MOLECULAR BASIS OF ENZYME CATALYSIS AND CONTROL

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ABSTRACT

Regulatory processes in living systems can be shown to be caused by ligand induced changes in the shape of a protein. By dividing the protein shape changes into molecular parameters a quantitative analysis of the regulation process can be obtained which is shown to correlate with actual data in living systems. The complexity of the analysis increases as the number of subunits in the enzyme and the number of ligands considered in the regulatory process increases. However, the principles are seen to be fundamentally similar in all cases. The orientation of reaction orbitals may be a key factor in the catalytic power of enzymes and in its regulatory control features.

When enzymes were first identified Berzelius and others recognized them as chemical catalysts. As their properties emerged, however, it was clear they had some special features not typical of other chemical catalysts such as acids and bases. One of these unusual properties was their specificity. The second was their enormous catalytic power. Conceptually these two processes are distinct. Experimentally there are some enzymes with high specificity and low catalytic power and some enzymes with high catalytic power and low specificity. Recent studies to unravel the fundamental nature of these processes have led us to the conclusion that there is an intimate connection between these unusual properties which may help to explain some of the unusual chemical and biological roles of these unique catalysts.

To explain the specificity of enzymes Emil Fischer proposed the fitting of a substrate to the surface of a protein in a template or keylock arrangement¹. The juxtaposition of a catalytic group with the bond to be formed or broken was essential and the rest of the molecule had to fit into a complementary surface to allow this juxtaposition. A combination of steric and electrostatic repulsions plus attractive forces in the substrate–enzyme contacts not directly involved in the bond breaking would thus explain this specificity of the enzyme. Some years ago it occurred to us that this classical theory was not sufficient to explain all the properties of enzymes and an induced fit theory was postulated². This theory retained the idea of a fit between substrate and enzyme but added the requirement that the substrate must
induce changes in the shape of the protein molecule to bring the catalytic groups into proper alignment for reaction. The role of the substrate as shown in Figure 1 was therefore more extensive and more active than that postulated earlier. If it did not induce the proper alignment of catalytic groups, it would not lead to reaction even if it were bound tightly to the protein surface.

Evidence to support this theory was obtained from many sources\(^3\) and has finally culminated in elegant crystallographic evidence of Lipscomb and co-workers in the case of carboxypeptidase\(^4\). In that enzyme direct movements of the catalytic groups involved in bond breaking have been observed—a tyrosine residue moving by as much as 15 Å and a carboxylic acid group by 2 Å.

One of the most important consequences of these ligand induced changes in the protein structure was the realization that such properties could explain a role for small molecules not themselves involved in the catalytic action of the enzyme but which could induce changes helping to regulate the velocity.
of the enzyme. If these molecules prevented or slowed down the enzyme activity, they were inhibitors; if they accelerated or allowed the action of the enzyme, they were activators. The role of such molecules is shown in Figure 2.

The manner in which such molecules can play a vital role in metabolism has now been documented extensively. It explains both the feedback inhibition of synthetic pathways and hormonal activation. It appears that ligand induced changes of the same type play a key role in the regulation of enzyme synthesis. Thus, inducers peel the repressor off DNA by ligand induced changes and co-repressors induce a conformation change which causes the repressor to bind to DNA. Ligand induced changes appear to be important in biological transport processes and have been postulated as a key source of the turn-on and turn-off signals of nerve receptors. Thus, there is evidence that these ligand induced changes play a wide variety of roles in biological systems. All these observations were consistent with the qualitative reasoning of a flexible enzyme but as always in science, one wishes ultimately to place qualitative arguments on a quantitative basis. Recently we have developed a quantitative theory to explain these ligand induced changes and I should like to describe that in the next part of my talk.

The keys to the way to express these changes in a quantitative manner arose from the phenomenon of cooperativity, a characteristic of regulatory proteins and a property originally discovered in haemoglobin. In Figure 3 is shown the difference between a classical Michaelis–Menten saturation curve or Langmuir isotherm and the sigmoid cooperative curve characteristic of haemoglobin and other regulatory proteins. The sigmoid nature of this curve was recognized by Bohr who discovered the phenomenon to be a
relationship in which the first molecule of ligand made it easier for the next molecule to bind. Hence the 'cooperative' process.

