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Destabilization is as Important as Binding

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Destabilization is as important as binding

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Enzymes make use of non-covalent interactions with their substrates to bring about a large fraction of their catalytic activity. These interactions must destabilize, or increase the Gibbs energy, of the substrate in the active site in order that the transition state can be reached easily. This destabilization may be brought about by utilization of the intrinsic binding energy between the active site and the bound substrate by desolvation of charged groups, geometric distortion, electrostatic interactions and, especially, loss of entropy in the enzyme–substrate complex. These mechanisms are described by interaction energies and require utilization of the intrinsic binding energy that is realized from non-covalent interactions between the enzyme and substrate. Receptors and coupled vectorial processes, such as muscle contraction and active transport, utilize binding energy similarly to avoid large peaks and valleys along the Gibbs energy profile of the reaction under physiological conditions.

1. Introduction

It is well known, although not always remembered, that the most important step in scientific discovery is to ask the right question. A useful question in science is one that is susceptible to experimental examination and that will increase our understanding of a process, if it can be answered. We would like to understand how enzymes work to bring about extraordinary increases in the rates of chemical reactions, usually without making use of highly reactive chemical reagents or catalysts, how enzymes catalyse the production of *movement*, as in muscle contraction or the active transport of ions across membranes, and how physiological processes are regulated by the interaction of small molecules with receptors.

First of all, it is essential to separate the contributions of kinetics and thermodynamics to these processes. This is complicated by the fact that kinetics can be described in terms of transition state theory by thermodynamic activation parameters, but the basic distinction remains clear.

In general, enzymes bring about enormous accelerations of the reaction rates of their substrates to generate products that are useful to the organism. However, coupled vectorial reactions bring about the movement of structures, as in muscle contraction, or movement of small molecules, as in the ATP-driven active transport of ions across membranes. The synthesis of ATP from ADP and phosphate by the transport of protons across the mitochondrial membrane in oxidative phosphorylation is an example of the same process in the reverse direction.

All of these processes are brought about by the highly specific binding interactions of one or more reagents, usually small molecules, with one or several proteins. These interactions are usually non-covalent, and in the cases in which they are covalent,

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specific non-covalent interactions of the effector with the protein are essential to mediate the reaction.

It is often thought that the most effective substrates, effectors and drugs, are those which have the highest affinities for binding to the binding site of the enzyme or effector. Pharmaceutical companies spend billions of dollars to develop drugs with high affinities for specific receptors, and it is certainly desirable for both physiology and pharmacology that small molecules have a high affinity for their receptors. However, the theme of this discussion is that although high affinities of a substrate or effector for an enzyme or receptor are obviously necessary or desirable, the most important property of these systems usually requires that the observed binding of the small molecule to the protein be relatively weak.

To make something happen, in biological systems as well as in biochemical and chemical reactions, it is necessary to drive a reaction from its initial state to a new state. When this is brought about by the binding of a small molecule to a protein, the binding energy is *utilized* to force a change in the state of the system that causes the reaction to occur. This change usually arises from non-covalent interactions of the small molecule with the protein that force the reaction to proceed toward the transition state or product.

The driving force for this process is the development of strong binding interactions between the small molecule and the protein in the transition state or product of the reaction. The larger the difference between the binding energy in the transition state or product of the reaction, compared with the binding energy for the reactants, the larger will be the rate acceleration or the driving force for a change in state of an enzyme–substrate complex or a receptor.

2. Enzyme catalysis

This is illustrated in figure 1 for catalysis by enzymes. If the enzyme stabilizes the transition state of a reaction, but stabilizes the substrate by the same amount, there is no decrease in the activation barrier for reaction of the enzyme–substrate complex, compared with the free substrate (figure 1*a*). For catalysis to be observed, it is essential that the substrate bind relatively weakly to the enzyme, so that the transition state can be reached easily. The dissociation constant of the enzyme–substrate complex should be close to the concentration of substrate that exists under the conditions in which the enzyme functions.

