A template-based approach to the modification of binding properties of globular proteins I: *Background and motivations.*

Design and production of polypeptide/proteins with desired bind site properties involves utilization of the ligand of interest in selection of those bind sites that yield the desired properties from an assortment bind sites with different structures and properties. Thus, as opposed to an “instructive” role and therefore involvement in determination of these bind site properties, the ligand instead functions in selection of bind sites with preexisting properties. Although there is consensus over this role for ligands, this was not always the case. A debate over the role of cognate ligands dominated discourse during the early- to mid- 20th century. On one hand were proponents of an instructive role (as a direct template), and on the other hand were those in support of “selective” role. Haurowitz and Pauling proposed a role for ligand in determination of bind site properties, whereas Jerne and Burnet proposed selection. Experimental studies and theoretical considerations in immunology –by Nossal and Tonegawa– and in enzymology –by Anfinsen and Haber– provided results in support of the selection theory. Thus, an acceptance of selection- over instruction theory. However, both the uniqueness of antibody production and polyclonality draw questions to the applicability of such generalization of selection theory to other non-immune proteins. It is based on these notions that we advocate reconsideration of the instruction (direct template) theory as [at least] a partial explanation of origins of ligand binding properties of peptide molecules. Such reconsiderations are especially relevant when considering some of the current challenges regarding optimization of catalytic rates of artificially engineered enzymes. In such cases, the instruction theory may stand as part of a solution. In addition, this may create an avenue for optimization of: antibody-based therapeutics; and quantitative and qualitative immunoassays; for all of which binding interactions is a crucial determinant of affinity, specificity as well as sensitivity of agent.

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DRAFT

A TEMPLATE-BASED APPROACH TO THE MODIFICATION OF BINDING PROPERTIES OF GLOBULAR PROTEINS I: *Background and motivations*.

Imadol V. Jeff-Eke[1]

Abstract

Design and production of polypeptide/proteins with desired bind site properties involves utilization of the ligand of interest in selection of those bind sites that yield the desired properties from an assortment bind sites with different structures and properties. Thus, as opposed to an "instructive" role and therefore involvement in determination of these bind site properties, the ligand instead functions in selection of bind sites with preexisting properties. Although there is consensus over this role for ligands, this was not always the case. A debate over the role of cognate ligands dominated discourse during the early- to mid- 20th century. On one hand were proponents of an instructive role (as a direct template), and on the other hand were those in support of "selective" role. Haurowitz and Pauling proposed a role for ligand in determination of bind site properties, whereas Jerne and Burnet proposed selection. Experimental studies and theoretical considerations in immunology –by Nossal and Tonegawa– and in enzymology –by Anfinsen and Haber– provided results in support of the selection theory. Thus, an acceptance of selection- over instruction theory. However, both the uniqueness of antibody production and polyclonality draw questions to the applicability of such generalization of selection theory to other non-immune proteins. It is based on these notions that we advocate reconsideration of the instruction (direct template) theory as [at least] a partial explanation of origins of ligand binding properties of peptide molecules. Such reconsiderations are especially relevant when considering some of the current challenges regarding optimization of catalytic rates of artificially engineered enzymes. In such cases, the instruction theory may stand as part of a solution. In addition, this may create an avenue for optimization of: antibody-based therapeutics; and quantitative and qualitative immunoassays; for all of which binding interactions is a crucial determinant of affinity, specificity as well as sensitivity of agent.

Keywords: *Direct template theory; Indirect template theory; Instruction theory; Selection theory; Bind sites; Cognate ligand.*

[1] **Contact information: ivjeffeke@gmail.com**

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One of the primary objectives of protein engineering is improvement of design and production of ligand-binding proteins with desirable binding properties such as selectivity, affinity, catalytic efficiency, etc[1,2]. In order to realize the stated end, a typical approach has been to study natural proteins, in an attempt at determination of both the extent of their properties and how these properties derive. With such information, replication of involved processes is performed in order to yield artificial forms. For example, antibody-based therapeutics and diagnostics (including screening and monitoring)[3] have both emerged as important areas in medicine. Antibody-based therapeutics are currently utilized in the treatment of a number of medical conditions[4]. Antibody-based diagnostics are currently used for early detection of pre-disease states by screening methods and early diagnosis of disease. The ability to produce antibodies in large quantities and with desired properties can be attributed to work by Kohler and Milstein: Hybridoma technology. From their understanding of i*n vivo* antibody production, they were able to create a platform for i*n vitro* antibody production. An array of modifications to this original platform have been developed, and if used in combination can yield an approximation of properties desired by the developer[4]. These are Yeast-display libraries; Human hybridomas from patients; Antibody cDNA cloning from lymphocytes selected on antigen; Ribosome-mRNA libraries[5]; phage-display technologies[3,6-8] and transgenic mice that express human immunoglobulin genes. As stated, desired features may be one or more binding properties of these antibodies. These current technologies do not utilize the intended ligand as template in the acquisition of bind sites or their desired properties. Instead, the ligand is merely used to select for those bind sites that satisfy the given requirements[9].