In explaining this phenomenon it must be remembered that haemoglobin is a protein composed of four subunits comprising two α chains and two β chains, respectively. Early workers recognized that some type of site–site interaction was probably involved in this cooperative process and postulated electrostatic or similar effects to explain this phenomenon. There were indeed four haeme groups in the molecule but a major difficulty arose as it became clear that the haeme groups were far from each other and therefore effects over great distances would have to be transmitted. The ligand induced conformation changes in the proteins allowed a more ready explanation for this anomaly and the model based on such changes is shown below. In Figure 4 are shown the changes which a monomer might undergo under the influence of a substrate molecule. Exactly the same type of changes, of course, could occur generated by either an inhibitor or an activator so we shall use the term ligand to refer to all three. We shall call the parameters K_s and K_i, 'molecular parameters' since they can be related directly to molecular events, i.e. the change in shape of the protein molecule and the binding of ligand to one or other of the two conformations. If the two steps proceed rapidly, it will, of course, not be possible to separate one pathway from the other. The product K_sK_i will of necessity equal K_s'K_i' and these will appear together as the single Michaelis–Menten constant, K_m, in the kinetics of simple enzymes.

In a multisubunit protein, however, a ligand induced distortion in one subunit can affect the interactions with neighbouring subunits as shown in Figure 5. The ligand induced changes may increase, decrease or leave unchanged the forces between neighbouring subunits. If there is no change
Figure 4. Illustration of energetic relationships in a monomer undergoing conformation changes induced by substrate: (a) Extreme pathways are $K_S K_f$ (initial protein conformation change followed by binding of substrate to the new conformation) and $K'_S K'_f$ (initial binding of substrates followed by protein conformation change). $K''_S K''_f$ is an intermediate type pathway involving simultaneous conformation change and binding followed $(K''_f)$ by further isomerization to final conformation. (b) Illustrative example of changes shown in Figure 4(a) in which $H$, $+$ and $-$ represent hydrophobic and electrostatic attractions between protein chains, substrate molecule and solution ions. It should be noted that the net bond formation involves only a single hydrophobic bond. Formation of product will be followed by dissociation of product and refolding of protein to initial conformation by an unfolding process similar to the folding.

in these forces, each subunit will act as if it were a monomer. If on the other hand the forces are changed, an added complexity is introduced and thus must be explained by the introduction of a 'subunit interaction term', $K_{AA}, K_{AB}, K_{BB}$ etc. to designate the change in interactions between adjacent peptide chains as the conformation of the individual subunits is changed. The equilibrium constant for the binding of a molecule of ligand to a dimer is thus shown in equation 1,

$$K_1 = 2K_{tAB} K_{SB} (K_{AB}/K_{AA}) [S]$$  

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utilizing the molecular parameters $K_t$ and $K_s$ but adding now a third molecular parameter to express the change in the interactions between adjacent subunits. In each of these designations the circle (or A conformation) will refer to the subunits in the absence of bound ligand and the square (or B conformation) refers to the subunit with ligand bound to it. $K_{AB}$ therefore describes the change in energy in the protein as the conformation changes from A to B. $K_{SB}$ describes the affinity constant of the substrate for the B conformation, and $K_{AB}$ the strength of interactions between two adjacent A and B subunits relative to adjacent A subunits ($K_{AA}$).

Exactly the same reasoning can be applied to proteins containing four subunits or any number of subunits and equations using these molecular
parameters to explain experimental data have been derived and are explained elsewhere\textsuperscript{10,11}. The fit of such curves to the haemoglobin data is shown in Figure 6. As can be seen, an excellent agreement between theory and data is obtained\textsuperscript{10}.

