i - R_f$ [13,14]. A confidence interval (*cnf*) of $\Delta R/R_f$ can be calculated using the following equation:

$$\mathrm{cnf}\left(\frac{\Delta R}{R_f}\right) = \left(\frac{\Delta R}{R_f}\right) \pm \frac{\left(t_{\alpha/2,\ n_1+n_2-2} \times \hat{\sigma}_{total} \times \sqrt{2/n_1 + n_2}\right)}{R_f} \tag{2}$$

$t_{\alpha/2}$ is the t-value of the probability 0.975 ($\alpha/2 = 0.025$) for a given degree of freedom. The n_1 and n_2 are the two data numbers of the series to estimate R_i and R_f. The value $n_1 + n_2 - 2$ is the degree of freedom. The total standard deviation of the two series data of R_i and R_f is shown in Eq. (3), where f_1 and the f_2 are the numbers of freedom $(n-1)$ while $\hat{\sigma}_1$ and $\hat{\sigma}_2$ are the standard deviations of the two series data for R_i and R_f, respectively.

$$\hat{\sigma}_{total} = \sqrt{\frac{2 \times \left(f_1 \times \hat{\sigma}_1^2 + f_2 \times \hat{\sigma}_2^2\right)}{(f_1 + f_2)}} \tag{3}$$

A $\Delta R/R_f$ absolute value ≥ 0.01 is typically sufficient to indicate significant interactions. Furthermore, the *cnf* of $\Delta R/R_f$ should not intersect the zero line for a significant interaction. The $\Delta R/R_f$ values can be positive or negative depending on the formed complex. Normally, the overall charge of a protein could get less negative after binding with a metal ion and thus can easily be detected by positive $\Delta R/R_f$ value. In another case, the bound metal ion on a protein could further bind coordinatively with the surrounding anions of the buffer leading to a more negative overall charge of the protein. This can then be detected by negative $\Delta R/R_f$ values.

The aim of this work was to study the influence of different metal ion groups on the various globular proteins using ACE–UV and provide reference values as well as a comprehensive, generic platform to characterize metal ion interactions in general.

2. Materials and methods

2.1. Chemicals and reagents

Bovine serum albumin (BSA, 99%), human serum albumin (HSA, 97%), ovalbumin (OVA, 98%), β-lactoglobulin (β-LG, 85% bovine milk) and myoglobin (MB, 90%), also $CrCl_3{\cdot}6H_2O$, $CoCl_2{\cdot}6H_2O$, CuCl, $GaCl_3$, AuCl, $AuCl_3$, $IrCl_3$, LiCl, $NiCl_2{\cdot}6H_2O$, $OsCl_3$, Pd $(NO_3)_2{\cdot}2H_2O$,