ROLE OF FLEXIBILITY IN THE SPECIFICITY, CONTROL AND EVOLUTION OF ENZYMES

D. E. KOSHLAND, Jr.
Department of Biochemistry, University of California, Berkeley, California 92740 USA

Introduction

In 100 years of enzyme research the importance of specificity has not changed at all but its explanation in terms of enzyme structure has changed enormously. Enzyme specificity is essential to function, not only to maintain the faithful reproduction of metabolic pathways but also to prevent unwanted side reactions at a particular active site. Specificity must involve a fit between enzyme and substrate but this fit turns out to be a dynamic one. A particular conformation of substrate is selected in most cases from among a number of conformational isomers and the substrate induces a change in the conformation of the protein. Simple binding provides specificity but binding plus a conformational change gives added specificity and with it new features of regulatory control.

To explore the new assessment of specificity it may be valuable to examine three aspects of this process: (a) the advantages of flexibility in enzyme function (b) the role of flexibility in co-operativity and (c) the evolution of enzyme function.

Advantages of flexibility in enzyme function

To begin such a section, one must first define what is meant by flexible and what is meant by rigid. Certainly no one expects protein structure to be totally rigid. There are vibrations within chemical bonds in the simplest compounds and rotation about single bonds of groups such as lysines on the surface of a protein which might be considered classical 'template' structures. What might be a good modern definition of rigid is one in which there is no significant change in the average position of residues on binding of ligand. The protein may breath, or surface groups may rotate, but the protein in the presence of bound ligand is essentially congruent with the protein alone. On the other hand, a flexible protein is one in which significant conformation changes are induced by binding ligand, changes which may be sufficient to turn a protein from 'off' to 'on' in regard to its catalytic action. Some advantages of such a flexible enzyme as compared to a rigid one are given below.

Kinetic specificity

A flexible protein can readily explain the exclusion of molecules which are similar in structure to the substrate, but which lack structural features needed to induce catalysis [1]. This can explain many features of specificity, e.g. deoxyglucose versus glucose or propionate versus butyrate, but its greatest importance probably lies in the ability to exclude water in an active site designed for a hydroxylic molecule. The specificity in these cases is kinetic since it is failure to react after binding not steric exclusion which controls the reaction.

Ordered binding

An ordered binding of substrates indicates that one substrate makes it easier for a subsequent one to bind [2]. Sequential binding can be rationalized on a rigid enzyme if it is postulated that there is steric blocking of active sites or if the first substrate itself provides structural features that attract the second substrate. These mechanisms can be excluded in most cases and evidence for conformational changes is extensive where ordered binding is observed. An obligatory order of binding is particularly important if a highly reactive intermediate is formed. If the highly reactive compound binds only after a conformation change induced by the acceptor, the reactive compound need only exist transiently on the enzyme surface. Hence wasteful side reactions are avoided.

Non-competitive inhibition

The kinetics of non-competitive inhibition have been known for a long time but the requirements for a molecule that can bind, not affect the binding of substrate, and inhibit enzyme action is almost impossible to visualize on a rigid enzyme. The phenomenon is simple to explain in a flexible one in which the binding of the inhibitor at a second site induces a conformation which disorganizes catalytic groups without affecting binding groups [3].

Control by molecules which are not themselves consumed in enzyme action

The suggestion that some analogs of substrate are not substrates because they are not large enough to induce the proper alignment of catalytic groups means that the 'deficient' substrate could be supplemented by a non-reacting molecule containing all or part of this missing structure [4]. Thus water, which lacks sufficient function to react significantly with ATP in hexokinase, can be supplemented by added xylose as shown by Sols and co-workers [5]. The xylose does not react itself but promotes the hydrolytic reaction because water plus xylose is almost structurally equivalent to glucose. Non-competitive inhibition, competitive inhibition, activation by distant sites all fit readily into a flexible enzyme in which control is identified with conformational change. The extensive application of this property in feedback regulation of biochemical pathways has marked a landmark in our understanding of such types of control. The flexible enzyme offers the advantage that regulators do not have to look in any way like substrate molecules in order to be competitive inhibitors. They allow further the possibility of many sites of control on a single protein, all controlling the same active site [4].