It is not sufficient to obtain an agreement between theory and experiment. One must in addition demonstrate that other theories are incompatible with the data. In the case of the haemoglobin binding the saturation curve correlating ligand bound to oxygen pressure is not sufficient to distinguish between theories since an alternate model derived by Monod, Wyman and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{comparison_of_square_case_with_4_experimental_haemoglobin_binding_curves.png}
\caption{Comparison of square case with 4 experimental haemoglobin binding curves}
\end{figure}

Comparison of square case with 4 experimental haemoglobin binding curves

Curves calculated using only $R_S$ and $S_{0.5}$

A= Rossi, Fanelli et al.- Human Hb- low $\mu$. C= Roughton et al.- Sheep Hb.
B= Rossi, Fanelli et al.- Human Hb- high $\mu$. D= Lyster et al.- Horse Hb.

Figure 6. Application of the saturation equation to the 'square' geometry to the oxygen binding equilibrium of a haemoglobin.

Changeux\textsuperscript{12} gives an equally good fit to the oxygen binding data. Other tools must then be applied to distinguish between the models and this has been done in several ways. A spin label attached to haemoglobin molecule was the device used by McConnell and co-workers\textsuperscript{13}. Direct observation by nuclear magnetic resonance was utilized by Shulman and co-workers\textsuperscript{14} and study of the reactivity of sulphhydryl groups was pursued by Brunori \textit{et al.}\textsuperscript{15}. In all cases the same conclusion was reached, i.e. that the ligand induced model as shown above was correct for this protein.

In our laboratory we have also devised a diagnostic test which we applied to haemoglobin and I would like to describe it to you because it is capable
of distinguishing between the models and also can reveal some features of
the strengths of the subunit interactions.

The rationale for this approach is shown in Figure 7. By converting $\alpha$
chains to a cyanomet form which has the same structure as the oxygen
bound form, one can 'freeze' two of the subunits in the square or B con-
formation as shown in line 1. It can readily be seen that the binding of
oxygen to the remaining two subunits will produce a ratio between the two
constants which is related by the product of the statistical factor four and
the change in the energy of the interactions between the like subunits
($\alpha-\alpha$ or $\beta-\beta$) to which the oxygen is bound. Thus, in the case in which the
$\alpha$ subunits are 'frozen', the $K_1/K_2$ ratio is related to the $\beta-\beta$ interactions.

\[
\begin{align*}
(a) & \quad \frac{K_1}{K_2} = \text{constant} \\
(b) & \quad \frac{K_1'}{K_2'} = \text{constant}' \\
(c) & \quad \frac{K_1}{K_2} = \text{constant} \\
(d) & \quad \frac{K_1'}{K_2'} = \text{constant}' \\
(e) & \quad 1 = \alpha_1 - \beta_1 \\
& \quad 2 = \alpha_1 - \beta_2 \\
& \quad 3 = \beta - \beta \\
& \quad 4 = \alpha - \alpha
\end{align*}
\]

Figure 7. Schematic illustration of the use of frozen conformational states to determine subunit
interactions. On line 1 is shown a tetrameric protein with $\alpha$ subunits oxidized (illustrated by +)
so that they are frozen in a conformation which mimics the oxygen liganded state. Binding of
oxygen to this protein will involve the same $\alpha-\beta$ subunit changes in each step but will involve
different $\alpha-\alpha$ interactions in step 1 than in step 2. On the second line is the same protein in which
the $\alpha$ subunits are frozen in the form which mimics the oxygen liganded state in which case the
$\beta-\beta$ interactions vary from step 1 to step 2. In line 3 is the situation if the concerted model is
followed in which case the constants for the bonding of ligand will be related simply by statistical
factors. In line 4 the same situation is shown when the other subunits are frozen. The $K$s in lines
3 and 4 do not necessarily have to be equal since the net affinity $\alpha$ subunits may not be identical
to $\beta$ subunits but the Hill slopes in both of these plots must be 1.0 whereas on the two lines they
may differ from 1.0.
In the case in which the β subunits are frozen the change in interaction energy occurs between the α-α subunits. If on the other hand the haemoglobin follows the symmetry model of Monod, Wyman and Changeux, it can be readily seen (on the next two lines of Figure 7) that the binding constants for the first and second molecules of oxygen should be related by the statistical factors four only. The appropriate haemoglobins were synthesized by Dr Haber and appropriate oxygen binding curves were observed. The experiments led to the following conclusions. (1) The protein must follow the sequential model since the $K_1/K_2$ ratios were different from four, a situation incompatible with the symmetry model. (2) A direct β-β interaction occurs whereas there is no change in α-α interactions. The x-ray crystallographic data of Perutz and co-workers found that there could be contacts between the β subunits. Our results indicate that there is no change in energy in the α-α interactions. We do not say whether there are any α-α contacts, merely that if there are such contacts, they do not change during the oxygen binding phenomena. (3) From an evaluation of the binding curve a value of 700 calories is calculated for the change in the β-β energies. (4) Using similar procedures one can evaluate the strength of the $α_1-β_1$, $α_1-β_2$, α-α and β-β interactions.

