Analytical Biochemistry 433 (2013) 19–27

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

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

Quantitative analysis of affinity enhancement by noncovalently oligomeric ligands

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ARTICLE INFO

Article history:
Received 21 May 2012
Received in revised form 30 September 2012
Accepted 3 October 2012
Available online 12 October 2012

Keywords:
Receptor–ligand interactions
Noncovalent interactions
Self-association
Oligomerization
Multivalency
Linkage
Binding models
Numerical analysis

ABSTRACT

Designed ligands that self-assemble noncovalently via an independent oligomerization domain have demonstrated enhancement in affinity for a variety of chemical and biological targets. To better understand the thermodynamic linkage between enhanced receptor binding and self-assembly, we have developed linkage models for the three commonly encountered types of noncovalently oligomeric ligands: homofunctional oligomeric ligands, heterodimeric ligands that target a single receptor, and bispecific ligands that crosslink noninteracting receptors. Expressions and numerical approaches for exact analysis as a function of total ligand concentrations are provided. We apply the linkage models to the binding data for two published noncovalently oligomeric ligands: one targeting a small molecule (phosphocholine) and the other targeting a soluble protein (tumor necrosis factor α). The linkage models provide a quantitative measure of the potential and realized enhancement in affinity that could inform and guide design optimization efforts, and they reveal physical insight that would elude model-free analysis. Incorporation of the linkage models, therefore, is expected to be valuable in the rational engineering of noncovalently oligomeric ligands.

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Considerable interest in bioconjugate research is focused on receptor-targeting ligands that mimic monoclonal antibodies (e.g., target specificity, multivalency, recruitment of cytotoxic immune responses) while incorporating self-assembly and novel functionalities not associated with native antibodies. These systems commonly use self-assembling substrates (e.g., polypeptides, polynucleotides, phospholipids) that present one or more types of binding moieties in a multivalent manner. An important class of such polymers consists of oligonucleotides and oligomerizing peptides, whose stoichiometry, stability, and specificity are encoded in the primary sequence. They offer potential advantages over other chemical and polymeric systems in terms of defined stoichiometry, monodispersity, and chemical homogeneity (particularly if produced by recombinant techniques) [1]. By modularizing the oligomerization domain with respect to the binding moiety, noncovalently oligomeric ligands afford a level of design flexibility not accessible to native antibodies and many other chemical or polymeric bioconjugate systems.

The proof-of-concept of this type of design was a dimeric "miniantibody" in which single-chain variable fragments (scFvs)[1] were fused to a dimerization domain [2]. Many other examples, harboring a variety of ligands, have since followed [3–12]. In addition, several groups have extended this concept by incorporating different receptor-binding moieties to yield bispecific ligands [4–6] as well as combining with phage display technology to generate novel multivalent ligands to tumor markers [13,14]. Thermodynamically, the oligomeric stability of a multivalent ligand is expected to contribute to the observed affinity, and vice versa, in a linkage fashion. Although the quantitative treatment of thermodynamic linkage between binding and self-assembly is well established [15–18] and applied to various areas of inquiry such as hemoglobin [19,20] and protein–DNA interactions [21–23], no explicit treatment of noncovalently oligomeric bioconjugate systems currently exists. Specifically, there is a dearth of attention to the quantitative contribution of the oligomerization domain to the observed affinity and gain in biological activity. Instead, the importance of valency of the noncovalent scaffold and the conformational stability of the receptor-binding moiety (in the case of scFvs) are generally emphasized [24–29]. The lack of quantitative analysis on the role of oligomeric stability represents a gap in their development. This is evidenced by published implementations that exhibit only modest affinity enhancement [10,30]. In one case, the tetrameric ligand exhibits lower apparent affinity than a dimeric one bearing the same receptor-targeting moiety [11]. Although strong affinity enhancements have been obtained using cystine-linked scaffolds [4,13,14], engineered disulfides often result in unintended crosslinking and misfolding of recombinant proteins [7,31] and may even be deleterious to receptor binding [2]. If oligomeric stability is a major contributor to receptor affinity and enhancement of biological activity, informed engineering of systems with appropriate oligomeric stability could significantly advance the development of noncovalently oligomeric ligands.

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[1] *Abbreviations used:* scFv, single-chain variable fragment; ELISA, enzyme-linked immunosorbent assay; p53tet, wild-type p53 tetramerization; TNFα, tumor necrosis factor α.

0003-2697/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2012.10.005

Here, we model the quantitative linkage between self-assembly of an oligomeric ligand and receptor binding. We address the three types of noncovalent ligands commonly encountered in the literature: homotypic ligands, heterospecific ligands that target the same receptor, and bispecific ligands that crosslink noninteracting receptors. Finally, we use a linkage model to analyze previously published data to gain physical insight into the properties of two designed ligands. Availability of these models should facilitate the analysis and design of noncovalently oligomeric ligands that effectively realize their potential for affinity enhancement.

