Molecular Immunology 46 (2009) 2395–2402

Contents lists available at ScienceDirect

Molecular Immunology

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

Short communication

Thermodynamic characterization of the interactions between the immunoregulatory proteins osteopontin and lactoferrin☆

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ARTICLE INFO

Article history:
Received 24 February 2009
Accepted 16 April 2009
Available online 23 May 2009

Keywords:
Lactoferrin
Osteopontin
Immunity
Inflammation
Cytokines
Immunodeficiency diseases
Calcium
Iron
Isothermal titration calorimetry
Differential scanning calorimetry
ITC
DSC

ABSTRACT

The immunoregulatory proteins osteopontin (OPN) and lactoferrin (LF) are both highly abundant in milk, with a conserved ratio between different mammalian species, suggesting that the role of each protein in infant development may be linked. In this study we used isothermal titration calorimetry and differential scanning calorimetry experiments to demonstrate that LF and OPN interact with each other through a complex mechanism involving multiple cationic LF molecules binding to a single anionic molecule of OPN. At least two classes of thermodynamically distinct LF binding sites were identified, with the higher affinity interactions (dissociation constants 10^{-6} M) being in the biologically relevant range. Ca^{2+} binding to OPN, or Fe^{3+} binding to LF, influenced the enthalpy and entropy of binding, but had little effect on the overall binding affinity. Considering that the regions of electrostatic complementarity between OPN and LF mediate the numerous biological functions of each protein, we suggest that OPN may act as a carrier protein for LF in milk, and modulate the potent antimicrobial and immunostimulatory activities of the LF protein.

1. Introduction

Human milk contains a wide variety of hormones, growth factors and other biologically important proteins with immunostimulatory or antimicrobial activities that help protect infants against infection. Many of these proteins are found in significantly higher concentrations in human milk than in bovine milk, suggesting that they are likely to be of particular importance in the innate immune defense and the development of the human infant (Hosea Blewett et al., 2008; Lonnerdal, 2003).

For example, the 80 kDa glycoprotein lactoferrin (LF) is present at levels of more than one gram per litre in human milk, which is roughly a ten-fold higher concentration than that found in bovine milk (Masson and Heremans, 1971). A major host defense protein and important component of the innate immune system, LF has a large number of biological activities including antibacterial, antiviral, antifungal, antitumor and anti-inflammatory activities (Pan et al., 2007; Ward et al., 2005; Legrand et al., 2008). In addition, LF can regulate the formation and resorption of bone (collectively known as bone remodeling), by stimulating osteoblasts and inhibiting osteclasts (Cornish et al., 2004, 2006). Several of LF's biological activities are associated with the protein's ability to bind Fe^{3+} with extremely high affinity ($K_a \sim 10^{20}$ M^{-1}), which can starve microorganisms of the metal that is required for metabolism and growth (Baker and Baker, 2004). Like other members of the transferrin protein family, LF binds one Fe^{3+} ion to each of its two homologous domains, the N-domain and C-domain. Several Fe^{3+}-independent modes of action have also been described for LF, which involve the interaction of the highly cationic (pI ~ 9.0) LF protein with negatively charged biological membranes, glycosaminoglycans, or DNA (Fleet, 1995; Valenti and Antonini, 2005). Human LF has recently also been shown to act as an 'alarmin' that promotes the recruitment and activation of leukocytes and dendritic cells (de la Rosa et al., 2008). In addition, LF can be proteolytically cleaved by pepsin under the acidic conditions of the stomach to generate the 25 amino acid peptide fragment lactoferricin, which retains very similar antimicrobial,

Abbreviations: OPN, osteopontin; LF, lactoferrin; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry.

☆ This work was supported by a CIHR emerging team grant in the area of novel alternatives for antibiotics. The calorimetry equipment used was purchased through grants provided by the Canada Foundation for Innovation and the Alberta Science and Research Authority. H.J. Vogel holds a Scientist award from the Alberta Heritage Foundation for Medical Research.

