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Cooperative Binding Mitigates the High-Dose Hook Effect

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Abstract

In the beginning of the last century immunologists discovered that increasing the concentration of antibody does not always increase the binding of antigen. On the contrary, at a high enough concentration range the amount of antibody-antigen complexes decreased. This phenomenon was later referred to as the "prozone effect" or the "high-dose hook effect" and seemed to exist in many multivalent proteins. In a later study it was discovered that allosterically regulated proteins are less susceptible to this effect. Our aim is to investigate the mathematical basis of how allostery mitigates the prozone effect.

A combinatorial account of the prozone effect and its behaviour under allosteric conditions was developed and illustrated in MATLAB. Kinetic simulations were done by formulating reactions with the mass action law and running parameter scans in COPASI.

We developed a combinatorial theory that provides an explanation of the impact of cooperativity on the prozone effect. The mitigation of the prozone effect under these conditions reappeared in simulations of ligand binding to dimeric and tetrameric proteins thereby confirming the validity of the theory.

Keywords: prozone effect, hook effect, mechanistic model, linker protein, cooperativity, allostery

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Introduction

Since the early 20th century, immunologists have noted that more is not always better: Increasing the amount of antibody in an antibody-antigen reaction could reduce, instead of increase, the amount of precipitating antibody-antigen complex [1]. Similarly, mice receiving larger doses of anti-pneumococcus horse serum were not more, but less protected against pneumococcus infection [9, 10]. There was clearly a range of antibody concentrations above the optimum at which no effects (or negative effects) were obtained. This region of antibody concentrations was named the prozone, and the related observation the "prozone effect"[1, 9, 10] or (after the shape of the complex formation curve) the "high-dose hook effect" (reviewed in [5, 11]).

Over the following decades, the prozone effect became better understood beyond its first application in immunology, and as a more general property of systems involving multivalent proteins. In 1997, Bray and Lay showed using simulations of various types of protein complexes that the prozone effect is a general phenomenon in biochemical complex formation, and occurs whenever one protein acts as a "linker" or "bridge" between parts of a complex [3]. This was corroborated using a mathematical model of an antibody with two antigen-binding sites by Bobrovnik [2].

The prozone effect thus results from partially bound forms of the "linker" proteins competing with each other for binding partners, and as a consequence, there is a regime of concentrations where adding more linker protein will decrease the amount of fully formed complexes, rather than increase it.

Are all complexes with a central multivalent "linker" protein equally susceptible to the prozone effect? In a simulation of allosterically regulated proteins using the Allosteric Network Compiler (ANC), Ollivier and colleagues found that allostery can mitigate the prozone effect [16].

In this case, ligand binding to the linker protein is cooperative (reviewed in [19]), and the simulations by Ollivier et al. showed that the higher the cooperativity, the less pronounced the prozone effect [16].

This agrees with what we know about cooperative binding: If ligand binding to one site is conducive to ligand binding to other sites, this will favour the formation of fully assembled complex over partial complexes, and thus increase the total amount of fully formed complex at a given linker concentration, compared to the non-cooperative case. In other words, partially bound forms of the linker protein still compete among themselves for binding partner, but cooperative binding skews the competition in favour of the

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forms that have more binding sites occupied and are thus closer to the fully bound form.

In this paper, we formalise these ideas. We first provide a mathematical description of the principle behind the prozone effect and show that it is indeed smaller for proteins that display cooperative ligand binding. We illustrate the point using simulations of linker proteins that are either dimeric or tetrameric.

Mathematical Treatment

We start by looking at a case in which a linker protein L binds perfectly (i.e. with an infinitely small K_d) to one molecule each of A and B to form a ternary complex (LAB). The binding sites for A and B are separate and have the same affinity for the linker L.

In the following, we will denote amounts or numbers of molecules with lower-case letters: a will be the number of molecules of A, b the number of molecules of B, and λ the number of molecules of L. Without loss of generality, we will assume that $b \leq a$. A system (before binding) where $\lambda = 7$, $a = 4$, and $b = 3$ is shown in figure 1.

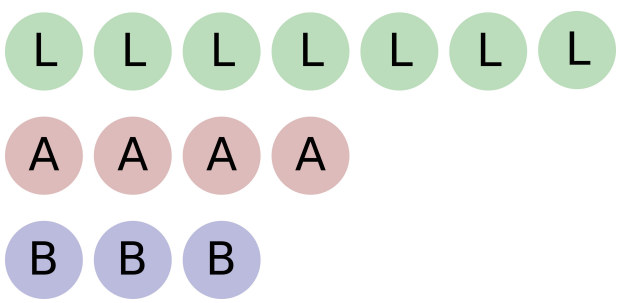

Figure 1: Free molecules of L, A and B

Assuming perfect binding and no cooperativity, the molecules of A and B will be distributed randomly across molecules of L. At the end of the binding phase, any given molecule of L will be either free, bound to A only, bound to B only, or part of a complete LAB complex. Clearly, this is a combinatorial problem that can have a variety of possible outcomes in terms of the numbers of complete LAB complex, partial complexes (LA or LB) and free (unbound) L. One possible configuration for the case where $\lambda = 7$, $a = 4$, and $b = 3$ is shown in figure 2.