What is the chemical and physical basis for these differences in binding? It is well known that enzymes stabilize transition states; this is true by definition. The active site is complementary to the transition state and binds it very strongly (Pauling 1968).

Furthermore, in many enzymes the transition state involves direct chemical reactions with groups on the enzyme, such as the transfer of protons between acidic or basic groups on the enzyme and the substrate.

The reasons for the relatively weak observed interaction between the enzyme and the substrate in the ground state are less obvious. Desolvation of basic and acidic groups, especially if they are charged, can cause very large increases in the energy of the enzyme–substrate complex that are relieved in the transition state. Physical distortion of the bound substrate in an active site that is complementary to the transition state is the most obvious mechanism for ground state destabilization. Perhaps the best known example is the suggestion by Phillips (1967) that catalysis

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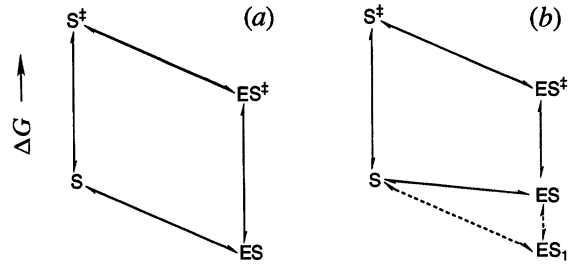

Figure 1. (*a*) There is no decrease in the barrier for reaction if an enzyme stabilizes the transition state and the substrate equally. (*b*) Catalysis is observed when the enzyme stabilizes the transition state much more than the ground state, so that the observed binding of the substate is weak.

of the hydrolysis of glycosides by lysozyme is brought about by stabilization of a planar, carbocation-like transition state at the anomeric carbon atom for glycoside cleavage by the enzyme. This transition state is also stabilized by the electrostatic interaction between the carboxylate anion at the active site and the developing positive charge on the oxocarbenium ion in the transition state. Development of this positive charge relieves the destabilization caused by desolvation of the carboxylate anion in the enzyme–substrate complex.

Desolvation of charged groups provides a particularly attractive mechanism for ground state destabilization (Lipscomb *et al.* 1969). It is relatively easy for an enzyme to protect charged groups in the enzyme or the substrate from contact with the solvent when they are bound in the enzyme–substrate complex. This can cause an increase of several orders of magnitude in the basicity of the catalytic group.

One of the most obvious and attractive mechanisms for bringing about catalysis is simple approximation of the reacting and catalysing groups that brings them into exactly the correct position to react; i.e. the enzyme can reduce the entropy loss that is required to make a new covalent bond by fixing the bound substrate in precisely the correct position for reaction.

The advantage that can be realized from this fixation is much larger than often believed. It is a factor of as much as 10^8 for the loss of entropy in the reaction of two molecules in 1 M solutions, and it is still larger for the more dilute solutions in which the enzyme functions under physiological conditions (Page & Jencks 1971). This number may be surprising, because it is enormously greater than the factor of 55 M that corresponds to the replacement of a solvent molecule by the reacting molecule. The large number reflects the fact that water and other liquids have a very large entropy, i.e. a great freedom to take up slightly different positions. The exact fixation of a molecule that occurs when it forms a covalent bond with another molecule corresponds to a very large loss of entropy, on the order of -30 entropy units. This large change in entropy is responsible for the very fast rates that are observed for most intramolecular reactions, compared with their bimolecular counterparts.

The difference between 10^8 M and 55 M reflects the fact that the fixation of a molecule relative to another molecule by a covalent bond is far more rigid, and correspondingly less probable to occur spontaneously, than the location of a molecule relative to another molecule in a liquid, where it is free to rotate and take up an extremely large number of conformations that are not correct for the formation of the new covalent bond. Thus, the exact fixation of reacting groups relative to each other is the simplest, and possibly the most important mechanism that enzymes can use to bring about rate accelerations (Jencks 1975).