Co-operativity

A flexible protein made up of more than one subunit means that alterations in one subunit can affect the reactivity of neighboring subunits [6]. This allows the properties of positive and negative co-operativity discussed in the section on Co-operativity below.

Covalent modification

Induced conformational changes can expose previously buried amino acid residues many angstroms away. This altered reactivity of groups was utilized in the early studies of conformational changes to provide the evidence that such changes occurred. They have their important corollary in addition in the regulation of enzyme activity by covalent modification. Obviously, the modification of a residue outside the active site which 'freezes' or alters the conformation of the active site will affect enzyme activity. The conformation of the covalently modified protein can cause it to be activated or inhibited relative to the unmodified protein. The cell successfully uses phosphoryl, adenyl, pyridoxal and other groups in such control [7].

Catalytic power

Conformational energy can be used for catalytic power [8]. The protein can be programmed to produce strain within the same subunit as in lysozyme or between neighboring subunits as in flip-flop models or reciprocating dimer models. It can also lead to exclusion of water or desolvation which may have catalytic potentials.

Thus, a variety of properties are available to a flexible enzyme which are not possible for a rigid enzyme. Not all of these properties are needed by every enzyme and therefore it is not surprising that some enzymes seem very close to classical template-type behavior. Even enzymes which seem close to template behavior such as chymotrypsin and ribonuclease do appear to exhibit small conformational changes on the binding of substrate, and these may be crucial to enzyme function. Conformational changes, therefore, are the usual concomitant of ligand binding and have many advantages, but they are certainly not required in all cases.

Co-operativity

The phenomenon of co-operativity deserves a special discussion since it has revealed insights into the nature of conformational transitions and is a property of major importance in regulation. Moreover the two major theories of co-operativity involve fundamentally different assumptions about protein structure.

The Monod–Wyman–Changeux, or MWC model, postulates that a protein exists in two conformational states and that co-operativity arises from the displacement of the equilibrium between the symmetrical stabilized states. This model takes the view that

distortions which break the symmetry of the molecule will be energetically unattractive. This is a model of elegant simplicity and one which appears to apply to hemoglobin [9].

The Koshland-Nemethy-Filmer, or KNF model, is based on induced conformational changes transmitted through subunit interactions. It assumes that the capacity of the protein to be distorted is a key programmed feature of the protein structure. In this model the initial and final states may be symmetrical but symmetry is destroyed in the partially liganded states. It follows from the mathematics of this model that both positive and negative co-operativity would be expected depending on the ligand and the programming of the protein. Since the MWC model predicts only positive co-operativity of binding, this model applies to enzymes involving negative co-operativity, and of course to positive co-operativity as well in many cases. It is particularly appropriate in cases in which the enzyme exhibits both negative and positive co-operativity. The list including both negative and mixed co-operativity now extends to at least 20 enzymes [10].

Most importantly the transmission of the conformational change can be highly specific and focussed [11]. This means the protein can be programmed to send conformational signals over long distances and have different ligands induce different responses (cf. fig.1).