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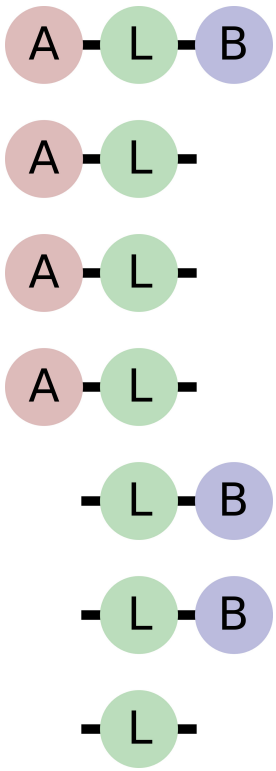

Figure 2: One possible configuration of binding of four molecules of A and three molecules of B to seven molecules of L

We are interested in expressing the expected number of full complexes (LAB) formed as a function of λ. We will denote this quantity as $E_{\mathrm{LAB}}(\lambda)$

As long as the number of linker proteins L is limiting, then the total number of ternary complexes formed will be λ.

$$E_{\mathrm{LAB}}(\lambda) = \lambda \qquad \text{if } \lambda \leq b$$

If the amount of linker protein is larger than the amount of protein B, but smaller than the amount of protein A, then all of L will be bound to A at least, and the amount of completely formed LAB complex will depend on b alone.

$$E_{\mathrm{LAB}}(\lambda) = b \qquad \text{if } b < \lambda \leq a$$

Finally, if the amount of linker protein is larger than both a and b, then we have to consider all possible binding scenarios. Figure 3 shows a probability

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tree for each molecule of L. For reasons of convenience, we show binding as a two-stage process (A binds first, then B), but this is not meant to represent a temporal order. The resulting probabilities for each end state would be the same if the order of binding was switched.

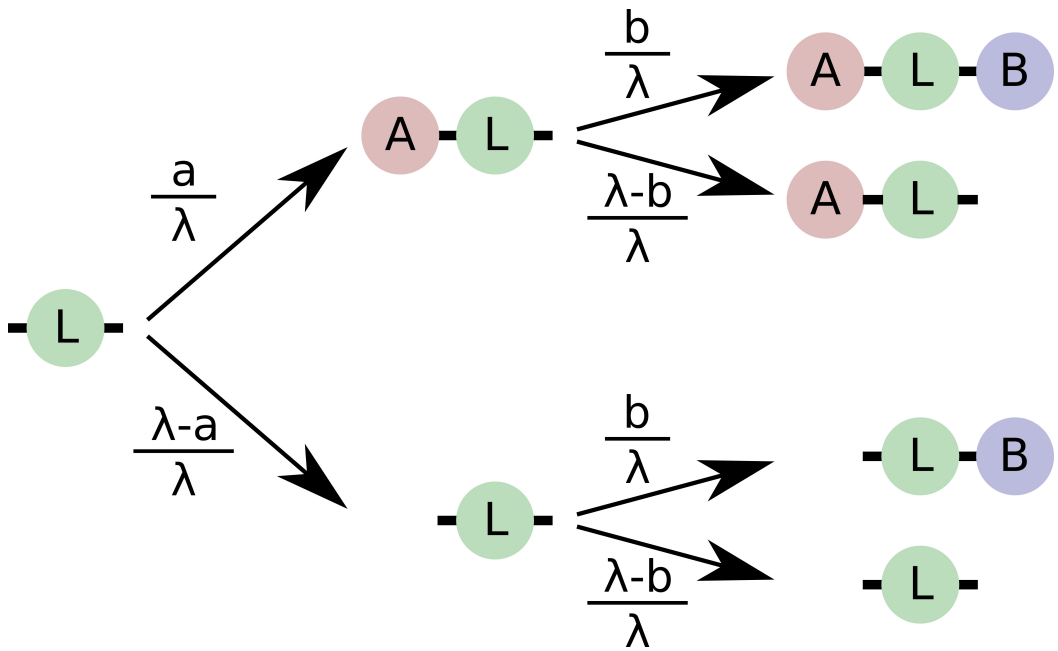

Figure 3: Probability of binding events when both a and b are smaller than λ. For each L, the arrows are marked with the probabilities of the associated binding event.

The expected number of LAB complex can be computed by taking the probability of each L to become an LAB complex, and multiplying with the amount of L:

$$E_{\mathrm{LAB}}(\lambda) = \frac{a}{\lambda}\frac{b}{\lambda}\lambda = \frac{ab}{\lambda} \qquad \text{if } a < \lambda$$

Thus, for fixed amounts of A and B (with $b \leq a$), we can write the expected amount of LAB as a function of λ as a three-part function:

$$E_{\mathrm{LAB}}(\lambda) = \begin{cases} \lambda & \text{if } \lambda \leq b \\ b & \text{if } b < \lambda \leq a \\ \frac{ab}{\lambda} & \text{if } a < \lambda \end{cases}$$

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A plot of the above function for a = 80, b=50, and $\lambda = 1$ to 400 is shown in figure 4 (black line). For each value of λ, the figure also shows the result of 100 simulations of the probabilistic binding events (grey dots).