Thus, this method of freezing subunit conformations and then comparing the properties of the molecule is a way in which models can be identified and also the strength of subunit interactions can be placed on a quantitative basis. It is particularly important to evaluate these molecular parameters in proteins like haemoglobin since the detailed x-ray crystallography allows us to use these quantitative interactions to understand the nature of protein design.

The procedures utilized above can then be extended to more complex systems in a slightly different way. In Figure 8 the binding of a molecule of ligand to a tetrahedral protein is shown. In this case the equilibrium constants, $K'_1$, $K'_2$, $K'_3$, $K'_4$, are the intrinsic constants in which the statistical factors are corrected out to make the arguments simpler. On the first line is shown the case in which there are no conformational changes and therefore the binding constants to each subunit will be the same if those constants are corrected for statistical factors. A Michaelis–Menten type binding curve would be obtained. On the other hand the ligand induced model will give binding constants which are related to each other by the molecular parameters discussed above. Only if these constants become unity will the four values equal each other. In the very simple case which we call our simplest sequential model it can be seen there is a relationship between the constants, $K'_1K'_4 = K'_2K'_3$. Similarly in the Monod model the four constants have a fixed relationship with each other, i.e. $K'_1 < K'_2 = K'_3 = K'_4$. Similar derivations can be made for other cases.

Dr Cornish-Bowden has recently developed a method for statistical curve fitting utilizing such equations and thus evaluating the individual constants. Even in a cooperative protein where the binding of a second molecule may start before the binding of the first molecule is completed, the method factors out the four constants. Data for one case, yeast glyceraldehyde 3-phosphate dehydrogenase, are shown in Figure 9. In this case neither the simplest sequential model as shown above nor the symmetry model of Monod or
the classical Michaelis–Menten equations fits the data. The model which fits is the general ligand induced model in which the ligand induces conformation changes but instead of changing the subunit to which ligand is bound only it changes the subunit to which ligand is bound but also distorts neighbouring chains as shown in Figure 1019. In this case the evaluation of the individual constants showed a mixture of positive and negative cooperativity in which the first molecule makes it easier for the second to bind, the second more difficult for the third, and the third more difficult for the fourth. We have now applied this method to a number of different proteins and have found all together at least thirteen proteins which fit our general ligand induced model. In one protein, haemoglobin, the simplest ligand induced model fits the data. It is quite clear therefore that this general model is widely applicable and it appears that the quantitative behaviour of most regulatory proteins can be explained on the basis of these molecular parameters. Moreover, the methods developed to explain the regulatory proteins which are catalysts seem to be applicable to other types of regulatory proteins such as the repressors, the transport proteins and sensibly the nerve receptors.

