Protein-Ligand Interactions

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Proceedings of a Symposium held at the University of Konstanz, West Germany, September 2–6, 1974

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Walter de Gruyter · Berlin · New York 1975

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Cover design: R. Hübler. – Printing: Druckerei Gerike, Berlin. – Binding: Mikolai, Berlin. – Printed in Germany
ISBN 3 11 004881 7

Preface

The present volume contains the proceedings of a symposium on "Protein-Ligand Interactions" held at the University of Konstanz, West Germany, on September 2–6, 1974.

Protein-ligand interactions are of great biological significance and cover a wide spectrum of systems. They range from respiration to the action of receptors and repressors and to the subtle influence of electrolytes on proteins. The last decade has witnessed great progress in a molecular approach to biopolymer-small molecule interactions, which includes the application of thermodynamics, quantum mechanics and modern kinetics to protein-ligand interactions, particularly to enzymatic reactions.

Symposia or congresses usually are devoted either to a large variety of subjects which are only loosely connected, or they are highly specialized in a narrow field. The basic idea of the present Symposium has been to assemble both biologically and physicochemically oriented scientists in order to discuss current topics in the wide field of protein-ligand interactions. It has been the organizers' hope to promote an interdisciplinary interaction and stimulation of ideas between scientists looking at specific problems from different viewpoints. It is for this reason that the discussions at this Symposium have had no time limit placed on them, as far as this is possible.

Photoprints of the submitted typescripts were circulated among the participants before the Symposium in order to facilitate the progress of the proceedings and discussions. The discussions were not directly recorded. The participants were requested to provide a written report of what they considered worth including in the publication. This information is presented in this volume.

As usual for symposia, there was no refereeing of the papers submitted, the contents of which are solely the authors' responsibility.

As a natural consequence of the aims of this meeting, the number of participants had to be severely limited. A large number of outstanding workers in the fields discussed, unfortunately, could not be heard directly. We hope that the reading of these proceedings will, nevertheless, provide some stimulus and inspiration for future research also for those who did not attend the meeting.

Our appreciation is extended to all participants who have cooperated so effectively in order to promote this Symposium and publication of its proceedings. Special thanks are due to the Stiftung Volkswagenwerk, whose generous financial support has made this Symposium possible. The support given by the University of Konstanz, where the meeting took place, by the Gesellschaft der Freunde und Förderer der Universität Konstanz and by the Byk Gulden Lomberg Chemische Fabrik GmbH, Konstanz, is gratefully acknowledged.

We would like to acknowledge the cooperation of the staff of the Verlag Walter de Gruyter, Berlin. We are also very grateful for Mrs. S. Lau's devoted assistance in all matters concerning the Symposium.

In conclusion, the editors would like to express their hope that meetings of a similar kind on various biological and biophysical topics of interest will be held in the future and will be instrumental in the promotion of new ideas and approaches in the natural sciences.

Konstanz, September 8, 1974

H. Sund
G. Blauer

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Section I. General

Chairman: *J. P. Changeux*

Rome, June 21, 1974

To Members of the Konstanz Symposium:

Allow me to say how much I appreciate having been asked to participate in the Konstanz Symposium on Protein-Ligand Interactions, and how much I regret not being able to be present. My absence is due to involvement in a program of IUPAB lectures in Eastern Europe which come just at the same time and for which I am responsible. Not being able to take part in person, I was planning to submit a general introductory paper to be read by someone else. Unfortunately, however, I fell ill just at the time I had reserved for preparing it; as a result, I am substituting the rather more specialized article enclosed. It represents a joint effort with two of my colleagues in Rome, Prof. M.Brunori and Mr. A. Colosimo.

As it is, I like to think of some of the things I might have talked about had I been present. I might, for instance, have developed and amplified the concept of linkage, both homotropic and heterotropic, which is so basic to the question of regulation in biological macromolecules, showing how very simply it emerges from the concept of the Binding Potential. Thence I might have gone on to point out that the Binding Potential itself, in which the variables are the chemical potentials μ of all the components except the reference component (e.g. the macromolecule), actually corresponds to only one member of a family, or set, of potentials in each of which the variables consist of an arbitrary combination (or mixture) of μ's and the corresponding n's (n denoting the total amount of a component). These mixed potentials are applicable to experiments in which certain components are maintained at constant μ (this means that the system is open with respect to those components), while others are maintained at constant n (this means that the system is closed with respect to those components). They can all be derived from the total energy of the system (or from one another) by a Legendre transformation, just as the Hamiltonian function can be derived from the Lagrangian one in classical mechanics. They are $2^r - 1$ in number, where r is the total number of components (including the reference component) and the -1 arises from the special property of the energy as a first order homogeneous function of its variables (entropy, volume, and the n's).

Another line of thought, possibly of greater interest at this meeting, would have been a review of the allosteric concept as a mechanism to account for the homotropic and heterotropic linkages exhibited by large working proteins and other macromolecules in which the sites are so far apart that the possibility of direct interactions between them can be ruled out. In nearly all cases of polyfunctional molecules the <u>binding potential</u> may be formulated in terms of the logarithm of a <u>binding polynomial</u>, which is in essence a macroscopic form of the grand canonical partition function of statistical mechanics: it shows how the ligands are partitioned among the

various forms which the molecule assumes in their presence and provides an admirable means of getting an overall view of the response of the molecule to its environment. In the case of an allosteric system the binding polynomial assumes a rather special form, whose factorability or non factorability is a criterion of heterotropic or homotropic interaction between sites (as indeed it always is). And then, passing on from this, it might have been interesting to point out how the binding polynomial can be formulated equally well either in terms of the free energy changes and equilibrium constants of individual binding events or alternatively in terms of probabilities and conditional probabilities of site occupation. (The probability approach is the subject of a recent paper by Paul E. Phillipson and myself which will soon appear in the Proceedings of the National Academy of Sciences).

Still one more topic might have been the linkages which can arise in a polyfunctional macromolecule, such as an enzyme, which is not in equilibrium but only in a steady state in relation to its environment. Such a system can be represented formally by a multidimensional cube in which various closed pathways have an element in common. This provides a picture of how, as a result of the steady flow of matter through the system and the consequent steady liberation of energy, one process can drive another.

Of course, even had I been present, I could not have hoped to deal with all these topics, but at least I could have touched on some of them in a more lively and personal way than is possible on paper. All the same I do hope that the somewhat more specialized communication I am submitting may not be wholly without interest.

With best wishes for the success of the meeting and deep regrets at not being there.

Jeffries Wyman

Concerted Changes in an Allosteric Macromolecule

Alfredo Colosimo, Maurizio Brunori and *Jeffries Wyman*

ABSTRACT

This paper reports an analysis of the behaviour of allosteric macromolecules in the framework of the simple Monod-Wyman-Changeux model. The emphasis is on the analysis of the reciprocal influence of the various parameters which enter into the model, with particular reference to the relationship among a number of sites in the macromolecule and the Hill parameter n. This appears of interest for the behaviour of some of the extremely large respiratory proteins containing up to 100, or more, oxygen binding sites, where a very large value of n is often coupled with a relatively small value of the total interaction free energy.