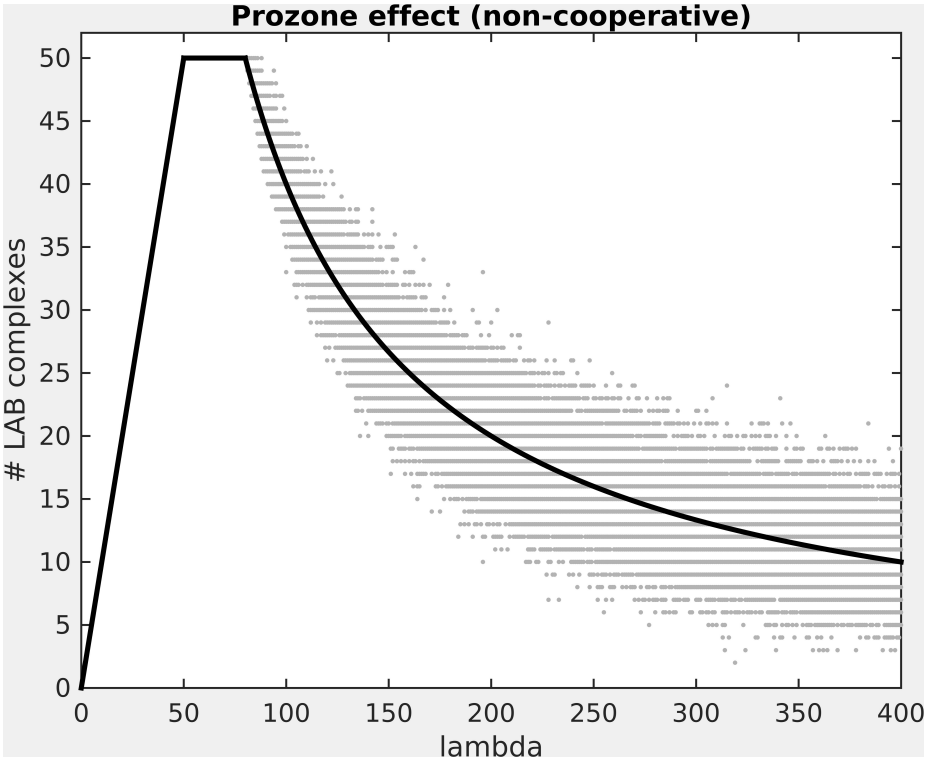

Figure 4: The Prozone effect for a Linker protein without cooperativity, assuming perfect binding. Black line: Expected value. Grey dots: Results of 100 stochastic simulations. Amount of linker protein (lambda) varied from 1 to 400, amounts of proteins A and B were 80 and 50, respectively. Simulations were run using MATLAB [20].

As we can see, the amount of fully bound complex will first increase with increasing amounts of L, then stay constant (at b) until the amount of L exceeds the amounts of both A and B, and then go down again as L increases further. In other words, for large enough L, adding L will decrease the expected amounts of fully bound complex LAB. This is the prozone effect.

Now, how does the situation change if binding to L is cooperative, i.e. if binding of L to a molecule of A (or B) is more likely when a molecule of B (or A) is already bound?

Again, as long as λ is smaller than both a and b, the amount of linker L

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will be limiting, and we thus have:

$$E_{\mathrm{LAB}}(\lambda) = \lambda \qquad \text{if } \lambda \leq b$$

If the amount of linker protein is larger than the amount of protein B, then there can be at most b fully bound complexes, just like in the non-cooperative case. Thus, b is the maximum possible value for $\mathrm{E_{LAB}}$.

If λ exceeds both a and b by a sufficient amount, we can again follow a probability tree (displayed in figure 5) to determine the probability of a single linker protein being fully bound. Again, this is computed as the probability of A binding ($\frac{a}{\lambda}$, as before) times the probability of B binding, given A is already bound, which will depend both on $\frac{b}{\lambda}$ (as before) and on the cooperativity coefficient c. This gives us an expected value for the number of fully formed LAB complexes:

$$E_{\mathrm{LAB}}(\lambda) = \frac{abc}{\lambda}$$

What do we mean by "a sufficient amount"? Clearly, λ must be bigger than both a and b. But remember also that $\mathrm{E_{LAB}}$ is limited by b. So, the question is, when is $\frac{abc}{\lambda} < b$? This is the case when $ac < \lambda$.

Thus, the complete function for $\mathrm{E_{LAB}}$ is as follows:

$$E_{\mathrm{LAB}}(\lambda) = \begin{cases} \lambda & \text{if } \lambda \leq b \\ b & \text{if } b < \lambda \leq ac \\ \frac{abc}{\lambda} & \text{if } ac < \lambda \end{cases}$$

How is this cooperative case different from the non-cooperative case? It is easy to see that the maximum number of bound complexes is still the same, because this is determined by b (in other words, the availability of the scarcer of the two ligands). Two things, however change: First, the range of concentrations at which this maximum number of complexes is formed, becomes larger, i.e. we can increase λ further without seeing a detrimental effect on LAB formation. Second, after the maximum is reached, the decline in the expected number of LAB complexes as a function of λ is less steep. There is still a prozone effect, but the effect is less drastic, and it sets in at higher concentrations of L. This is how cooperative binding works to counteract the prozone effect. Figure 6 shows the cooperative case for the same values of a, b, and λ as the noncooperative example shown above.