The KNF model provides a mathematical framework which is very general and can be applied to almost any case of conformational distortion. The key constants used in the mathematical description of conformational changes in this model are the ligand affinity constant, Kx_A the intrinsic affinity of ligand X for conformation A of an individual subunit, the conformational transition constant (Kt_{AB} records the energy needed to convert an individual subunit from conformation A to conformation B) and a subunit interaction constant (K_{AB} refers to the affinity of a subunit of conformational state A with a subunit of conformational state B). With these three constants each closely related to the structural properties of the protein essentially any conformational situation can be described. For illustrative purposes, the original description of the model applied the mathematics to

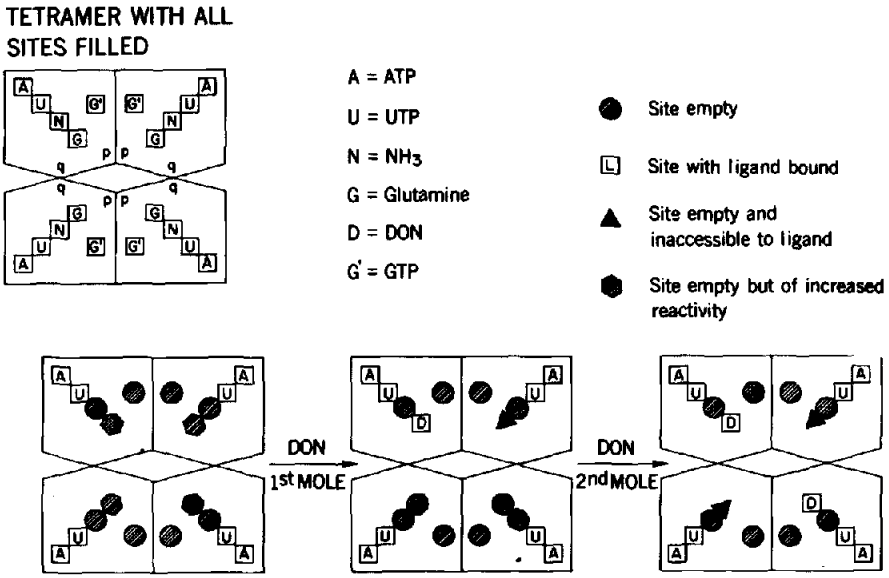

Fig.1. An illustration of the focused and specific nature of induced conformation changes. The binding of ATP and UTP to CTP synthetase increases the reactivity of the glutamine substrate of the active site by an induced conformational change. Reaction of an SH group at the glutamine site with the reagent DON can induce a conformation change which turns off the equivalent SH group in the neighboring subunit many angstroms away. The DON has essentially no effect on the binding constants for ATP and UTP at the active site.

the simplest case, i.e., when there were only two conformations of an individual subunit and only the subunit to which ligand is bound is distorted. This simplest model may provide a good first approximation in many cases, but it was clearly stated to be an illustrative calculation, a situation overlooked by some subsequent utilizers of the model. The important feature of the model is that it provides the mathematical apparatus to deal with appreciably more complex cases such as the rabbit muscle glyceraldehyde 3-phosphate dehydrogenase in which distortion of neighboring subunits occurs in a strong negative co-operativity pattern. The extensive work on hemoglobin [9] and the emerging crystallography of multi-subunit enzymes [12] should ultimately produce a correlation between the mathematical parameters and the detailed movements of atoms.

Evolution of function in enzymes

Although the study of evolution of enzyme structure [13] is a subject of intense activity, the evolution of function is a relatively uninhabited area. The extensive data from X-ray crystallography and sequencing delineate structure; and function follows as an afterthought. Nevertheless, function must be the driving force for evolution and the role of flexibility described above suggests a logical scenario for the evolution of function in enzymes [14].

In asking how function may have evolved, it would seem that two essential and interrelated criteria would have to be met. Firstly, the function must be subject to improvement by small changes in structure. Secondly, the structure must be capable of incremental modifications in a random manner. It is, of course, conceivable that a sudden all-or-none appearance of a perfect enzyme could occur by pure statistical chance. The evidence that we have, however, suggests step by step changes in amino acid residues leading to improved function. Hence one needs at each turn some functional property of the protein which can be subject to such 'fine-tuning'.