Cooperative binding of a ligand by a macromolecule is conveniently described by a Hill plot in which $\ln(\bar{x}/1-\bar{x})$ is represented as a function of $\ln x$, where $\bar{x}$ is the fractional saturation of the macromolecule with ligand X and x is the ligand activity. Such a graph must always have an asymptote of unit slope at each end unless the interaction between the sites is infinite. The minimum value of the total interaction free energy realized per site in saturating the macromolecule with the ligand is then given by RT $\Delta\ln x$, where $\Delta\ln x$ is the horizontal distance between the asymptotes. Similarly and more generally, the minimum value of the interaction free energy realized in passing from *any* degree of saturation $\bar{x}_1$ to $\bar{x}_2$ is given by the horizontal distance between the lines of unit slope drawn through the two points $[\ln x_1, \ln(\bar{x}_1/1-\bar{x}_1)]$ and $[\ln x_2, \ln(\bar{x}_2/1-\bar{x}_2)]$. At the same time the point value of the interaction free energy at any degree of saturation is given by

$$\frac{RT}{\bar{x}(1-\bar{x})}\left(1 - \frac{1}{n}\right) = \Delta F_I$$

where n is the slope of the Hill plot at the point in question [1]. The earlier propositions just enunciated are obtained from this by integration; and it will be seen that whenever $n > 1$ anywhere, there must be positive (or stabilizing) interactions between sites, *i.e.* the system is cooperative. The parameter n can of course never exceed the number of sites, and when it is equal to the number of sites it means that the interaction free energy between the sites is infinite, *i.e.* that the sites are so strongly coupled that they all react simultaneously to give an n-th order reaction. It should perhaps be pointed out that n is a statistical quantity and that even when n goes to infinity the point value of the interaction free energy is only $\Delta F_I = RT/\bar{x}(1-\bar{x})$.

Cooperativity in biological macromolecules is often the result of conformational (or allosteric) changes induced by the ligands; in other words, it is the expression of ligand-linked conformational equilibria. It is of interest to see how this shows up in the Hill plot, and what the actual behaviour of some of the very large macromolecules as represented in their Hill plots implies as to the underlying conformational changes. Of particular interest here are some of the extremely large respiratory proteins, containing up to 100 or more sites for oxygen, where a very large value of n is often coupled with a relatively small total interaction free energy, *i.e.* where the Hill plot shows a rather sharp upward bend or kink in the middle range of saturations associated with rather closely spaced asymptotes.

The two most commonly invoked allosteric models are the Monod-Wyman-Changeux (M.W.C.) and the Koshland-Nemethy-Filmer (K.N.F.) models, both of which can be shown to be special cases of a more general parent model [2]. Of these, the M.W.C. (or concerted) model is the simplest and involves the smallest number of parameters (only 2). At the same time it offers the greatest possibility of a large but localized value of n coupled with a relatively small value of $\Delta F_{I(total)}$. It is this model, therefore, which we shall consider in what follows.

In the M.W.C. model [3] it is assumed that the macromolecule exists in only two forms (T and R), in each of which the ligand binding sites are all alike and independent. The binding polynomial (or generating function) for this model is given by

$$(1) \quad P = (1+x)^r + L_0(1+\alpha x)^r$$

where r is the number of sites, the same in each of the two conformations, L_0 is the equilibrium constant for the T→R transition in the absence of ligand (*i.e.*, L_0 = R/T when $x = 0$) and α is the ratio of binding constant K for a site in the R form to that of a site in the T form ($\alpha = K_R/K_T$).

In this model the value of $\bar{x}$, the fractional saturation of the macromolecule with ligand, is given by

$$\bar{x} = \frac{1}{r}\frac{d \ln P}{d \ln x} = \frac{1}{r}\frac{P'}{P}$$

where

$$(2) \quad P' = \frac{d P}{d \ln x} = r[(1+x)^{r-1} x + L_0(1+\alpha x)^{r-1}\alpha x]$$

At the same time n is given by

$$(3) \quad n = d \ln(\bar{x}/1-\bar{x})/d \ln x = \frac{P''}{P'} - \left(\frac{rP'-P''}{rP-P'}\right)$$

where

$$P'' = \alpha P'/d \ln x = P' + r(r-1)[(1+x)^{r-2}x^2 + L_0(1+\alpha x)^{r-2}\alpha^2 x^2]$$

Equation (3) may also be written as

$$(4) \quad n = \left(\frac{P''}{P'} - r\bar{x}\right)/(1-\bar{x})$$

Further, the total apparent interaction energy is given by

$$(5) \quad \Delta F_{I(total)} = RT \ln C$$

where

$$(5.1) \quad C = \frac{(1+L_0)(1+L_0\alpha^r)}{(1+L_0\alpha)(1+L_0\alpha^{r-1})}$$

In carrying out the analysis it is convenient to treat C and α as the independent variables and then, on the basis of (5.1), to obtain L_0 by the relation

$$(6) \quad L_0 = (B \pm \sqrt{B^2 - 4A})/2A$$

where

$$(6.1) \quad A = \alpha^r, \; B = \frac{1-C(\alpha+\alpha^{r-1})+\alpha^r}{C-1}$$

These equations make it possible to calculate the Hill plot (or the corresponding binding curve), of the macromolecule for various sets of values of r, C, α, and L_0. What we are primarily interested in is of course the sharpness of the transition in relation to the spacing of the asymptotes, *i.e.* n in relation of $\Delta F_{I(total)}$ as given by C. As we shall see, for the sufficiently large values of r the transition can be extremely sharp for any value of $\Delta F_{I(total)}$, even a very small one, with the result that we run into something approaching a phase change. Although detailed calculations can be made very easily on the basis of the above equations with the aid of a computer, still, as a first step, it is convenient to get an analytical solution for the special case where we impose the conditions of symmetry of the binding curve. This brings the transition into the middle of the Hill plot, where $\bar{x} = 1/2$; also it reduces the number of parameters to one (α) and greatly simplifies the equations.

The conditions of symmetry of the binding curve (or of the Hill plot, for they are the same) can be shown to be met provided only we set

$$(7) \quad L_0 = \alpha^{-r/2}$$

For this value of L_0, the value of the median ligand activity, x_m, now equal to $x_{1/2}$ (for which $\bar{x} = 1/2$), is given by

$$x_m = x_{1/2} = \alpha^{-1/2}$$

TABLE I

Dependence of n_m on the number of binding sites r, calculated from Eq. (11) for two values of α.

a) $\alpha = 9, \frac{1+\alpha}{(1+\alpha^{1/2})^2} = 0.625$

r	n_m	C	$\Delta F_{I(total)}$[cals]
4	1.75	7.5	1,195
6	2.26	8.8	1,290
8	3.76	9	1,315
50	11.2	9	1,315
100	24.0	9	1,315

b) $\alpha = 100, \frac{1+\alpha}{(1+\alpha^{1/2})^2} = 0.835$

r	n_m	C	$\Delta F_{I(total)}$[cals]
4	3.01	98	2,700†
6	4.35	100	2,730
8	5.69	100	2,730
50	33.8	100	2,730
100	70.0	100	2,730

†This corresponds very closely with the case of human hemoglobin, for which the binding curve is approximately, if not exactly, symmetrical (see Antonini and Brunori for review [4]). The result is an argument for the applicability of the concerted model to that molecule. The idea that symmetry of function might be associated with symmetry of structure was suggested many years ago [5].