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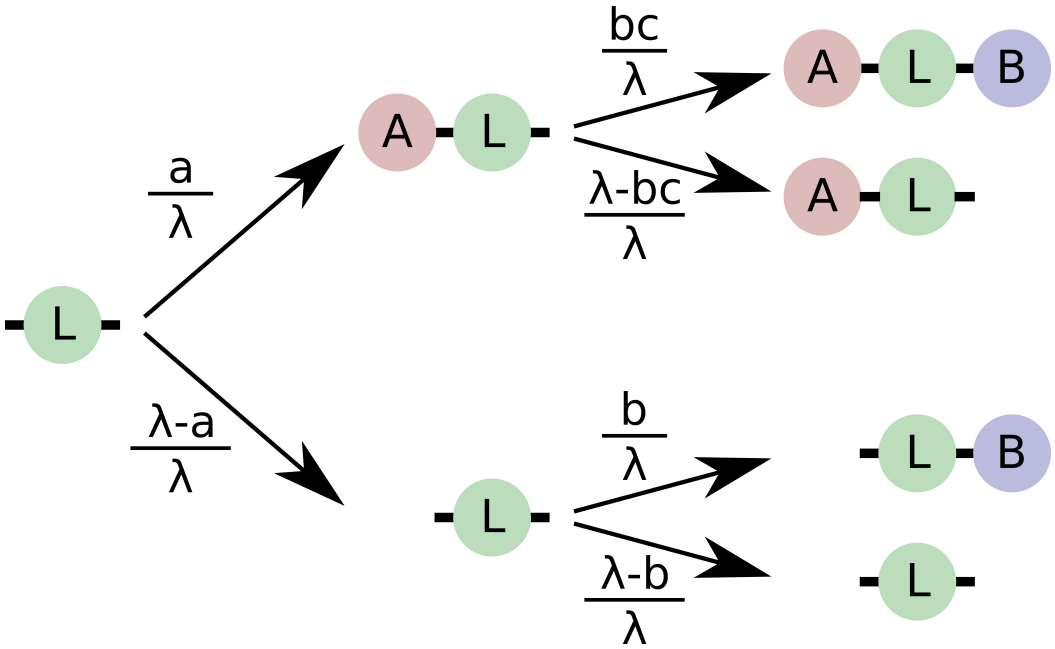

Figure 5: Probability of binding events for a cooperative linker L when both a and b are smaller than λ. For each L, the arrows are marked with the probabilities of the associated binding event. The amount of cooperativity is indicated by a multiplicative factor c, where $c > 1$ denotes positive cooperativity, and $c = 1$ in the absence of cooperativity.

The above analysis assumes that binding of A and B to L is perfect, in the sense that if there is a free molecule of ligand and there is an unoccupied binding site, then binding will happen with a probability of 1. In real biological systems, of course, such certainty does not exist. The probability of a binding event depends not only on the availability of ligand and binding sites, but also on their affinities, usually measured in terms of association or dissociation constants.

This will affect the expected number of fully bound complexes, the range of concentrations at which certain behaviours can be observed, and the way we think about cooperativity. An analytical analysis is complicated by the fact that, unlike in most other binding scenarios that are well described in theoretical biochemistry, we are operating under conditions of "ligand depletion", where the limited availability of ligand will affect the dynamic behaviour of the system [7].

Therefore, the scenario of real-life biological systems with non-zero dis-

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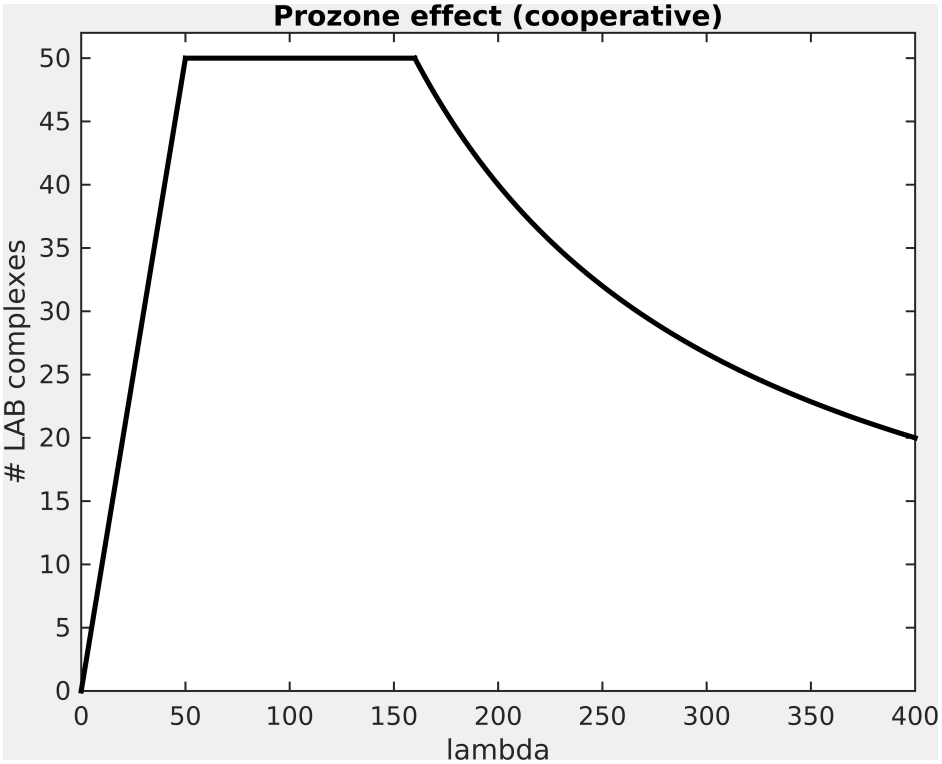

Figure 6: The Prozone effect for a Linker protein with cooperativity, assuming perfect binding (expected values). Amount of linker protein (lambda) varied from 1 to 400, amounts of proteins A and B were 80 and 50, respectively. The cooperativity constant c was set to 2. Plot was drawn in MATLAB [20].

sociation constants lends itself well to simulation approaches. In simulations of biochemical systems, one possible way of representing cooperative binding is as a decrease in dissociation constants (i.e. an increase in affinity) if one or more of the binding sites on the receptor are already occupied [19].