Enzymes owe their unusual power to the juxtaposition of catalytic groups and substrates in a complex which would be unlikely to form by a random collision of the individual components. Anyone who has tried to synthesize enzyme analogs knows how difficult it is to arrange reactive groups on an organic molecule (for example a norbornane or a phenanthrene) which could duplicate orientation of catalytic groups. To arrange groups in a simple organic molecule to duplicate specificity also is essentially impossible. The enzyme has solved this problem by creating a fairly large structure, e.g. molecular weight of 10 000–50 000, in which many of the residues are simply serving as scaffolding for the final orientation of a few critical residues in appropriate alignments. Once this organization is achieved, catalytic factors of the order of 10^{12}, 10^{15}, 10^{30} are observed depending on the particular reaction and the manner in which the calculation is made [15].

A protein molecule made up of twenty amino acids in a relatively large structure, however, is an ideal identity for incremental improvement. Once a very primordial catalytic function was observed, possibly by the chance juxtaposition of two catalytic residues, further optimization could easily arise from mutations of amino acids quite far from the catalytic site. These small increments would lead to random improvements in catalytic function which would then be fixed by selection. Moreover, the size of the protein molecule observed today need not have been achieved overnight. A gradual evolution to larger structures as a general way of optimizing structure would of necessity have evolved. What is important is that one could create a catalytic function in a small molecule and the evolution of improved function would be achievable by small increments in the protein structure.

Such a template-type enzyme, however, would have certain limitations. It could not provide any of the functions listed in the section on the Advantages of flexibility in enzyme function and most importantly, it could not eliminate the wasteful side reaction with water for any reaction involving a molecule approximately as nucleophilic as water, such as a sugar. The advantage of a 55 M water would be too great. A flexible enzyme with an induced conformational change which arose by fortuitous mutation would then provide the organism with an increased survival value by increasing the ability of these enzymes to exclude unwanted side reactions. This would also allow an enzyme designed for maltose to exclude glucose, etc. In this way a new species of catalyst would arise involving induced fit behavior and having all the other properties described above, i.e. ordered

binding, the protection of higher energy intermediates, etc.

It is obvious that once these flexible type molecules were being synthesized, by pure chance a binding site for an effector not precisely at the active site might arise by random probability and could, of course, be refined again by mutational selection. If this effector were a metabolite which was itself controlled by feedback regulation, it would provide that organism survival value and again be selected over evolutionary time. It should be emphasized that product inhibition could exist with a template type enzyme, but the advantage of a flexible enzyme is the possibility of regulatory molecules which do not look in any way like substrates or products at the active site. Most hormones, inhibitors, and metabolic feedback regulators then become candidates for regulation even if they do not fit into, or have an affinity for, the active site.

Once the enzymes are flexible, co-operativity would inevitably arise. In a multisubunit enzyme distortion of one subunit would in most cases probably affect the conformation of neighboring subunits. Sometimes these would make subsequent molecules easier to bind and in other cases more difficult.

The mathematical consequences in enzyme activity of these ligand-induced changes are shown in fig.2. By plotting the binding (or activity) curves on a log plot, a Michaelis–Menten curve becomes sigmoid (which is confusing to some) but the steepness of the curve becomes an index of the co-operativity of the system. It is seen that altering the subunit interactions alone can give curves which are more co-operative (positive co-operativity) less co-operative (negative

