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Ligand binding complexes in lipocalins: Underestimation of the stoichiometry parameter (n)

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ABSTRACT

The stoichiometry of a ligand binding reaction to a protein is given by a parameter (n). The value of this parameter may indicate the presence of protein monomer or dimers in the binding complex. Members of the lipocalin superfamily show variation in the stoichiometry of binding to ligands. In some cases the stoichiometry parameter (n) has been variously reported for the same protein as mono- and multimerization of the complex. Prime examples include retinol binding protein, β lactoglobulin and tear lipocalin, also called lipocalin-1(LCN1). Recent work demonstrated the stoichiometric ratio for ceramide:tear lipocalin varied (range n = 0.3–0.75) by several different methods. The structure of ceramide raises the intriguing possibility of a lipocalin dimer complex with each lipocalin molecule attached to one of the two alkyl chains of ceramide. The stoichiometry of the ceramide-tear lipocalin binding complex was explored in detail using size exclusion chromatography and time resolved fluorescence anisotropy. Both methods showed consistent results that tear lipocalin remains monomeric when bound to ceramide. Delipidation experiments suggest the most likely explanation is that the low 'n' values result from prior occupancy of the binding sites by native ligands. Lipocalins such as tear lipocalin that have numerous binding partners are particularly prone to an underestimated apparent stoichiometry parameter.

1. Introduction

Members of the lipocalin superfamily are linked by structural homology. Eight antiparallel β-strands form a fold or calyx, which accommodate small hydrophobic ligands (Fig. 1). Accurate quantification of binding generally requires calculation of the stoichiometry binding parameter, 'n'. In the analysis of binding studies for lipocalins, 'n' is often not reported [1–4]. In other reports the assumption is made that the association reaction is bimolecular and that the ligand binds to a single binding site, i.e., n = 1 [1,5–7]. This is a reasonable assumption in cases where the binding site has been well studied for the particular ligand. This may not be the case for heterogeneous ligands and binding sites especially in situations where multimeric complexes are formed. For example lipocalins are known to form higher order oligomers [8–10]. For apolipoprotein-D, crustacyanin and β lactoglobulin the oligomeric state has been linked to ligand binding functions [11,12]. Apolipoprotein-D was initially claimed as monomeric. Later dimerization was discovered when the protein binds lipids through conversion of Met 93 to methionine sulfoxide [13,14]. Lipocalins may form transient multimeric complexes induced by ligand binding, changes in pH, as well as increases in concentration [15–17]. The importance of an accurate stoichiometry parameter is evident in controversies over multimerization in the lipocalin family. β lactoglobulin and tear lipocalin (LCN1) have both been variably reported to exist as monomers and dimers in solution and crystal form [18–24]. Several authors noted that tear lipocalin (monomer mass = 17,446) [25] elutes in size exclusion chromatography at the apparent molecular mass for a dimer complex and even tetrameric complexes [21,26]. Gouveia and Tiffany suggested that apo-lipocalin was monomeric but holo-lipocalin was dimeric implying ligand induced dimer formation [22]. Later, multi-angle laser light scattering and rotational time constants (measured by both fluorescence anisotropy and electron paramagnetic resonance) indicated that both holo- and apo-tear lipocalin generally exist as monomers with a small percentage of dimer [24]. Most published studies of ligand binding in the lipocalin family assume or show a stoichiometric parameter of one [1,5–7,27–36]. However in the case of tear lipocalin calculations may be lower. Redl et al. calculated 0.3 for the ratio of retinol to tear lipocalin at saturation [21]. A recent study with ceramide revealed stoichiometric ratios of TL:ceramide ligand from n = 0.32–0.67, accompanied by a very low dissociation constant [37].

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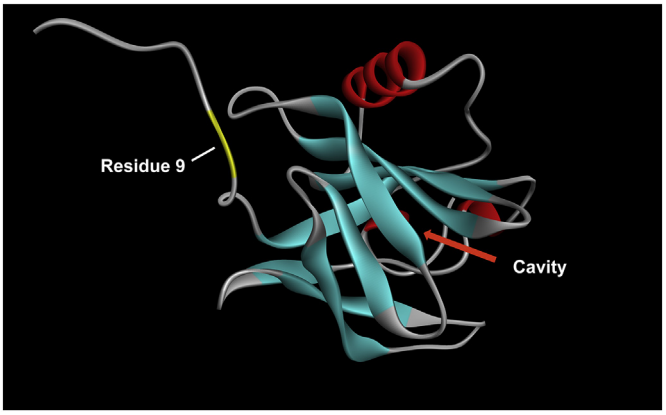

Fig. 1. Cartoon of the backbone structure of tear lipocalin showing the 8 antiparallel β strands that form the lipocalin fold. Orange arrow indicates cavity entrance. Residue 9 from the N terminus was chosen for the cysteine substitution because of its exposed position. (modified from published solution structure of tear lipocalin) [42].

Several explanations are possible including the formation of a multimeric complex with 2 lipocalin molecules flanking the 2 alkyl chains of the ceramide or a binding site pre-occupied by other ligands. The current study sought an explanation for the low 'n' in this case with an overall analysis of the lipocalin family.