Simulations

In order to investigate whether we could detect a prozone effect in a simple linker protein under conditions found in biochemical systems (with finite association constants), we ran simulations using the biochemical simulator Copasi[12]. The simulation included a linker protein L and two ligands A and B, and we followed the formation of LAB over time. We initially varied the free linker protein amounts between 1 and 1000, by keeping all other

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reactant concentrations at 100. This showed an increase in the fully bound linker protein until it reached a peak at 100 and started decreasing (see figure 7), as predicted by our theoretical model.

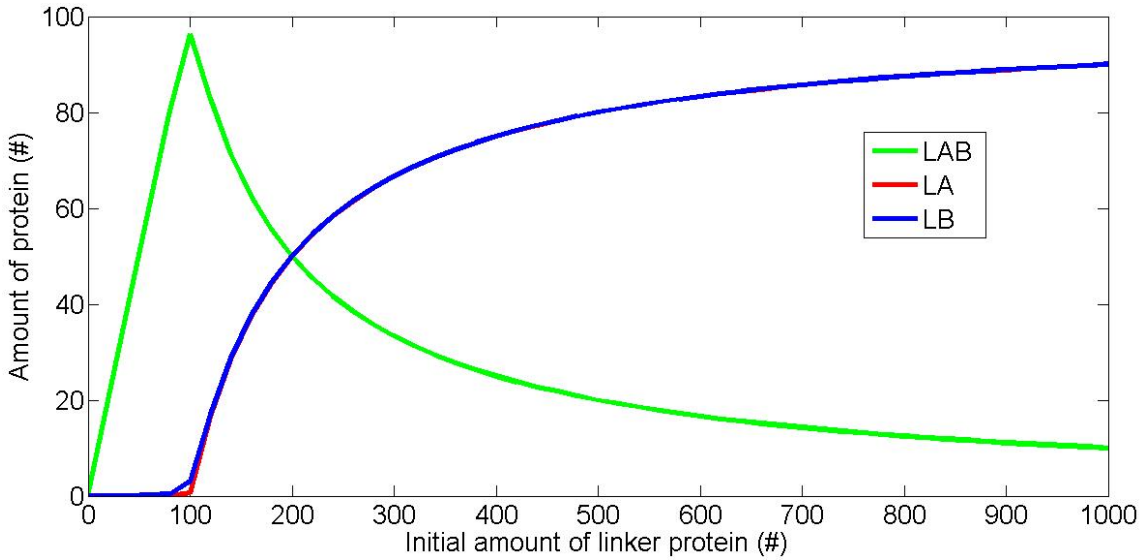

Figure 7: Steady-state amounts of a fully bound dimeric linker protein and partial complexes upon increase of free linker protein in a COPASI simulation.

Importantly, this is a property specific to the linker protein: When we varied the concentration of one of the ligands in the same way, the steady-state concentration of the fully bound linker protein increased and then remained at a plateau (see figure 8).

To determine whehter the simulated system behaved as predicted when the concentration of one ligand is lower than that of the other, we also simulated a case where the amount of ligand B was double the amount of ligand A, i.e. 200 and 100 respectively. Again, we could see the same pattern as in the (theoretical) case of perfect binding: Upon increase of the free linker protein, the amount of fully bound complex LAB was first limited by the amount of L until the free linker concentration reached the amount of A and stayed at this level until it finally attained the same value as the amount of B, and finally started decreasing (see figure 9). Hence, in accordance with the perfect binding case, we saw a prozone effect in this simple dimeric linker protein.

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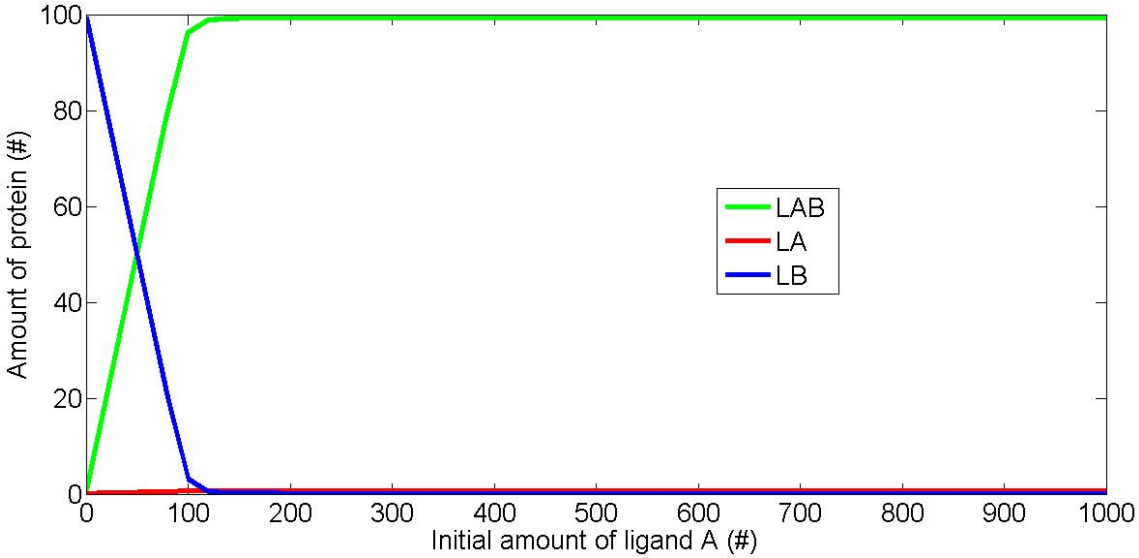

Figure 8: Steady-state amounts of a fully bound dimeric linker protein and partial complexes upon increase of free ligand in a COPASI simulation.