The question of whether or not natural proteins utilize cognate ligands as templates for formation of bind sites dominated debate during the early- to mid- 20th century. In general, two camps with respective theories emerged. The first argued for a template role for the cognate ligand (instruction theory) and the second, argues against the template role for the cognate ligand (selection theory). We shall briefly discuss preliminary theories which eventually gave rise to or motivated the current conceptions regarding instruction and selection theories. It is also important to mention here that although these preliminary theories were originally intended to explain antibody specificity for antigen; modern conceptions of these theories have however been generalized to also apply for binding between all peptides and their cognate ligands. With the binding peptide being analogous to the antibody, and the cognate ligand analogous to the antigen.

For instruction theory, we emphasize the antigen-template theory[10] or direct template theory[11] proponents of which Pauling and Haurowitz presented the most detailed attempt at a mechanism[11-13].

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Motivations for their positions were attempts at explaining the remarkable specificity of antibodies for antigens. Pauling posited that such specificity derives from the initial antibody folding process, which involves an antigen that acts as a framework on which its associated antibody molecule folds during synthesis[12]. Thus, the resultant specificity of the antibody for an antigen is reliant on antigen structure as a template. Haurowitz held a similar position, although he believed that synthesis and folding occurred separately[13]. Of foremost relevance to this work, is that the Haurowitz-Pauling hypothesis addresses an overlap of the two primary domains of protein studies: protein folding to a native structure, and binding interactions of the protein in its native state. That is, they both attempted explanation of specificity between antigen and antibody, when at physiologically folded state, as resulting from prior interactions during the folding process. In other words, the history of the protein determines the future function.
An alternate hypothesis, which advocates a [clonal] selection model, was proposed by **Burnet** –albeit as a successor to both a previously discarded theory (Burnet and Fenner: indirect template theory) and a modified theory (Jerne: the natural-selection theory). Although not explicitly stated for molecular correlations between antigen presence and antibody specificity [14], both the indirect template theory and its immediate successor (the natural-selection theory) posit that production of antibodies by immune cells, albeit in small quantities, pre-exist the presence of antigen with which it binds[10]. Thus unlike the direct template theory, antibody folding can occur in an absence of antigen; and the specificity that results can be said *not* to have derived from an antigen framework[14].

Experimental findings both by Nossal and Tonegawa helped solidify the "selection" viewpoint of antibody specificity as being independent of antigen, and indirectly revealed that antibody molecules can assume their functional structures in an absence of antigen. Nossal, 1958, 1965; attempted determination of the antigen content of "antibody-forming cells" following exposure to radiolabeled [flagellar] antigens at later moments of the immune response. Only a small amount of these were found within cells. Although such experimental endeavors may not have been motivated by the Haurowitz-Pauling hypothesis, the results, nonetheless, are in disagreement with the requirement for intracellular antigens as posited by the direct template model[15,16]. Tonegawa showed that the large variety of antibody binding specificities derive from DNA recombination events. That is, the specificity of a given monoclonal antibody derives from random genetic recombination events at gene segments that code for respective portions of the antibody molecule[17,18]. Thus, not only is this finding in support of the clonal selection model, it provides a mechanism for antibody formation. Another area where selection theory has prevailed over instruction theory is in the origins of the native state(s) of enzymes. Results from experimental studies on the renaturation of bovine pancreatic ribonuclease, as was carried out by Anfinsen et al[19, 20], were in support of the notion that the stated enzyme, following denaturation, could refold to a physiologically functional state. They showed that ribonuclease could undergo folding in an absence of external assistance (ex: molecular chaperones) and in an absence of its cognate

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substrate. Based on this finding, they proposed that the amino acid sequence of a polypeptide determines the folded, native structure for the given polypeptide; and that the given amino acid sequence is [naturally] selected for from a number of sequences. In other words, information required for folding to native state is carried as the sequence of amino acids for the given peptide. Although not directly related to antibody-antigen relationships, it has been presented –at least in its modern translations– as a universal property of [globular] peptide. Thus, the current view is a nontemplate-based acquisition of protein bind site structures and properties.

However, there are reasons why such generalizations may not be appropriate. Of these reasons, we shall state two of the most obvious. Firstly, the antibody-production component of biological systems from which such generalizations on selection theory arise is both unique and unparalleled by any known process. That is, the share quantity of the molecule synthesized, in addition to the means of production (gene reshuffling, hypermutability) are unique to the immune system. Even the most similar to this, T-cell receptor production, pales in comparison to that of immunoglobulins when considering their respective array of binding specificities[21]. Thus, protein folding phenomena as applies to antibody molecules, may not be generalized for all other peptide molecules. Secondly, such clonality in the binding behavior of secreted peptides, although shown for antibodies, has not been shown for other cell types. For example, it has not been shown that different cell populations of same cell type (ex: hepatocytes) can produce proteins (ex: enzymes, receptors, e.t.c.) with different substrate specificities. In essence, although evidence supports one side of the debate (selection), it fails to exclude the other (instruction). That is, there isn't substantial evidence against the instruction theory. Thus instead of a wide acceptance of selection theory as the sole means by which the given biological phenomenon derives, a better alternative is to consider selection theory as, at least, one of such possible means. Thus, leaving open the possibility of phenomena that may best be explained by the instruction theory.

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