At the same time the equilibrium constant L for the T→R transition, which in the general case is given by

(8) $L = L_0[\frac{1+\alpha x}{1+x}]^r$

becomes

$$(9) \quad L = \left[\frac{\alpha^{-1/2} + \alpha^{1/2}x}{1+x}\right]^r$$

and at the mid point of the binding curve, where $x = \alpha^{-1/2}$, L is unity. At this point the value of n given by Eq. (4) is

$$(10) \quad n \equiv n_m = 2\left[1+(r-1)\frac{(1+\alpha)}{(1+\alpha^{1/2})^2}\right] - r$$

Furthermore

$$(11) \quad C = \frac{2 + \alpha^{r/2} + \alpha^{-r/2}}{2 + \alpha^{(r-2)/2} + \alpha^{-(r-2)/2}}$$

We adopt the convention that $\alpha > 1$, which means that $L_0 < 1$†. It follows from (11) that for sufficiently large values of r, C becomes equal to α. Indeed for $\alpha = 10$ this is already approximately true even for $r = 4$. At the same time the expression $(1+\alpha)/(1+\alpha^{1/2})^2$ in the equation for n_m increases rather slowly from 0.625 to 1 as α goes from 9 to ∞. It is clear therefore that under conditions in which we are interested n_m will be linear in r regardless of the value of C [or ΔF_I^*] and will increase without limit as r increases, with the result that the transition becomes a true phase change. This is brought out by the figures shown in Table I (a and b). Another aspect of the situation is revealed in Fig. 1, which shows how the value of n_m/r, *i.e.* the value of n_m, normalized with respect to its maximum value r, increases toward 1 as $\Delta F_{I(\text{total})}$ increases indefinitely. It will be seen that the curves for different values of $r \geq 4$ all lie close together and approach a common bounding curve as r gets bigger and bigger.

It is a simple matter, with the aid of a computer+, to construct full Hill plots for various cases. Such plots (up to r = 36) are shown in Fig. 2. It is also a simple matter to construct Hill plots for more general cases where the constraint of symmetry is relaxed. Such plots are shown in Fig. 3, where the transition (or switch over point, defined as the point

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† The opposite assumption would of course give the same results.

* total

+ Computations were made with a Hewlett Packard Model 9830A computer.

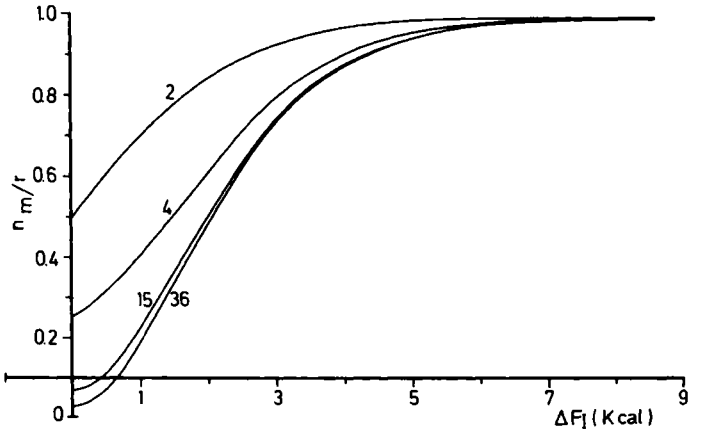

Fig. 1. Values of n normalized to its upper limit r (corresponding to $\Delta F_{I(total)} \to \infty$), *i.e.* n_m/r as a function of $\Delta F_{I(total)}$[cals] for the different values of r shown on the curves. This is for the symmetrical case.

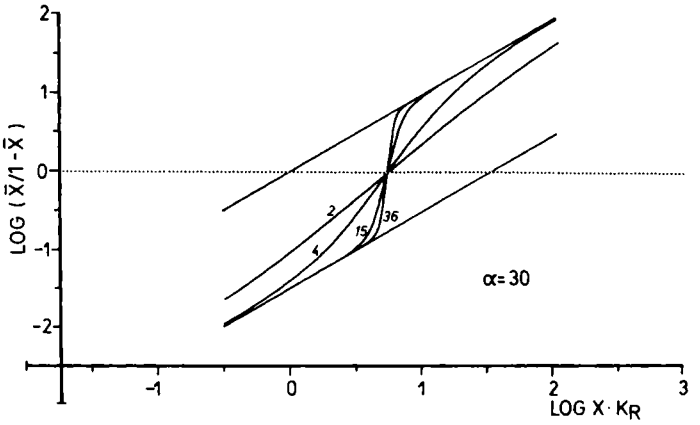

Fig. 2. Hill plots for the symmetrical case calculated for the constant $\alpha = K_R/K_T = 30$ for the different values of r shown on the curves. Except for r = 2, where $\Delta F_{I(total)} = 1235$ cals, these curves all correspond to $\Delta F_{I(total)} = 2000$ cals within 1 per cent.

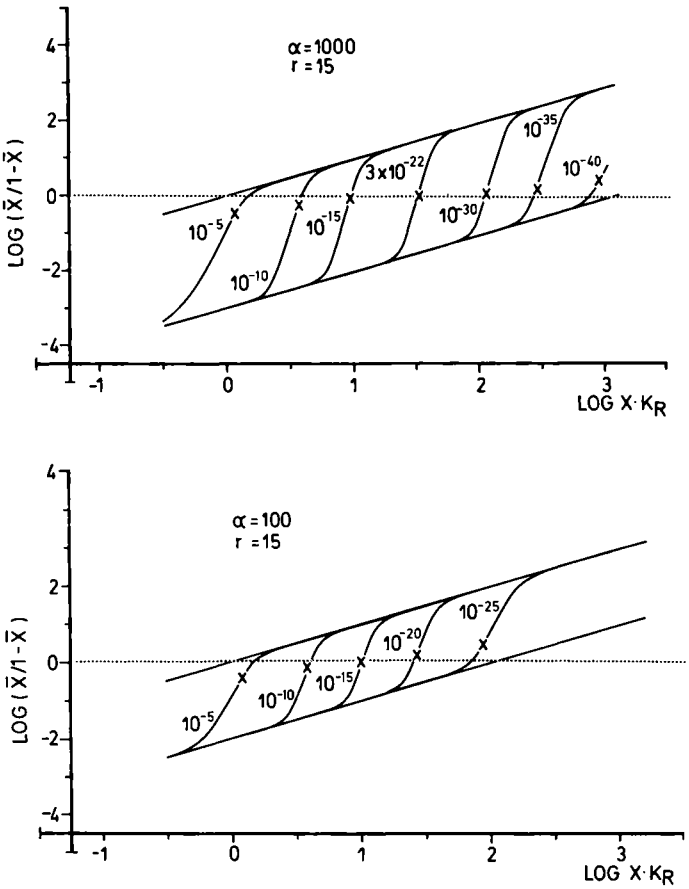

Fig. 3. Hill plots for the more general case where the symmetry condition is relaxed. α = 1000 for top curves and α = 100 for bottom curves. In both cases r = 15 and the values of L_0 are those shown on the curves. Asterisks indicate switch over point. Here we have defined the switch over point as the point where the amounts of the T and R forms are equal. Alternatively we might have defined it as the point where the amounts of *ligand bound* by the T and R forms are equal. The two points are not the same. The former corresponds to L = 1, the latter to $Y_T = Y_R L$.* The latter definition is more apposite to the analysis of kinetic experiments.