Next we wanted to look into the action of cooperativity on the prozone effect when there is imperfect binding. To study the prozone effect in a more complex case, we investigated a tetrameric linker protein we denote by A, which has four binding sites for the ligand G. In this case we saw that the partially bound linker protein concentration increases, whereas the fully bound linker complexes decreased gradually upon increase of free linker concentration (see figure 10). We simulated data sets with different cooperativities, which showed that the fully bound complex GGGGA decreases less after the critical linker protein concentration is reached when there is higher cooperativity. Moreover the low liganded complexes did not increase as fast as when there was no cooperativity. In essence, these simulations tell us that cooperativity, which is a given property of allosteric proteins, negatively regulates the prozone effect in these proteins.

Methods

Theoretical complex formation curves

The complex formation curves under the assumption of perfect binding shown in figure 4 were generated using MATLAB [20]. The MATLAB script

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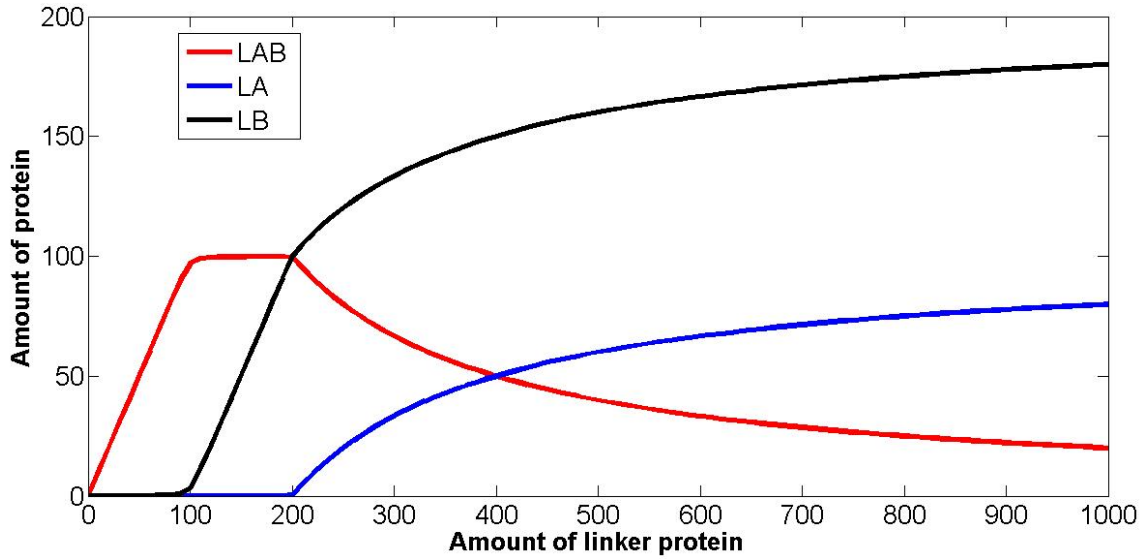

Figure 9: Steady-state amounts of a fully bound dimeric linker protein and partial complexes upon increase of free linker proteins in a COPASI simulation with 200 molecules of ligand B and 100 molecules of ligand A.

used to generate the plots is provided as Additional File 1.

Dimer and tetramer simulation

Models of dimeric and tetrameric proteins were specified as systems of chemical reactions and simulated using COPASI[12]. Binding of ligands to linker proteins followed mass action law. The simulations were performed using the parameter scan function in COPASI[12] varying the free linker amount between 1 and 1000 with 10000 intervals and keeping other reactants at 10 in the teramertic case (see below). The results were plotted in MATLAB[20].