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This exact fixation does not come easily. The very small probability of a pair of reacting atoms being in exactly the correct position to form a new covalent bond with each other provides the very large entropic barrier for reaction. However, enzymes are very dense, as a consequence of their close-packed structure and covalent peptide chain, and are able to bind substrates with a high degree of precision. The resulting decrease in entropy provides a major contribution to catalysis and to the spontaneous formation of an acyl phosphate from inorganic phosphate and a carboxylate group at the active site of an enzyme, as described below.

3. Coupled vectorial processes

The semanticist A. J. Korzybski concluded in his remarkable book *Science and sanity* (Korzybski 1958) that most of the problems of this world arise from poor understanding and communication because multiple meanings and interpretations are ascribed to words. This failure of communication has surely contributed to much of the controversy (and may have heightened the interest) in the interpretation of the mechanisms of enzyme catalysis and of coupled vectorial processes. The several different meanings of the term *coupling* provide an example of this problem.

These points, and the nature of coupled vectorial processes, are nicely illustrated by the calcium ATPase of sarcoplasmic reticulum from rabbit muscle. This enzyme brings about the relaxation of contracted muscles by initiating a series of events that arise from a decrease in the concentration of free calcium in the muscle. The enzyme transports two calcium ions from the cytoplasm to the lumen of sarcoplasmic reticulum vesicles in the muscle when one molecule of ATP is hydrolyzed, according to the reaction of equation (1). Muscle contraction is initiated by the release of Ca^{2+} ions from

$$2\ Ca^{2+}_{cyt} + ATP \rightleftharpoons 2\ Ca^{2+}_{lumen} + ADP + P_i \qquad (1)$$

sarcoplasmic reticulum vesicles. This results in the movement of tropomyosin relative to myosin in such a way that actin can combine with myosin and initiate the contraction cycle. The calcium is then pumped back into the sarcoplasmic reticulum by the calcium pump to decrease the calcium concentration and bring about muscle relaxation. The system is then poised to initiate another contraction cycle.

(a) *Coupling of reactions*

The essential steps of the ATP-dependent pumping of Ca^{2+} ions into sarcoplasmic reticulum vesicles are shown in the scheme below, which was proposed by Makinose (1973).

This scheme does what it is supposed to do: each cycle of the enzyme results in the transport of two Ca^{2+} ions into the vesicle and cleaves one molecule of ATP, according to equation (1). The reaction is readily reversible; ATP is synthesized from ADP and inorganic phosphate when Ca^{2+} moves out of the vesicle in the presence of EGTA, a chelating agent for Ca^{2+}.

The calcium ATPase illustrates nicely how the hydrolysis of ATP, a chemical reaction, is coupled to a vectorial reaction, the transport of two Ca^{2+} ions against a large gradient, from a concentration of less than micromolar Ca^{2+} outside to more than millimolar Ca^{2+} inside the vesicles. This is a coupling of *reactions*: the vectorial reaction of calcium pumping into the vesicle is driven by the chemical reaction of ATP hydrolysis, and the synthesis of ATP from ADP and inorganic phosphate is

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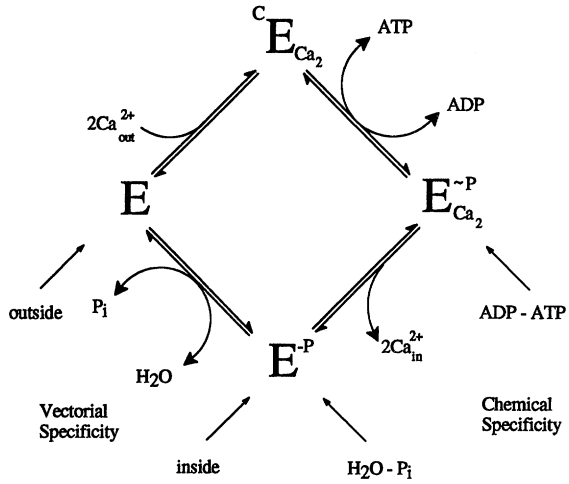


driven by the movement of calcium ions at a millimolar concentration inside the vesicle to submicromolar concentrations in the cytoplasm.