* Y_T and Y_R are the fractional saturation of the T and R states.

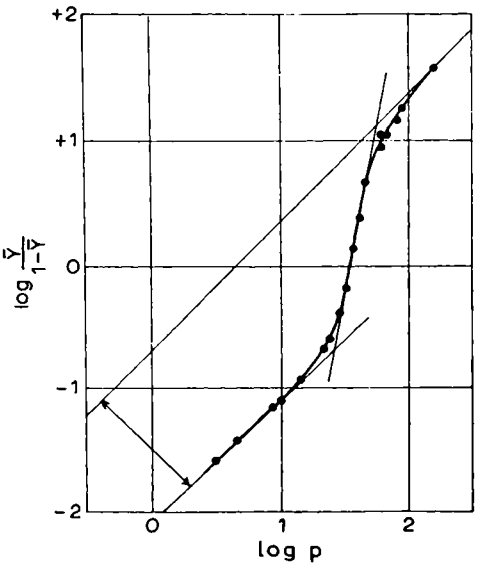

Fig. 4. Hill plot for oxygen binding by the blood of Spirographis spallanzanii at 20° and pH 7 (from Antonini *et al.*, ref. 6).

where R/T = L = 1) is no longer centered at $\bar{x} = 1/2$. It is interesting to see how, as we depart more and more from the condition of symmetry, the value of n, even at its greatest, decreases slowly but progressively while the region of high values of n moves farther and farther from the point $\bar{x} = 1/2$, following the switch over point, and eventually comes to be outside the experimentally accessible range of saturation.

The chlorocruorin from the worm Spirographis spallanzanii, which has a molecular weight of ∿ 2,500,000 and contains approximately 72 oxygen binding sites (hemes) per molecule, provides a good example of one of the larger respiratory proteins [6]. Its Hill plot, given in Fig. 4, shows a maximum value of n = 5, which occurs at $\bar{x} \simeq 0.64$, and a value of $\Delta F_{I(total)}$ = 1800 calories, corresponding to C = 25. A glance at Fig. 1 shows that these results are incompatible with the assumption that the whole molecule with its 72 sites behaves like a simple M.W.C. model. If so, the value of $\Delta F_{I(total)}$ = 1800 cals would imply a value of $n_m/r = 0.45$ or $n_m \simeq 72 \cdot 0.45 = 32.4$ as compared with the observed value of 5. We might, as the next

simplest hypothesis, assume that the sites interact in independent constellations, each containing r sites. The value of n_m would then be the same as that for a constellation, and we could calculate the value of r, assuming the M.W.C. model to be applicable to each constellation, either with the aid of Fig. 1 or, more directly, by rewriting Equation (10) in the form

$$(12) \quad r = (n_m+2\lambda-2)/(2\lambda-1)$$

where $\lambda = (1+\alpha)/(1+\alpha^{1/2})^2$

The answer is 10. An alternative hypothesis would seem to be that the interactions involve all the sites, diminishing progressively in some manner with distance. A special case would be that of a number of small constellations of strongly interacting sites with secondary interactions between the different constellations - a kind of hierarchy of interactions. This is an idea which finds an echo in the complicated symmetrical structures revealed by electron microscopy [7].

Very similar considerations apply to an even larger molecule, namely the hemocyanin of Helix pomatia, whose molecular weight is of the order of $9 \cdot 10^6$ and which contains $\sim$ 180 oxygen binding sites. Here the value of n_m is approximately 5. In this case it is found that when, in the absence of calcium, the molecular weight drops to (1/10) its full value, all cooperativity is lost although the (1/10) subunits each still contain 18 sites [10]. It would seem that the integrity of the whole molecule is a condition of cooperativity.

It will be seen from this analysis that the M.W.C. model carries, buried within it, the prediction of a ligand linked phase change which is realized more and more completely as the number of sites increases indefinitely. That this is so should not be altogether surprising when it is realized that one of the primary features of the model is the exclusion of all mixed conformations; in applying the model we are in a sense treating the macromolecule as a crystal, and in this connection it is worth recalling that the liganded and unliganded derivatives of hemoglobin crystallize in different forms [8] which are also characterized by different solubilities

[2]. The analysis given here may be compared with an earlier and rather different one [9], which, though physically somewhat unrealistic, is nevertheless suggestive in also predicting a phase change as the number of sites becomes infinite. Actually, although no completely sharp phase change has ever been observed, even in the case of the largest proteins studied, and although, as shown in the last paragraph, the simple M.W.C. model is not as such applicable to them, nevertheless data like those shown in Fig. 4 often reveal a very sudden transition, or switch over, which occurs within a limited critical range of liganding even when the total free energy of interaction is relatively small. At any rate, one thing is clear, and that is that the interactions responsible for the cooperativity in these giant molecules must radiate out to cover a large number of sites, almost certainly substantially larger than the minimum number calculated from Equation (12).

ACKNOWLEDGEMENT

A grant from the National Science Foundation to Jeffries Wyman is gratefully acknowledged.

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Received July 10, 1974

Ligand Interactions in Globular Proteins

Gregorio Weber

FREE ENERGY COUPLING BETWEEN LIGANDS.

The investigations of the last ten years have shown that the functions of proteins as catalysts, specific binding agents or mechanical entities can be greatly modified by the binding of small molecules, so that their properties may best be described as those of complexes of the protein with the small ligands. The functions of proteins as catalysts or as mechanical agents are often too complex to permit at present a complete analysis of the changes in function that result from the binding of different ligands. On the other hand the influence of one ligand upon the binding of another ligand in an isolated protein in solution may be described in simple fashion and its study can provide some of the fundamental information indispensable for the understanding of the more complex cases. I have given elsewhere[1,2] a simple thermodynamic formulation that permits one to extract from binding data the standard free energy coupling, ΔF_{xy}, between two ligands, X and Y, simultaneously bound to the protein. The free energy coupling is positive ($\Delta F_{xy} > 0$) if the binding of one ligand decreases the affinity of the protein for the other ligand, and negative ($\Delta F_{xy} < 0$) if binding of one ligand increases the affinity for the other. These relations between ligands are always mutual, since the conservation of the free energy of binding implies reciprocity of the effects. Few investigations have been directed to determine the free energy coupling between ligands, and from these and from the larger number of, so to speak, unintentional observations recorded in the literature[2] it is possible to draw some general conclusions: 1. Both positive and negative values of ΔF_{xy} have been observed. 2. None of them is larger than 2 kcal./Mole and a figure of 1-1.5 kcal/Mole may be quoted as typical for the absolute value of the free energy coupling between ligands.

LIGAND INTERACTIONS AND CHANGES IN PROTEIN STRUCTURE.