Reactions

Dimeric model

$$[L] + [A] \rightarrow [LA]$$

$$[LA] \rightarrow [L] + [A]$$

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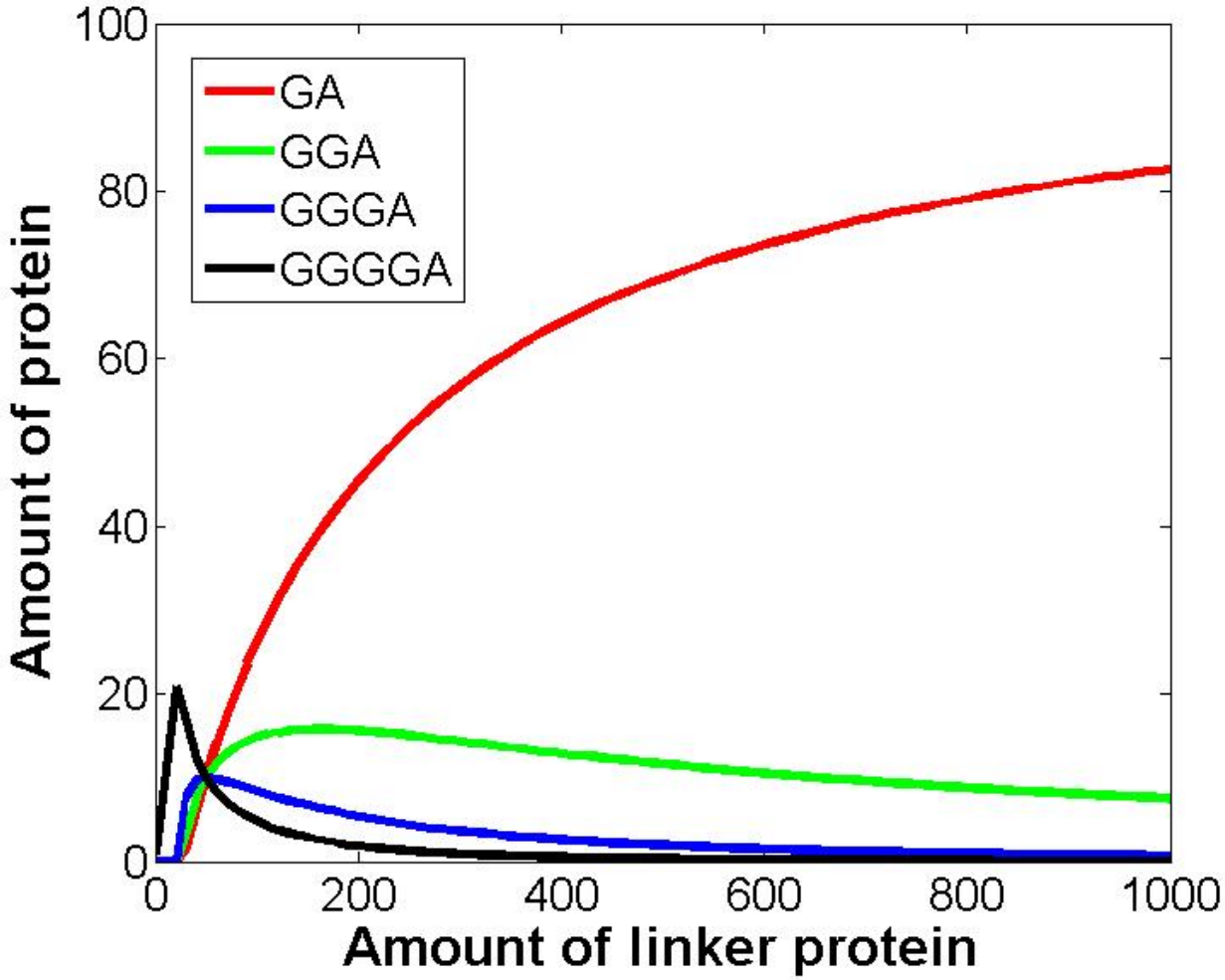

Figure 10: The plot shows the steady-state concentrations of the different liganded states of a tetrameric linker protein at cooperativity 10. The fully liganded state decreases while the less liganded ones increase.

$$[L] + [B] \rightarrow [LB]$$

$$[LB] \rightarrow [L] + [B]$$

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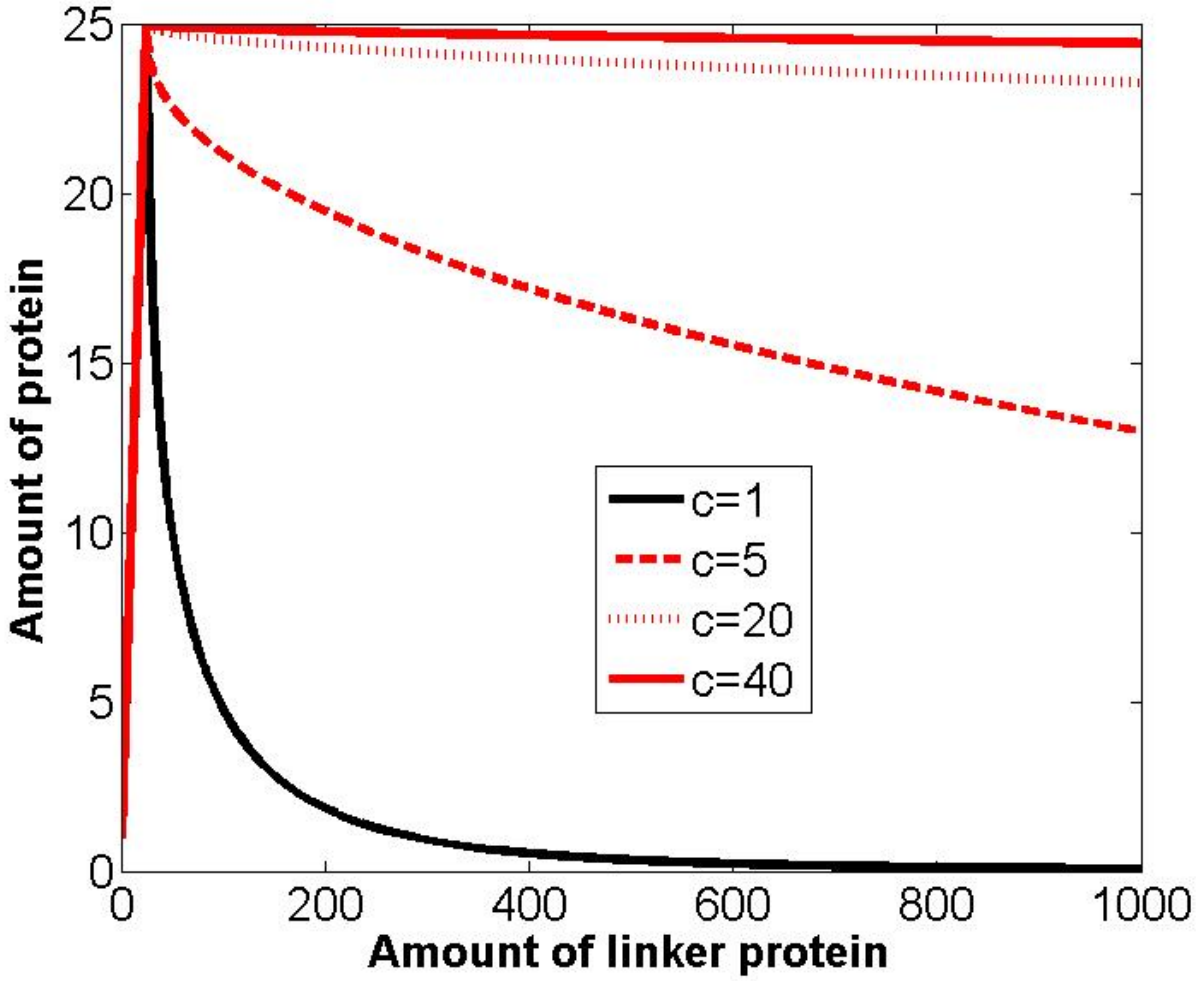