Another question which arose as the study of these conformational changes progressed was: ‘How big does a conformational change have to be in order to turn-off or turn-on an enzyme?’ The conformational changes observed by Lipscomb on carboxypeptidase were very large but changes in some other proteins have been small. Small changes or non-detectable changes might indicate that some proteins follow the template hypothesis of
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Figure 9. Illustration of curve fitting procedures for binding data of NAD to yeast glyceraldehyde 3-phosphate dehydrogenase. Heavy line indicates computer selected best fit assuming both positive and negative cooperativity during binding. Dotted line indicates best fit assuming concerted model of Monod, Wyman and Changeux. Dashed line indicates best fit with simplest sequential model using square geometry.

Figure 10. Ligand induced sequential model in a tetrameric protein. Ligand distorts neighbouring subunit as well as subunit to which it is bound.

Fischer. It was also possible, however, that all of these proteins were undergoing ligand induced changes but some of these changes were escaping detection because of the low resolving power of the methods available to study protein conformation changes. The study of the required size of the conformation change has led us into an exciting area which has revealed a clue to explain the source of the special catalytic power of enzymes.

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The size of this catalytic power is illustrated in Table 1 where the turn-over numbers of several enzymes are compared with a known organic mechanism. The enormous factors are a typical feature of enzymes. In fact almost all enzymes share this enormous discrepancy between the turnover number of enzyme and the catalytic power of the best known non-enzymatic catalysts.

| Enzyme         | Type of reaction    | $V_0/V_0$ | Contribution of
type of reaction | Unaccounted factor |
<table>
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<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>Acetal hydrolysis</td>
<td>$10^6$</td>
<td>0.3</td>
<td>$3 \times 10^6$</td>
</tr>
<tr>
<td>Aldolase</td>
<td>H exchange</td>
<td>$10^3$</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>Fumarase</td>
<td>Hydration of olefin</td>
<td>$10^{11}$</td>
<td>300</td>
<td>$3 \times 10^8$</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Hydrolysis of an amide</td>
<td>$10^2$</td>
<td>$5 \times 10^{-3}$</td>
<td>$5 \times 10^5$</td>
</tr>
</tbody>
</table>

Of course, it is well known that enzymes attract substrates to the surface of the protein and therefore it might be argued that increases in the number of collisions may explain the enormous catalytic power. A method has been devised for tabulating the increased effect of such maintained juxtaposition which we call the proximity effect. This proximity factor will be simply 55 times the velocity calculated at 1 M concentrations of the reactants. A factor of 55 will, of course, be introduced for each additional substrate after one and for each catalytic group. Even after this correction is made, however, the enzymes are still many orders of magnitude more reactive than the organic analogue.

It occurred to us that the big gap between the velocity of enzymes and the velocity of the best organic analogue could be rationalized if, in addition to proximity effects, a very delicate orientation of catalytic groups had to be maintained. The pie-shaped wedges in Figure II indicate schematically the portion of the reaction atoms over which reaction could occur. If these symbolic pie-shaped wedges are very large as indicated in this figure, the improved orientation on the enzyme surface relative to the orientation in bimolecular reactions in solution will not be very great. In that case the orientation factors might be in the neighbourhood of two to ten, factors which would reduce the discrepancy between enzymatic and non-enzymatic velocities but would fall far short of explaining them. On the other hand, if these pie-shaped wedges were very small, the orientation factors in a precisely oriented enzyme could be very large.

How could one evaluate the size of these orientation factors? The method devised was to study a very well known reaction and control the angle of approach of the reacting atom. In this case the compounds shown in Table 2 were investigated by Dan Storm and the relative rates of esterification in a bimolecular reaction and the intramolecular reaction of the synthesized compounds were studied. Studies of Pauling, Woodward, Pitzer and many others have suggested a directional nature to electron orbitals. Since
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Schematic representation of the proximity and orientation of substrates and catalytic residues at a hexokinase active site

Figure 11. Schematic illustration of two substrates and two catalytic groups reacting at an active site. For illustrative purposes the reaction is illustrated as though the substrates were ATP and glucose and the catalytic groups were histidine acting as a base and aspartic acid acting as an acid. The reacting atoms are circled. The pie-shaped wedges illustrate in two dimensions the fraction of solid angle of the atom designated over which reaction can occur.