2. Materials and Methods

2.1. Reagents

C18-ceramide (ceramide- N-(octadecanoyl) sphing-4-enine, also known as N-Stearoyl-D-erythro-sphingosine), was obtained from ACROS Organic (Pittsburg, PA). C6-NBD ceramide (N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-sphingosine) and C12-NBD ceramide (N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] dodecanoyl]-D-erythro-sphingosine) were obtained from Avanti Polar Lipids (Alabaster, AL). 4-Chloro-7-nitrobenzofurazan (NBD−Cl) and dimethyl sulfoxide (DMSO) were obtained from MilliporeSigma (St.Louis, MO).

2.2. Collection of Human Tears

Stimulated human tears were collected from healthy volunteers in accordance with the tenets of the Declaration of Helsinki and approved by the institutional review board. Informed consent was obtained from donors after explanation of the nature and possible consequences. Collection was performed as previously published [38–40] with polished glass tips and glass transfer pipettes. Tears were pooled in polytetrafluoroethylene-lined glass vials and stored under nitrogen at −80 °C until use.

2.3 Size-exclusion chromatography.

Fractionations of proteins, including tears, synthetic dimers and ligand binding complexes, were performed by gel filtration liquid chromatography using the AKTA purifier Versatile FPLC with a HiLoad Superdex 75 column (GE Healthcare, Piscataway, NJ). In general, one ml column fractions were collected with elution in 50 mM Tris-HCl, 100 mM NaCl pH 8.4 run at 1 ml/min as previously described [37]. For purification of tear lipocalin monomers peak fractions from gel filtration of 1 ml of tears were pooled and applied to an anion exchange column using DEAE-Sephadex A-25. Purity was confirmed by tricine polyacrylamide gel electrophoresis as published [40,41]. The appropriate absorbance wavelengths for NBD (480 nm) and protein (280 nm) were monitored simultaneously for analyzing NBD-derivatized proteins and NBD ligand complexes as necessary.

2.3. Expression of tear lipocalin and construction of tear lipocalin dimer

Tear lipocalin, wild type, was expressed and purified as previously described [23,25]. The free cysteine at residue position 101 in native tear lipocalin is buried in the G strand and is relatively inaccessible to chemicals for derivatization [25,42]. Tear lipocalin was altered by site directed mutagenesis to create a single free cysteine in the exposed portion (amino acid position 9) of the A strand (Fig. 1). The cysteine at position 101, was replaced with leucine as previously published [25]. A dimer of tear lipocalin was constructed by crosslinking the cysteine substituted monomers with 1,11-bismaleimido-triethyleneglycol, BM (PEG)3, (Thermo Scientific™ Pierce) according to the manufacturer's protocol. The product was a cysteine linked three unit polyethylene glycol spacer arm joining two tear lipocalin molecules. The reaction in brief included TL, 0.3 mM final concentration, incubated for 16 h 37 °C with a three fold molar excess of BM(PEG)3, 0.9 mM, final concentration in 10 mM Na−P, 130 mM NaCl, 5 mM EDTA pH 7.0. Formation of the dimer was confirmed with tricine gel electrophoresis stained with Coomassie blue followed by silver nitrate as previously described [40]. The crosslinker added 352 Da of net mass to the two monomers. Fractions, containing dimer were combined, concentrated on 10,000 MW cutoff centrifugal membrane filter by centrifugation at 14,000 *g* for 30 min.

2.4. Tear lipocalin labeling with NBD

Recent studies have indicate that at pH 7.0 NBD-Cl preferentially labels the N terminus of proteins [43]. In an attempt to verify specific labeling, NBD-Cl was reacted with both monomeric and dimeric tear lipocalin. A 900 ul reaction mixture comprised of tear lipocalin (10 μM) and NBD-Cl (100 μM) was incubated in 10 mM Na−P buffer at pH 8.0 overnite at 37 °C as previously described for NBD labeling of proteins [44]. In addition, identical reactions were run at pH 6.2 in sodium citrate buffer, pH 7.0 in sodium phosphate buffer. The reaction mixture was concentrated with a centrifugal membrane filter (Amicon 10 m 10,000 MW cutoff) and washed with 5 × 500 μl of the respective buffers. The final retentate was adjusted to 800 μl in their respective buffers. Labeling efficiency was estimated from absorbance spectrophotometry using the molar absorptivity, ε, for NBD-lysyl residues and TL at λ = 480 and λ = 280 nm as 26,000 and 13,760 M-1 cm-1 respectively [37,45,46]. The molar ratio of NBD label/tear lipocalin was multiplied by 100 to determine percent efficiency. The dimer was considered to have 2 fold the moles of tear lipocalin. .

2.5. Delipidation of tear lipocalin

Lipids were extracted from purified native tear lipocalin (0.8 ml, 5 mg/ml) using chloroform/methanol extraction as previous published [41]. C6-NBD labeled ceramide bound to tear lipocalin bound was extracted in parallel and served as a control. Delipidation was confirmed by the disappearance of fluorescence at 523 nm in the control sample (see Section 2.7.1). Two extractions in series were required. .

2.6. Spectroscopy

2.6.1. Absorption spectrophotometry

Absorption spectrophotometric measurements were obtained with a Shimadzu UV-2401PC instrument, (Kyoto, Japan). The concentrations of proteins and fluorescent lipids were calculated from molar extinction coefficients that have been published [37].

2.6.2. Steady State Fluorescence

Fluorescence measurements were made at 20 °C in a Jobin Yvon-SPEX Fluorolog-3 spectrofluorimeter (Jobin Yvon, Edison, NJ); bandwidths for excitation and emission were 2 and 4 nm, respectively. For NBD measurements, the excitation wavelength was 465 nm. Raman and