Much attention has been directed towards relating the changes in function of a protein upon ligand binding to the possible changes in structure that are then assumed to take place[3,4]. Energetic and structural changes will bear a direct relation to each other if there is complete reversal to the original properties upon removal of the ligand, a demonstration provided in most, if not in all the cases studied. We can expect each separate ligand, X or Y, to produce changes in the protein structure as compared to the original protein structure in the absence of the ligands. The structural changes relating directly to the ligand interaction are not these, but the probably much smaller changes by which the singly liganded forms PX and YP differ from the doubly-liganded form YPX. The conservation of free energy in the system obliges us to relate directly the value of ΔF_{xy} to these structural changes characteristic of the ternary complex. A small value of ΔF_{xy} bespeaks an equally small change in the energy of interaction linking the protein parts. This is perhaps most easily visualized in the case of positive free energy couplings ($\Delta F_{xy} > 0$) which imply a mutual destabilization or repulsion between the two bound ligands. If this repulsive interaction were to exceed the value of the energy of interaction of the intervening protein parts a compensatory relaxation, or dislocation of the protein structure would then take place reducing ΔF_{xy} to the permissible upper limit. The free energy couplings thus far measured are typically 1-1.5 kcal. The total value of the internal protein interactions is difficult to estimate but assuming individual contributions no greater than 1 kcal/amino acid residue it would still amount to more than 100 kcal for a typical globular protein chain. We can see at once the magnitude of the problem of translating the existence of ligand interactions into structural changes, since the energy of the former would amount to no more than 1% of the total energy of the interactions that maintain the protein structure. To account for such a small change would require knowledge of the average atomic coordinates of the various ligand-protein complexes with a degree of precision which X-ray crystallographic analysis cannot provide today[5], and may never be able to provide. In a recent review Jensen[6] points out that one of the principles used today in the refinement of the X-ray structures of proteins is that of conservation of the 'normal' bond angles and distances.

Evidently if we are to have a structural map sufficiently detailed and accurate to carry out computation of all the energies of interaction among the protein residues, the atomic coordinates must be determined without recourse to auxiliary energetic assumptions if circular arguments are to be avoided. The classical indetermination of Physics arises in the case of large energies and very small particles. Here we seem to be in precisely the opposite case, of very small energies distributed among the many degrees of freedom of a very large particle, and we may have to face the unfeasibility--if not the impossibility--of determining all the distances, and thus all the energies with the required precision.

INSUFFICIENCY OF MECHANICAL MODELS OF LIGAND INTERACTION.

In attempting to explain the decrease in affinity for one ligand when another is bound, various authors have proposed mechanical models which picture the effects as due to the creation of steric barriers of virtually infinite energy. Such models are bound to be poor, and still worse, very misleading representations of the real situation in which a very modest additional free energy is required for the formation of the ternary complexes. The insufficiency of these mechanical models becomes patent when one compares the potential energy of interaction as a function of distance for a pair of atoms, with a similar potential curve describing

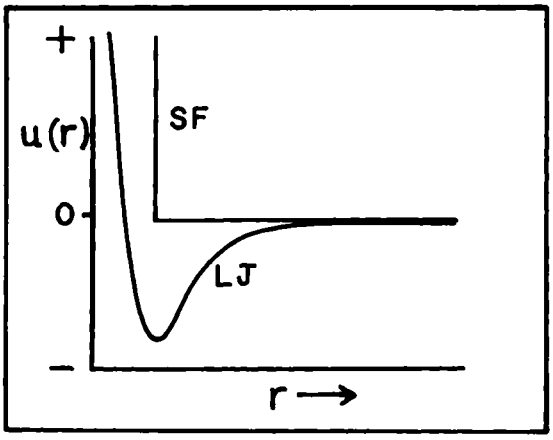

Fig. 1. Comparison of the potential energy of interaction u(r), as a function of the distance r, for a pair of atoms in a space filling model, SF with the Lennard Jones potential (LJ). The depth of the minimum would be 1-3 thermal energy units.

the behaviour of the commonly used space-filling models. As shown in figure 1 the latter cannot represent at all the changes in interaction of the order of a few times the thermal energy, which are the only ones involved in the interligand interactions.

PHYSICAL ORIGIN OF LIGAND INTERACTIONS.

Although the prospects for determining in each particular case the exact structural features responsible for the ligand interactions are almost nil we can nevertheless recognize the main molecular mechanisms that must be responsible for their existence. If two ligands not directly in contact influence each other's binding they must do so through the intervening protein structure. Conceivably such effects arise in two different ways: The first is by electrostatic interactions, which will be stronger across the low-dielectric-constant protein core than when the charged ligands are at the same distance in water solution. These electrostatic effects need not be confined to the case in which both ligands are charged. When only one of them is an ionic ligand it can interact with either charged groups or polarizable groups involved in the binding of the uncharged ligand. Thus 2,3-diphosphoglycerate and each of the four oxygens in hemoglobin are linked by a free energy coupling of +1.3 kcal/Mole[7]. The repeated observation that the most diverse polyanions have effects similar to diphosphoglycerate is one good example of this kind, and in view of the overall prevalence of charged ligands, this mode of influence may well turn out to be the commonest. A second mode of interaction may be envisioned for the case of two uncharged ligands: this will consist of small displacements of the peptide chains, or even only 'strains' in the protein structure, which can be considered for this purpose a portion of a 'hard sphere' fluid, as proposed by Klapper[8].

LIGAND INTERACTIONS IN SINGLE CHAIN PROTEINS AND IN OLIGOMERIC PROTEINS.

Most of the cases of ligand interactions described in the literature refer to proteins made up of several subunits, but in recent years ligand interactions have been conclusively demonstrated in single chain proteins. Kolb & Weber[9,10] have shown the presence of multiple interactions between anilino naphthalene sulfonate (ANS) and 3,5-dihydroxybenzoate when both

are bound by bovine serum albumin. Panagou, et al.[11] have observed allosteric behaviour in a single chain protein,ribonucleotide reductase from L.Leishmannii. Moreover, many of the effects of ligand interactions observed in oligomeric proteins appear to be of the type in which the interacting pairs within any single subunit are virtually independent of the others. Kolb[10] has observed this to be the case for the ternary complexes of chicken heart lactate dehydrogenase with NADH and oxalate. These observations are not surprising. From our previous considerations we expect ligand interactions to be characteristic of all compact globular polymers and not to require in any way the existence of an oligomeric aggregate for their presence. However, the prevalence of the observation of these effects in oligomeric proteins can be simply rationalized: The boundary between subunits constitutes a region where only weak interactions are present and binding of a ligand elsewhere in the protein is therefore likely to produce changes in it. In this manner two identical ligands bound to neighbouring subunits may exert influence upon each other. Cooperativity and antagonism in the binding of these two ligands will naturally appear[1] if some intersubunit bonds are broken or formed by the binding of a ligand at either subunit. Independent binding without ligand interaction, but with a change in the free energy of subunit association will follow, if binding at either subunit breaks equal and independent bonds at the subunit boundary (Figure 2). Evidently these subunit boundary changes, corresponding to free energy changes which are small compared to the total free energy of interaction of each peptide chain, or even to the total free energy of boundary interactions, need not introduce by themselves very conspicuous modifications in the structure. It is not therefore immediately obvious in what way the crystal structures observed for the liganded and unliganded forms are related to the mechanism of interligand coupling. Other places at the boundary, besides those involved in common bonds, may be independently modified by binding of each ligand. It is also increasingly recognized[12] that crystal forces arising out of the different packing of the molecules in the liganded and unliganded forms in the crystal must be taken into account.