Table 2. Relative rates of esterification with ethanol

<table>
<thead>
<tr>
<th>Acid</th>
<th>Observed rate $k_{eq}$, mole$^{-1}$ min$^{-1}$</th>
<th>Correction factors</th>
<th>Corrected $k$</th>
<th>Relative rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$COOH</td>
<td>1.09 x 10$^{-3}$</td>
<td>55.5 (proximity)</td>
<td>$6 \times 10^{-2}$</td>
<td>1</td>
</tr>
<tr>
<td>I</td>
<td>8.6 x 10$^{-2}$</td>
<td>64 (non-bonded interaction)</td>
<td>5.5</td>
<td>89</td>
</tr>
<tr>
<td>II</td>
<td>0.34</td>
<td>0.34 (non-bonded interaction)</td>
<td>32</td>
<td>540</td>
</tr>
<tr>
<td>III</td>
<td>7.2</td>
<td>4.4 (non-bonded interaction)</td>
<td>1120</td>
<td>18600</td>
</tr>
<tr>
<td>IV</td>
<td>1120</td>
<td></td>
<td>1120</td>
<td>18600</td>
</tr>
</tbody>
</table>

I

II

III

IV

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quantum mechanics is not yet in a state which allows us to calculate interactions with such complex molecules, we could not a priori know which of the molecules would give fast reaction or if any of them would. We could only postulate that, if the angles were constrained and different in the various cases, some of these compounds might show enormous velocities relative to others. We could show from model studies that indeed some were constrained and it was in fact found that some of them gave extraordinarily high velocities, $10^6$ times the bimolecular rate at 1 M concentration in one case. By appropriate controls it could be shown that ring strain, compression, solvation, torsional angles, non-bonded interactions, and conformational isomers could not explain the discrepancy. The remaining hypothesis which could explain the discrepancy in these intramolecular compounds was the fact that a very high precision in the angle of attack was required.

It should be emphasized that the orientation factor we have been considering right from the start of this particular discussion is not the orientation of two reacting atoms already placed in juxtaposition, it is not the orientation of the entire molecules, e.g. glucose, relative to the entire second substrate molecule, e.g. ATP, but rather the orientation of the reacting oxygen relative to the orientation of the reacting phosphorus. A precision of this order of magnitude therefore must reflect the electron orbitals of the atoms and is not due to an overall steric hindrance in the molecule itself.

It was further reasoned that if such high precision was obtained, substitution for sulphur versus oxygen might in some cases lead to a serious change in the rate, and this was indeed observed by the thiolactones relative to the oxygen content. Studies are now in progress with other reactions.

Further support that this 'orbital steering' plays a key role in enzyme action is obtained from the modification of an oxygen group in the enzyme subtilisin converting the serine at the active site to a cysteine residue. It was shown chemically that this was the only change occurring in the molecule and solution physicochemical studies showed no change in the shape of the protein. This has been confirmed and extended by the crystallographic study of Kraut and co-workers who find no change in any of the positions of the atoms except that the sulphur has replaced the oxygen at the active site. This simple change was found to turn-off enzyme activity completely. Such an inhibition of enzyme activity would not be expected from non-enzymatic reactions in which the sulphur analogue reacts as readily as the oxygen compound. It is expected, however, if the enzyme is designed precisely for the electron orbitals of oxygen but cannot tolerate a change to the larger sulphur atom.

The precise orientation of catalytic groups suggested by this 'orbital steering' hypothesis is a possible solution to the enigma of enzymes, the source of their enormous catalytic power. Interestingly enough it also explains how allosteric effectors can exert regulatory control and how specificity properties can also be explained. If the enzyme is flexible and the alignments required are precise, a very small induced change can make a big difference. A change which just brings the groups into proper alignment activates the enzyme. A change which destroys the alignment inhibits it. It appears therefore as if the catalytic power, the specificity and the control properties of enzymes are all closely related.
REFERENCES

26. J. Kraut, Personal communication.