These reactions are coupled as a consequence of changes in *specificity* of the enzyme for catalysis of chemical and vectorial reactions in different states of the system, as shown in the above scheme. The chemical reactions are controlled by the binding and dissociation of Ca^{2+}. The enzyme is phosphorylated by ATP only when it has two Ca^{2+} ions bound in high-affinity sites that are exposed to the cytoplasm. After phosphorylation by ATP the calcium ions can no longer dissociate to the cytoplasm; instead they dissociate into the lumen of the vesicle. The chemical reaction of phosphorylation controls the vectorial reaction of calcium binding and release on one or the other side of the membrane. Conversely, the vectorial reaction of calcium binding controls the chemical reactions of phosphorylation and dephosphorylation by changing the chemical specificity of the enzyme for catalysis of phosphorylation by ATP or by inorganic phosphate (Pickart & Jencks 1984).

Thus, the binding and dissociation of substrates and ions are responsible for control of the chemical and vectorial steps of the reaction in such a way that neither the chemical reaction of ATP hydrolysis nor the vectorial reaction of calcium transport can occur unless the other one also occurs. Here we are not so interested in the strength of the binding; what is important is the change in specificity for chemical and vectorial reactions that is brought about as a consequence of the binding. This is important because the binding serves as a *switch* that changes the chemical and vectorial specificities for catalysis. Binding of the chemical reagents changes the vectorial specificity and binding or dissociation of the vectorial reagent, Ca^{2+}, changes the chemical specificity.

This dependence of coupling on specificities is important because specificities of enzymes for catalysis, and in this case for vectorial reactions as well, are generally much larger than differences in their affinity for different substrates. There is no detectable phosphorylation of the calcium ATPase by ATP in the absence of calcium. Such phosphorylation would result in uncoupled hydrolysis of ATP. The transport of Ca^{2+} into or out of the sarcoplasmic reticulum vesicle in the absence of the phosphorylation–dephosphorylation cycle is many orders of magnitude slower than transport in the reaction sequence of the scheme above.

In this, and in many other biological systems, the consequences of binding can be

very large indeed and may have little relation to the observed strength of binding. The binding event can serve as a switch that causes a change of many orders of magnitude in the rate of a reaction.

(b) *Coupling of binding energies*

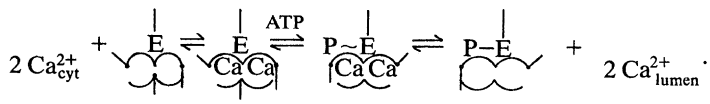
The coupling of reactions ensures that the chemical reaction of ATP hydrolysis will take place when, and only when, two Ca^{2+} ions are transported into the sarcoplasmic reticulum vesicle, but it tells us nothing about whether this will occur at a useful rate. For calcium accumulation to occur at a useful rate it is necessary that every step of the reaction can occur at a useful rate; there cannot be large kinetic or thermodynamic barriers along the reaction path. The kinetic barriers are reduced by stabilization of transition states and by avoidance of ground state stabilization that would result in energy wells along the reaction path, as in catalysis by other enzymes. The thermodynamic barriers are avoided by stabilization of chemically unstable intermediates and destabilization of stable intermediates in such a way that the Gibbs energies are similar for all of the intermediate species along the reaction path under physiological conditions. This is a difficult problem for the enzyme to solve because in aqueous solution the chemical intermediates that are formed along the reaction path, which include an acyl phosphate anhydride and inorganic phosphate, differ greatly in energy.

The remarkable fact is that evolution produces enzymes that equalize the energies of the intermediate species in the reaction sufficiently that there are no large thermodynamic barriers along the reaction path. This is analogous to the stabilization of transition states, except that it is also important to avoid large energy wells in the reaction profile that would result in large thermodynamic barriers for steps of the reaction.