An important energetic characteristic of oligomeric proteins is that a single ligand at the boundary (e.g., 2,3-diphosphoglycerate in hemoglobin) may couple with the several ligands bound each to one subunit. In this

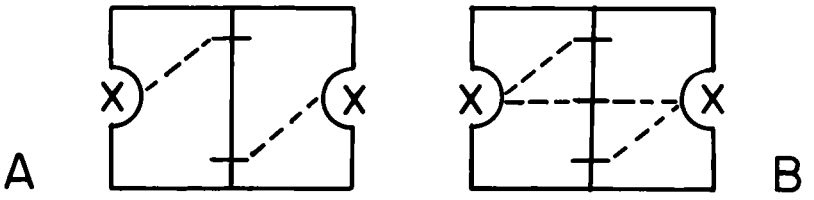

Figure 2. Ligand interaction effects and subunit association: In A binding of X at either side breaks (forms) independent and equal bonds. Ligand binding promotes dissociation (association) of subunits without appearance of ligand interaction. In B common bonds are broken or formed by binding at either side. Ligand interactions are observed together with changes in free energy of association of the subunits.

manner the total free energy coupling for the unique ligand at the boundary may increase to several times the value found for isolated ligands, and may therefore reach 5-7 kcal/Mole. I have discussed elsewhere the possible significance of these facts for the interconversion of chemical and osmotic energies[2,13]. It may well be that this energetic characteristic is responsible, more than any other, for the prevalence of oligomeric proteins.

ENERGY TRANSFER TO AND FROM THE PROTEIN STRUCTURE.

When two ligands are simultaneously bound to the protein with $\Delta F_{xy} < 0$, the ternary complex is stabilized with release of the corresponding free energy. When they are bound with $\Delta F_{xy} > 0$ free energy is stored in the ternary complex in the form of 'repulsive' interaction between the ligands. In either case the free energy coupling equals the change in chemical potential required to attain half saturation with one ligand in the absence, and in the presence of an excess, of the other ligand[1]. A change in chemical potential of 1.5 kcal/Mole results from a change in concentration of one order of magnitude. It follows that there is a direct relation between the strength of the energies of interaction of the protein parts that keep it in the globular form, and the physiological range of concentration of those ligands that regulate the metabolic functions of the protein. From the energetic point of view this relation may be considered to arise from the transfer of free energy to and from the protein structure. As in all other cases of energy transfer, impedance matching between the 'donor' and 'acceptor' systems is strictly required.

The donor system is here the protein, and the acceptor the surrounding solution, if ΔF_{xy} is negative, and conversely the donor is the surrounding solution and the acceptor the protein, if the free energy coupling is positive. Impedance matching corresponds here to chemical potential matching. On the protein side it is the standard chemical potential that changes, while on the ligand side it is the concentration, or variable part of the chemical potential that is involved. It will be noticed that actual 'storage' of free energy in the protein structure takes place only when $\Delta F_{xy} > 0$, that is in the case of repulsive effects between the bound ligands. I believe that this constitutes the only mode of free energy storage in the protein structure, apart from actual formation of covalent bonds.

STRUCTURAL FLUCTUATIONS IN PROTEINS.

While the structural details responsible for ligand-ligand interactions may be virtually inaccessible to our present-day methods, the protein dynamics that they imply and the changes of these dynamics under various conditions may furnish information of importance towards the understanding of biological processes. In assessing the dynamics of the protein structure relevant to our case we shall be governed by a principle which is supported by virtually every line of evidence, namely that nearest neighbour interactions are the most important factor in maintaining the conformation of the protein in its average state, and that there is no delocalized form of energy capable of furnishing additional stabilization. Given these premises we can expect that local fluctuations of the structure will recur with a frequency $Z = A \exp-(E/RT)$, where A is a pre-exponential factor and E the energy required to produce the structural fluctuation. We expect E to be of the same order as ΔF_{xy}, perhaps two or three times as large, while A is determined by the rate of energy exchange among interacting neighbouring structures in the protein. Various lines of thought[2] would suggest for A values of 10^{12}-10^{14} sec^{-1}. If the lower limit is used and E = 2-4 kcal/Mole we have $Z = 10^{8}$-10^{9} sec^{-1}. It is in fact possible to show by direct experimental measurements the reality of the rates and energies of activation that we have assigned to these fluctuations.

DEMONSTRATION OF PROTEIN STRUCTURAL FLUCTUATIONS BY FLUORESCENCE METHODS. The excited states for allowed emission of visible or ultraviolet light last for times of the order of 1-20 nsec. in the majority of cases. The postulated fluctuations of the protein structure should therefore modify the character of the emission and be readily detectable from the properties of the intrinsic protein fluorophores, or of fluorescent probes included in the protein structure. Both types of experiments have yielded positive results. Lakowicz & Weber[14] have studied the quenching of the fluorescence of proteins by oxygen. The fluorescence from the tryptophan residues of the twelve proteins studied was quenched by oxygen at rates equal to 1/2 to 1/5 of the diffusion controlled rate, which applies to tryptophan and simple indole derivatives in water solution. Observations of fluorescence lifetime have shown that the process is not due to static association of oxygen with the tryptophan residues and therefore that it requires the effective diffusion of oxygen within the protein structure to take place in the 2-6 nanoseconds which the excited state lasts. Such rapid diffusion process must necessarily require rapid breathing of the protein through the kind of fluctuations that we have deduced from the small values of the free energy coupling between ligands. More recently we have been able to obtain further evidence for the character of the energy fluctuations proceeding from an original observation of Brand & Gohlke[15]. These authors showed that the solvent relaxation, or rearrangement of the solvent molecules around an excited fluorophore, has a counterpart in the rearrangement of the surroundings when the fluorophore is anilino naphthalene sulfonate (ANS) bound to bovine serum albumin. This observation effectively shows that rearrangement of the protein structure about the excited fluorophore takes place in a time of the order of nanoseconds. To extend this observation we have used the method of differential phase fluorometry[16], which is particularly well adapted to the measurements of subnanosecond time intervals. We employed for the interpretation of the results a two-state model according to which the fluorophore may be effectively surrounded by only two kind of environment, one corresponding to equilibrium in the ground state, the other to equilibrium in the fluorescent, or lowest singlet, state. The experimental data permit the determination of the two rates for conversion of each state into the other. In ANS-Bovine serum albumin complexes[17] we found that conversion

from 'ground state' equilibrium surroundings to 'excited state' equilibrium surroundings took place at a rate of 10^9 sec^{-1} while the opposite rate was ten times slower. It may be noticed that the latter rate, which is the one most directly and accurately measured by the experiments, closely corresponds to a fluctuation about equilibrium, of the type that we expect to take place in the intact protein structure. Measurements at different temperatures have indicated for this rate an energy of activation of 3.5 kcal. It is of interest to notice that binding of a second ligand, 3,5 dihydroxybenzoate, which is known to couple with ANS[9], could be followed by its effect upon these relaxation parameters of the ANS fluorescence[17]. Similarly we have observed recently that a complex of bis-ANS and aspartate transcarbamylase[18] undergoes similar relaxation behaviour, and changes it upon the binding of carbamyl phosphate to the enzyme.

LIMITATIONS OF THE DYNAMIC OBSERVATIONS.