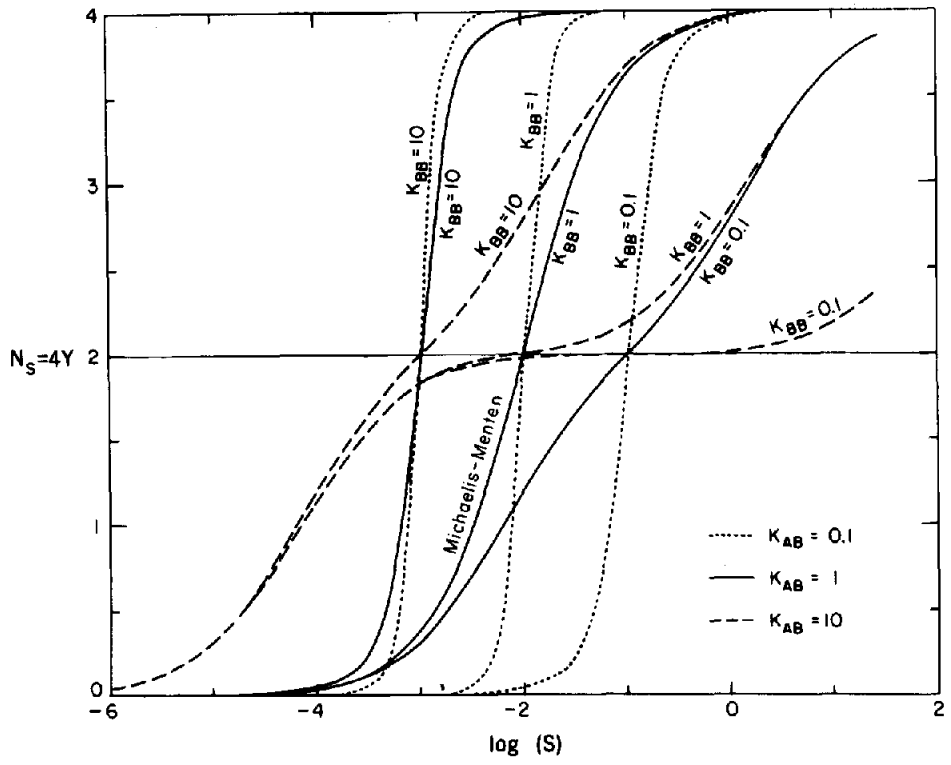

Fig.2. The effect of selection pressure on co-operativity. The curves whose computer calculated values for saturation of tetrameric protein for cases in which the *subunit interactions only* were altered. The energy of the conformation change for a single subunit (K_t) and the intrinsic affinity of the subunit (K_s) are held constant and the subunit interactions (K_{AB}, K_{BB}, etc.) are varied. Since amino acid alterations in the contact regions between subunits can change these subunit interactions a selection device for altering co-operativity and $S_{0.5}$ values exists without the need for any mutations at the active site. N_s indicates the number of molecules of substrate absorbed to the protein. Y is the fraction of the active sites which are occupied.

co-operativity) or equally co-operative to a Michael–Menten situation. Moreover, altering the subunit interactions can lead to changes in the midpoint of the curve as well as its co-operative pattern. The fine tuning of the regulatory properties of the protein can thus be achieved by changing the amino acid residues in the contacts between subunits. The curves of fig.2 apply to all ligands, activators and inhibitors as well as substrates. This means that an enzyme which has optimized its active site by selection pressure does not need to randomly alter that active site to achieve further fine tuning. It can do so by mutation in the subunit contact region. Experiments on hybridization of multisubunit proteins [16] and in the properties of hemoglobin mutants [17] have shown that the subunit contact regions are as highly selected as active sites.

Enzymes that show positive co-operativity for some substrates and negative co-operativity for others have a particular evolutionary advantage. They could, as in the case of CTP synthetase, be positively co-operative for a substrate such as ATP which is highly controlled in the medium. On the other hand, this same enzyme is negatively co-operative to GTP and is thus dampened in sensitivity to large fluctuations in GTP levels.

The various functions have been listed sequentially in order to indicate the probable order in which they first arose over evolutionary time. However, it would not be necessary that one of these functions be completely optimized before the second begins to be selected. Thus selection for flexibility could arise before specificity of binding was fully optimized. Similarly, flexibility for control need not be finalized before co-operativity started to arise. Nevertheless, it seems logical that functions would evolve in this order and that the chance modification of amino acid residues provides an ideal mechanism for the fine-tuning essential for the evolution in enzymes as we see them today.

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