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doi:10.1016/j.molimm.2009.04.024

antiviral, antitumor and immunological properties to those of the intact protein (Gifford et al., 2005).

It was recently shown that the cytokine osteopontin (OPN, also known as secreted phosphoprotein 1, bone sialoprotein 1, early T-lymphocyte activation protein 1, nephropontin, uropontin) is also present at roughly ten-fold higher concentrations in human milk than in bovine milk (Nagatomo et al., 2004; Sorensen et al., 2001). OPN is a 36 kDa, anionic (pI ~ 3.6) predominantly unstructured (Fisher et al., 2001) phosphorylated glycoprotein (Christensen et al., 2005; Sorensen et al., 1995). Like LF, expression of OPN is up-regulated in response to inflammation, infection and cancer (Johnston et al., 2008; Scatena et al., 2007), and the protein plays an important role in bone remodelling (Alford and Hankenson, 2006). The biological activities of OPN are generally achieved through direct interaction with cell surface receptors such as CD44 and integrins, which regulate the migration, adhesion, survival, differentiation and activation of monocytes/macrophages, T cells, and several types of cancer cells (Kazanecki et al., 2007). Recently OPN was also shown to play a role in mast cell degranulation (Bulfone-Paus and Paus, 2008; Nagasaka et al., 2008). Integrin binding by OPN is mediated by the common RGD integrin recognition motif, as well as by an adjacent SVVYGLR motif (Weber et al., 1996; Yokosaki et al., 1999). Thrombin cleavage at a site immediately following the integrin-binding motifs is commonly observed *in vivo*, and generates an N-terminal fragment of OPN that is capable of promoting enhanced cellular responses (Senger and Perruzzi, 1996). In addition, OPN binds to Ca^{2+} and hydroxyapatite, which are important interactions for the regulation of bone remodelling (Boskey, 1995; Chen et al., 1992).

In spite of its abundance, the role of OPN in milk is currently not well characterized. Interestingly, Kanno and co-workers recently used OPN-affinity chromatography to identify LF as a potential OPN binding partner in milk (Azuma et al., 2006). The parallel immunoprotective functions of OPN and LF, the high degree of homology between the human and bovine proteins, and the relative conservation of their protein ratios in bovine milk (OPN ~ 15 mg/L, LF ~ 150 mg/L) and human milk (OPN ~ 150 mg/L, LF ~ 1500 mg/L) suggests that an interaction between OPN and LF could be biologically important. To gain insight into the details of this interaction, we have examined the binding of OPN to LF in vitro using isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) experiments. ITC measures the heat that is generated or absorbed when two proteins bind to each other, and provides accurate information on the dissociation constants and the stoichiometry of the interaction (Perozzo et al., 2004). DSC measurements reveal the increase in the denaturation temperature of a protein when it interacts with a ligand or another protein (Bruylants et al., 2005). In addition to the high sensitivity of ITC and DSC, both techniques can characterize the binding between proteins free in solution, without a need to covalently attach any probes that could potentially influence the interaction. Collectively our ITC and DSC data reveal a complex interaction involving multiple LF molecules binding to a single molecule of OPN, with affinities in the biologically relevant range of $\sim 10^{-6}$ M. Investigations of the Ca^{2+}- and Fe^{3+}-dependence of these interactions have provided further insights into the potential role of OPN and its binding to LF in milk and other biological compartments where these two proteins are co-localized at high concentrations.