I have already pointed out the limitations that the small energies of ligand interaction impose upon the derivation of their causes from the structural X-ray data. Limitations of a different kind but of similar origin apply to the study of the protein dynamics by the fluorescence methods. From very general considerations we expect that the fluctuations in the protein properties will be increasingly difficult to reveal as their energy decreases and they become faster, in accordance with the exponential relation between the rate and the energy of activation. The possibility of direct measurement of low-energy, spontaneous fluctuations, after recognizing them among the many other low-energy changes taking place in the system seems well-nigh impossible. In our experiments this difficulty is circumvented by creating a high energy state within the protein by light absorption and deducing the existence of the protein fluctuations from the changed properties of such easily observable fluorescent state. However, the creation of such state is not without influence upon its surroundings and we are witnessing not so much a spontaneous process characteristic of the 'intact' proteins as an induced change from which the properties of the structure are indirectly deduced. We need not be unduly pessimistic or apologetic about our conclusions, but they must certainly take into account the particular properties of the

method of observation and the uncertainties that it necessarily introduces. For it is a characteristic of all observations that they interfere with the object of the observations, and for the time and energy ranges that we are considering the interference can no longer be considered as negligible.

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Received July 19, 1974

DISCUSSION

Steinhardt: By way of clarification, would you comment on the difference between the small interaction energies you have been discussing and the rather large free energy changes that accompany the binding of certain ligands, such as lipid-like substances, by proteins (ΔF up to about 9 kcal). With long chain fatty acids (Goodman) binding constants up to 10^8 have been measured. Would you say that such binding greatly alters the globular structure?

G. Weber: I was referring to the free energy of ligand-ligand interactions. These are only a fraction (10 - 20 %) of the total standard free energy of binding of either of the interacting ligands.

Helmreich: I just would like to add that the small energy changes involved in ligand binding to proteins of course agree with the very fast rates of ligand binding to proteins which are usually diffusion controlled.

G. Weber: It happens that - for very good reasons- the free energies of binding are directly determined by the rates of dissociation of the ligands, and that the rates of association are relatively constant and often close to diffusion controlled. This, whether the standard free energy of binding is small, like in fumarate-fumarase (Massey) or very large like in avidin-biotin complexes (N.M. Green).

von Hippel: What is molecular interpretation of impedance matching? Is it that something in the protein is capable of changing "one kcal worth"?

G. Weber: Exactly. You put it better than I did.

Antonini: What would be the size of the movements associated with the fast fluctuations?

G. Weber: Probably very small and localized - I imagine, a group rotating, or moving a few Å units, etc. Certainly nothing major.

Navon: In the experiment of fluorescence lifetime, when several ANS molecules are bound to each serum albumin molecule is it possible that there will be energy transfer between the unrelaxed, blue-shifted ANS molecules and the relaxed red-shifted ones? How will your results be affected by this phenomena?

G. Weber: Energy transfer does take place among the various ANS molecules, but since the free energy of binding of the first-bound four molecules is equivalent and the fluorescence spectrum does not change with number bound there is no reason to suppose that there is a class of molecules of ANS which relaxes and one which does not. The temperature dependence of the observed relaxation is another good argument to disregard the effect of energy transfer in the observed results.

Navon: In your lecture you have referred to the large fluorescence shift in the ANS molecule as due to solvent reorganization. What is your opinion on the possibility (E.M. Kosower, private Communication) that it is caused by a planar-non-planar conformational change in the excited state of the ANS molecule?

G. Weber: Similar relaxation effects are observed with 2-diethylaminonaphthalene-5-sulfonate and 1-8-naphthosultam. In the former only a rotation of the amino group could take place. In the latter there is no possibility of a non-planar conformation.

Veeger: Is it possible to discriminate between the two possibilities: a) Fluctuations between the two states as described in your paper. b) Fixed binding in two states with different microenvironment.

G. Weber: Two different microenvironments occupied by the ANS molecules would give the observed results if the "red-shifted environment"-ANS had a longer lifetime of fluorescence than the unshifted one. However we would not expect to observe a temperature dependence. The temperature-dependence experiment is crucial to distinguish between the two possible origins, but the presence of heterogeneity, as you suggest, could somewhat falsify the results. I believe that, if present, it plays here only a very minor part.

Ligand Binding to Enzyme Complexes

Kasper Kirschner, Wolfgang Weischet and *Robert L. Wiskocil*

MULTIENZYME COMPLEXES

The existence of specific aggregates of different polypeptide chains capable of catalyzing a number of distinct metabolic reactions indicates that heterologous protein-protein interactions can be important for expressing enzyme function (1). The oligomeric structure (i.e. multiple identical subunits) may be essential for the allosteric regulation of enzyme activity as, for example, in aspartokinase-homoserinedehydrogenase (2). When the different enzyme activities correspond to sequential metabolic steps, direct cooperation between distinct active sites becomes possible. Composite sites may be created by bringing different active sites into close contact at the subunit-subunit interface, thus providing for molecular "channels" or "compartments" for labile intermediates (3). Finally, the accumulating evidence suggests that heterologous protein-protein interactions may only be a step towards the evolution of more sophisticated enzymes. Gene fusion may provide a way for the conversion of multienzyme complexes to single polypeptide systems (4,5).

We have studied tryptophan synthase from E. coli as a typecase of simple multienzyme complexes (6). This enzyme is a complex of two α- and two β- chains. It is capable of catalyz-

zing the following reactions (6):

1) Indoleglycerol-P + L-ser $\longrightarrow$ D-glyceraldehyde-3-P + L-trp
2) Indoleglycerol-P $\rightleftharpoons$ D-glyceraldehyde-3-P + indole
3) Indole + L-ser $\longrightarrow$ L-trp

The complex may be dissociated reversibly to α- and β_2- subunits. They can also be obtained as individual proteins from suitable mutants (6). The interaction of α- and β- subunits is characterized by an apparent intrinsic dissociation constant on the order of 10^{-8}M, but depends on the presence of PLP* and serine (15). Reaction 2 is catalyzed also by the α- subunit alone but only 1 - 2 percent as efficiently as an equivalent amount of the $\alpha_2\beta_2$ complex. Similarly, reaction 3 is catalyzed by the β_2- subunit, which contains two equivalents of PLP. Again, the complex is approximately 50 times more efficient in this reaction than the isolated subunit (6). The physical basis for the phenomenon of mutual activation may be of general interest.

The syntheses of indoleglycerol-P and tryptophan from indole are Bi-Uni reactions. Steady-state kinetic studies may shed light on possible differences in the mechanisms as being responsible for the phenomenon of mutual activation. More specifically, the binding of the crucial intermediate indole to α- and β_2 - subunits as well as to the $\alpha_2\beta_2$ - complex might be used as a probe for mapping out the putative composite active site (3,8,9,16). Finally, the synthesis of indolepropanol-P, a nonreactive analogue of indoleglycerol-P (7), provided an additional tool for probing site-site interactions in the complex. The ease with which the complex can be reversibly dissociated into functional components (6) sets the stage for detailed equilibrium and kinetic studies of subunit assembly.