Figure 11: The plot shows the amount of fully bound tetrameric complex GGGGA at different cooperativities (c), and shows that cooperativity makes the fully liganded state decrease less.

$$[LB] + [A] \rightarrow [LAB]$$

$$[LAB] \rightarrow [LB] + [A]$$

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$$[LA] + [B] \rightarrow [LAB]$$

$$[LAB] \rightarrow [LA] + [B]$$

Tetrameric model

$$[A] + [G] \rightarrow [GA]$$

$$[GA] \rightarrow [G] + [A]$$

$$[GA] + [G] \rightarrow [GGA]$$

$$[GGA] \rightarrow [G] + [GA]$$

$$[GGA] + [G] \rightarrow [GGGA]$$

$$[GGGA] \rightarrow [G] + [GGA]$$

$$[GGGA] + [G] \rightarrow [GGGGA]$$

$$[GGGGA] \rightarrow [G] + [GGGA]$$

Parameter values

Discussion

In this study we looked into the prozone effect in allosteric proteins, and learnt that cooperative ligand binding contributes to balancing the amount of fully bound protein complexes. As a result, the concentration of fully bound complex does not decrease as much as in non-allosteric cases.

These results are likely to be relevant in a wide range of biological systems. For instance, neuronal signalling depends on a number of proteins with multiple ligand binding sites. including membrane receptors such as the AMPA receptor or postsynaptic calcium sensors such as calbindin and calmodulin. The existence of multiple ligand binding sites and, under some conditions,

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Table 1: The table lists the parameters for the dimeric (k) and tetrameric (q) models (c=10) following the order of the reactions above.

Parameter name	Parameter value
k_{1f}	$1\ s^{-1}$
k_{1b}	$0.1\ s^{-1}$
k_{2f}	$25\ s^{-1}$
k_{2b}	$0.1\ s^{-1}$
k_{3f}	$25\ s^{-1}$
k^1_{3b}	$0.1\ s^{-1}$
k^2_{4f}	$1\ s^{-1}$
k^2_{4b}	$0.1\ s^{-1}$
q_{1f}	$2.2\ s^{-1}$
q_{1b}	$0.1\ s^{-1}$
q_{2f}	$22\ s^{-1}$
q_{2b}	$0.1\ s^{-1}$
q_{3f}	$220\ s^{-1}$
q^1_{3b}	$0.1\ s^{-1}$
q^2_{4f}	$2200\ s^{-1}$
q^2_{4b}	$0.1\ s^{-1}$

the relative scarcity of ligands (e.g. of glutamate in the synaptic cleft, and of calcium in the postsynaptic neuron) makes those proteins, in principle, prone to the prozone effect. Interestingly, several of these proteins are allosterically regulated (this is the case, for instance, for AMPA receptors[6] and for calmodulin[18]), which could confer a sensitivity advantage at high receptor-to-ligand ratios [7].

The prozone effect is also a frequently discussed problem in medical diagnostics, because it can lead to false-negative effects if the levels of analyte to be detected are too high. Recent examples of this effect have been reported in the diagnosis of meningitis[13], malaria[8, 17], and even in pregnancy tests[15]. To avoid such cases, systematic dilution of the sample (and thus a reduction of analyte concentration) can help[4], but is not always practicable[14]. Given our results, another way to reduce the risk of false-negative results due to the prozone effect would be to somehow make analyte binding to the reporter in the assay cooperative. One way of achieving this in a sandwich immunoassay by making one of the receptors multimeric has been patented in 2001[14].

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Competing interests

The authors declare that they have no competing interests.

Author's contributions

Developed theoretical framework: MIS; designed and performed simulations: RDR; analysed and discussed results: RDR, MIS; wrote the paper: RDR, MIS.

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