For example, the formation of an acyl phosphate from inorganic phosphate and a carboxylate group at the active site of the calcium ATPase of sarcoplasmic reticulum in the absence of calcium occurs readily, with an equilibrium constant close to 1.0 at pH 7.0 (Hasselbach 1974; de Meis & Vianna 1979; Inesi *et al.* 1980; Pickart & Jencks 1984). The formation of an acyl phosphate in solution is extremely unfavourable with $\Delta G^{0\prime} = 10.3$ kcal mol^{-1} for 1 M reactants at pH 7.0 (de Meis & Vianna 1979; Jencks 1983). The enzyme reduces the entropy of the reactants and stabilizes the covalent acyl phosphate relative to the $E \cdot P_i$ complex by the expression of some 10 kcal of binding energy in the covalent species in order to overcome the unfavourable equilibrium constant for the formation of an acyl phosphate.

(c) *How is calcium transported?*

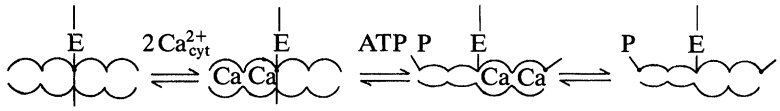
The binding of calcium to the cytoplasmic sites of the enzyme occurs readily, with high affinity. There are at least two ways in which the two Ca^{2+} can be transported into the lumen of the vesicle. The simplest mechanism occurs through binding of two Ca^{2+} ions to the high-affinity sites on the free enzyme, followed by phosphorylation of the enzyme and dissociation of the two Ca^{2+} ions to the lumen of the vesicle:



(2)

This mechanism involves a change in the gating of one pair of calcium binding sites upon phosphorylation.

The alternative mechanism involves transport of the two Ca^{2+} ions from one pair of high-affinity binding sites on the cytoplasmic side of the enzyme to a different pair of low-affinity binding sites on the lumenal end of a channel when the enzyme is phosphorylated



$$+ \ 2\ Ca^{2+}_{lumen}. \qquad (3)$$

We have carried out experiments to distinguish between these two mechanisms by measuring the formation of phosphoenzyme from inorganic phosphate at equilibrium in the presence of different concentrations of lumenal calcium:

$$E + Pi + Mg^{2+} \rightleftharpoons E - P \cdot Mg^{2+} \overset{Ca_{in}}{\rightleftharpoons} Ca_2 \cdot E \sim P \cdot Mg^{2+}, \qquad (4)$$

$$E + Pi + Mg^{2+} \rightleftharpoons E - P \cdot Mg^{2+} \overset{Ca_{in}}{\rightleftharpoons} Ca_2 \cdot E \sim P \cdot Mg^{2+}. \qquad (5)$$

$$Ca^{2+}_{in} \downarrow \uparrow$$

$$Ca_2 \cdot E$$

If there is only a single pair of calcium binding sites (2), then increasing the concentration of lumenal calcium will drive the reaction to the right and convert all of the enzyme to phosphoenzyme, even if the concentrations of phosphate and Mg^{2+} are less than optimal (4).

However, if there are lumenal binding sites for calcium on the free enzyme, as well as on the phosphoenzyme (5), the calcium can bind to the free enzyme instead of to the phosphoenzyme and increasing the concentration of lumenal calcium will not drive phosphoenzyme formation to completion when the concentrations of phosphate and Mg^{2+} are less than optimal.

The results of these experiments show that the maximum concentrations of phosphoenzyme that are formed at saturating concentrations of lumenal Ca^{2+} decrease when the concentration of Mg^{2+} is suboptimal; the maximal phosphoenzyme concentration levels off at progressively lower values as the Mg^{2+} concentration is decreased. The same result was obtained with subsaturating levels of inorganic phosphate: the maximum concentration of phosphoenzyme at saturating concentrations of lumenal Ca^{2+} decreases when the concentration of inorganic phosphate is less than optimal.

These results are consistent with the behaviour that is expected if there are lumenal binding sites for Ca^{2+} on the free enzyme (5), so that Ca^{2+} can bind to either the phosphoenzyme or the free enzyme; they are not expected if there are no lumenal binding sites for Ca^{2+} (Jenks *et al.* 1993).

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