Materials and methods

Numerical root-finding

Models are formulated as generally described by Wells [32]. When expressed in terms of total ligand concentration, the models are usually intractable to analytical solutions. Models in which the dependent variable cannot be solved analytically are formulated as systems of n nonlinear equations to be solved by numerical techniques of root-finding:

$$\mathbf{y} \equiv \begin{bmatrix} f_1(\mathbf{x};\mathbf{a}) = 0 \\ f_2(\mathbf{x};\mathbf{a}) = 0 \\ \vdots \\ f_n(\mathbf{x};\mathbf{a}) = 0 \end{bmatrix}, \tag{1}$$

where **x** and **a** represent the vectors of variables and parameters, respectively. For ease of subsequent manipulations, we generally choose **x** to be the unbound ligand and receptor species. One-dimensional and multidimensional root-finding was performed using routines from the NAG C Library (Numerical Algorithms Group, Oxford, UK). These routines combine iterative procedures based on bracketing and bisection with Newton's method. Convergence was typically taken when $|x_{i+1} - x_i| < \sqrt{\varepsilon}$, where $\varepsilon \sim 10^{-16}$ is the machine precision.

Nonlinear regression

For the purpose of model-fitting, reported ligands were selected on the basis of completeness of binding data required for application of the appropriate model as described in the text. In general, this includes companion data for the monomeric counterpart and/or a covalently linked analogue. Data in print were digitized at the highest available resolution. Data were fitted by nonlinear least-square minimization using Origin (Origin Lab, Northampton, MA, USA) on the same scale (logarithmic or linear) as presented in the source. Global fitting of multiple data sets was performed where possible with assigned shared parameters as described in the text. Fitted estimates of parameters $\hat{a}_i$ are given with 95% joint confidence limits as prescribed by the F test method for joint parameters [33]. For each parameter, we search for values of a_i such that

$$SS(a_i) = SS(\hat{a}_i)\left(1 + \frac{p}{n-p}\right)F^{\alpha}_{p,n-p}, \tag{2}$$

where SS is the residual sum of squares and $F^{\alpha}_{p,n-p}$ is the upper critical value at $\alpha = 0.05$ of the $F_{p,n-p}$ distribution (n data points, p parameters).

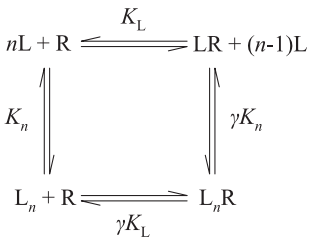
Results

Noncovalently oligomeric ligands typically employ oligomerization domains that undergo coupled folding and association in a two-state transition at equilibrium (Fig. 1A and B). The resultant enhancement in affinity for the target receptor relative to its monovalent counterpart may occur by several distinct mechanisms. One is classical avidity through binding to proximate receptors immobilized on a surface (e.g., cell membrane, chromatographic support, plastics; see Fig. 1C) or to nonoverlapping sites on a single receptor (Fig. 1D). Even in binding to a single sparse receptor, a multivalent ligand exhibits an avidity-like effect by presenting a locally high concentration of ligand near the receptor (Fig. 1E). Receptors that otherwise have no affinity for each other may be crosslinked by incorporation of distinct specificities (Fig. 1F), a concept realized in bispecific antibodies. These examples highlight the diversity embodied in the definition of "receptor" for a multivalent ligand. It may range from a single macromolecule to a section of cell membrane or artificial surface onto which targeted macromolecules are anchored. To avoid ambiguity, we borrow from the study of antibodies the terms "epitope" and "paratope" with reference to a binding site (on the receptor) and a binding moiety (presented by the ligand), respectively. The term "ligand" is reserved for designating the total molecule (monomeric and oligomeric).

Our goal is to establish the linkage relationships between the self-assembly for various classes of noncovalently oligomeric ligands and the resultant increase in affinity for their receptor at equilibrium. We first consider an n-meric homotypic ligand whose oligomerization domain undergoes a two-state transition between L and L_n at equilibrium (i.e., intermediates are not appreciably populated) with dissociation constant K_n, and the two species bind their receptor R with intrinsic *macroscopic* dissociation constants K_L and K_{Ln}:

$$K_n = \frac{[L]^n}{[L_n]}; \quad K_L = \frac{[L][R]}{[LR]}; \quad K_{Ln} = \frac{[L_n][R]}{[L_nR]} = \gamma K_L. \tag{3}$$

Note that the dissociation constant K_n captures both unfolding and dissociation of the oligomerization domain, which are coupled at equilibrium. The parameter $1/\gamma$ represents the intrinsic affinity enhancement due to oligomerization. The linkage between receptor binding and self-association is depicted in Scheme 1:



Scheme 1. Model for receptor binding for a two-state n-meric homotypic ligand.

The various species are also related by equations of state for the ligand and receptor:

$$[L]_t = [L] + n[L_n] + [LR] + n[L_nR] = [L] + n[L_n] + [L]_b \tag{4}$$

$$[R]_t = [R] + [LR] + [L_nR] = [R] + [R]_b. \tag{5}$$

The subscripts "t" and "b" denote total and bound concentrations of ligand and receptor, respectively. Substituting from the equilibrium expressions for the complexes into Eqs. (4) and (5), and eliminating [R], yields $[L]_b$ and $[R]_b$ in terms of [L]:

$$[L]_b = [R]_t \frac{\gamma K_n [L] + n[L]^n}{\gamma K_L K_n + \gamma K_n [L] + [L]^n} = [R]_t \Psi \tag{6}$$

$$[R]_b = [R]_t \frac{\gamma K_n [L] + [L]^n}{\gamma K_L K_n + \gamma K_n [L] + [L]^n} = [R]_t \theta_R. \tag{7}$$

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