2. Materials and methods

2.1. Proteins

Bovine lactoferrin (LF) was purchased from Sigma (Sigma-LF), and shown to contain a mixture of iron-free LF (apo-LF), iron saturated LF, $Fe^{3+}{}_2$-LF (holo-LF), and partially Fe^{3+}-saturated LF ($Fe^{3+}{}_1$-LF), see the DSC results section. To prepare homogeneous samples of apo-LF, or holo-LF for use in ITC and DSC experiments, Sigma-LF was treated by "iron-removal" or "iron saturation" protocols. Briefly, apo-LF was prepared by dialyzing Sigma-LF in 100 mM citrate overnight at room temperature to remove Fe^{3+} from the protein, followed by extensive dialysis against 10 mM ammonium bicarbonate and lyophilization. Holo-LF was prepared by dissolving Sigma-LF in 50 mM Tris (pH 7.5), 3 mM $FeCl_3$, 3 mM nitrilotriacetic acid, 3 mM sodium bicarbonate ($NaHCO_3$), and 100 mM KCl to a concentration of 10 mg/mL and incubating the mixture at room temperature for 18 h, followed by extensive dialysis against 10 mM ammonium bicarbonate, and lyophilization. Each procedure resulted in the production of more than 99% pure apo-LF or holo-LF samples as judged by the DSC data, see the results section below. The concentration of LF was determined using the extinction coefficients, $\varepsilon_{280} = 102{,}890\ M^{-1}\ cm^{-1}$ for apo-LF, and $\varepsilon^{1\%}{}_{465} = 0.58$ for holo-LF (Mazurier and Spik, 1980).

Bovine OPN was purified to >95% from the acid whey fraction of fresh cows milk by fractionation on a Q-Sepharose Big Beads (Amersham) chromatography column. SDS-PAGE, gel filtration chromatography, and matrix assisted laser desorption ionization mass spectrometry experiments showed that the final OPN sample consisted of a mixture of ~20% full-length OPN, and ~80% thrombin-cleaved OPN, with mass-spectrometry-determined sizes near 36.3 kDa and 23.7 kDa respectively. The cleavage of OPN occurs in the cow's udder prior to purification, and the two fragments could not be separated during purification due to their similar chemical properties. The concentration of OPN was determined in terms of the common region shared by the truncated and full-length forms of the protein, since both forms of OPN contain all of the UV–visible Trp and Tyr residues (Y20, W27 and Y142), with a predicted molar extinction coefficient of $\varepsilon_{280} = 8480\ M^{-1}\ cm^{-1}$.

2.2. Isothermal titration calorimetry

For Ca^{2+}-binding experiments, lyophilized OPN was dialyzed in 20 mM HEPES, 100 mM NaCl, pH 7.5 over 48 h at room temperature. The dialyzed protein was diluted to a concentration of 50 μM with dialysis buffer, and then titrated with 5 mM CaCl2 in 20 mM HEPES, 100 mM NaCl, pH 7.5 at 25 °C using a MicroCal VP-ITC microcalorimeter (MicroCal, Northampton, MA). The heat of dilution of Ca^{2+} titrated into buffer was shown to be negligible in separate control experiments. Data processing and analysis was performed using MicroCal Origin version 7.0 software. Ca^{2+}-binding data were best fitted using the "one set of sites" Origin fitting model, with the three fitting parameters, stoichiometry (N), enthalpy (ΔH) and association constant (K_a) each allowed to float during the curve fitting. The fitted Ka values were converted to the reported K_d values using the relationship $K_d = 1/K_a$, and the reported values represent the average and standard deviation of three independent titrations.

For LF-binding experiments, apo-LF or holo-LF and OPN were dialyzed in 20 mM HEPES, 100 mM NaCl, 10 mM EDTA for "apo-OPN" experiments, or 20 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$ for "Ca^{2+}-OPN" experiments. "Forward titrations" consisted of the sequential injection of 270–300 μM apo-LF or holo-LF into 10–15 μM OPN, in the respective Ca^{2+} or EDTA containing dialysis buffer at 25 °C. In reverse titrations, 300 μM Ca^{2+}-OPN was titrated into 30 μM apo-LF or holo-LF, or 300 μM apo-OPN was titrated into 50 μM apo-LF or holo-LF at 25 °C. The heat of dilution of LF or OPN was negligible under all conditions as determined in separate control experiments. The simplest best fit for each data set was obtained using the "two sets of sites" model supplied in the Origin 7.0 software. All parameters (N, ΔH, and K_a) were allowed to float during the fitting procedure for apo-OPN experiments, but the N value for the second binding event in the Ca^{2+}-OPN experiments was fixed at 1.0 due to the low affinity of the interaction. The ΔH