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* PLP, pyridoxal 5'-phosphate

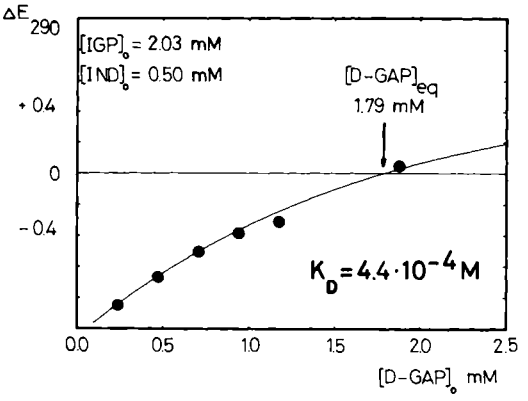


Fig. 1. Determination of the equilibrium constant of indoleglycerol-P cleavage (reaction 2). Various solutions with constant total concentrations of indoleglycerol-P (IGP) and indole (IND) and variable total concentrations of D-glyceraldehyde-3-P (D-GAP) as indicated in the figure were monitored at 290 nm after adding a catalytic amount of $\alpha_2\beta_2$ complex. The ordinate represents the total absorbance change with respect to a blank solution containing only indoleglycerol-P and indole after thermodynamic equilibrium has been reached. Buffer: 0.05 M Tris·HCl, 1 mM EDTA, 0.2 mM DTE, pH 7.8. Temperature: 25°C, d = 1 cm, $K = (IGP)^{-1}$ (IND) (D-GAP).

STEADY-STATE KINETICS (INDOLE ⟶ INDOLEGLYCEROL-P)

The condensation of indole with glyceraldehyde-3-P (reaction 2) can be followed at 290 nm. Using this convenient handle and the $\alpha_2\beta_2$ complex as catalyst, the equilibrium constant of the reaction was determined by "titration" of known fixed concentrations of indole and indoleglycerol-P with varying concentrations of glyceraldehyde-3-P (Fig. 1). That concentration of glyceraldehyde-3-P, at which the addition of enzyme causes no further absorbance change, corresponds to the equilibrium concentration. The magnitude of the equilibrium constant emphasizes the fact that synthesis of indoleglycerol-P is the thermodynamically preferred direction of reaction 2. Since the synthesis of tryptophan (reaction 3) is virtually irreversible (6), a transient accumulation of indole is ruled out if the

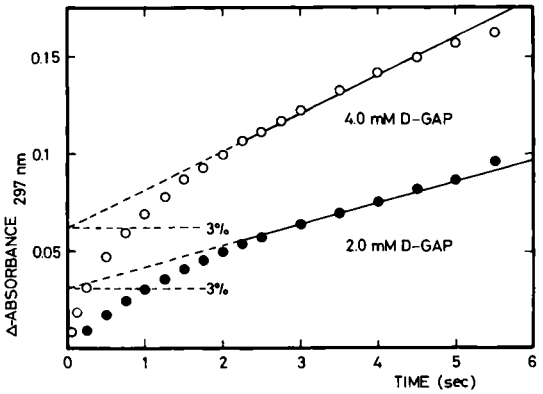


Fig. 2. Biphasic consumption of glyceraldehyde-3-P during synthesis of indoleglycerol-P (reaction 2). $\alpha_2\beta_2$ complex was mixed with solutions of D-GAP and indole and the synthesis of IGP was monitored at 297 nm. o - o: 4 mM D-GAP, 0.53 mM IND, 0.04 mM α- and 0.07 mM β-equivalents. ● - ●: 2 mM D-GAP, 0.53 mM IND, 0.02 mM α- and 0.035 mM β-equivalents. Buffer: 0.1 M K-phosphate, 1 mM EDTA, 0.2 mM DTE, pH 7.6. Temperature: 20°C. The concentration of GAP consumed in the transient phase was calculated from the ordinate intercepts with $\Delta\varepsilon_{297\ nm} = 0.457\ mM^{-1}cm^{-1}$.

"physiological" reaction 1 is indeed a sequence of reactions 2 and 3.

For chemical reasons, only the carbonyl form of glyceraldehyde-3-P is expected to participate in reaction 2. Fig. 2 shows a stopped-flow experiment in which indole and glyceraldehyde-3-P were mixed with high concentrations of the tryptophan synthase complex.

It is apparent that the observed "burst" corresponds to the consumption of only 3 % of the total concentration of glyceraldehyde-3-P. This amounts to the known fraction of the carbonylform in equilibrium with the hydrate (10). The subsequent zero-order phase is determined by the slow dehydration of the aldehyde hydrate (10).

Table 1. Selected steady-state and rate constants for the synthesis of indoleglycerol-P. Comparison of α-subunit and $\alpha_2\beta_2$ - complex as catalysts.

constant	α-subunit	$\alpha_2\beta_2$ complex
k_1 ($M^{-1}sec^{-1}$)	3.6×10^4	4.2×10^5
K_{IND} (mM)	5.0	2.2
k_{cat} (sec^{-1})	3.3	121
K_{IGP} (mM)	0.040	0.007
K_{IPP} (mM)	0.05	0.01

Detailed studies of the steady-state kinetics of reaction 2 catalyzed by the $\alpha_2\beta_2$ complex in the absence and presence of indolepropanol-P support previous conclusions (8) that the mechanism corresponds to strictly ordered addition with glyceraldehyde-3-P binding first:

Scheme 1 $$E\cdot IPP \rightleftharpoons E \overset{k_1}{\rightleftharpoons} E^{GAP} \rightleftharpoons E^{GAP}_{IND} \overset{k_{cat}}{\rightleftharpoons} E+IGP$$

The results of the analysis are summarized in the righthand column of Table 1. The mechanism implies that only the binary enzyme: glyceraldehyde-3-P complex is capable of binding indole in a productive mode. The second-order rate constant of glyceraldehyde-3-P recombining with the enzyme (k_1) yields another clue. Its value is 2 - 3 orders of magnitude below diffusion control, indicating that it represents an overall process involving at least one additional step. This could be an isomerization creating a productive indole binding subsite.

Similar studies employing the α- subunit cannot yet be interpreted in the same unequivocal manner. The values in the lefthand column of Table 1 have been obtained under the assumption that the same catalytic mechanism applies to the α- subunit.

This procedure appears to be justified by the fact that indolepropanol-P is a competitive inhibitor towards glyceraldehyde-3-P but a noncompetitive inhibitor towards indole in both cases (cf. scheme 1).

STEADY-STATE KINETICS (INDOLE ⟶ TRYPTOPHAN)

Kinetic data obtained for reaction 3 catalyzed by the $\alpha_2\beta_2$ complex under steady-state conditions surprisingly led to non-intersecting double reciprocal plots obeying equation 1a.

eq. 1a $$\frac{1}{v_i} = \frac{1}{V} \left(1 + \frac{K_{SER}}{SER} + \frac{K_{IND}}{IND} \right)$$

This is in contrast to an earlier report (14). The discrepancy is probably due to previously undetected substrate inhibition by indole at concentrations above 0.2 mM. In similar experiments the ratio of indole to serine concentration ($X = \frac{IND}{SER}$) was kept constant while the total concentration was varied (eq. 1b).

eq. 1b $$\frac{1}{v_i} = \frac{1}{V} \left(1 + \frac{1}{IND} \left(X \cdot K_{SER} + K_{IND} \right) \right)$$

The straight lines shown in Fig. 3 prove the validity of equations 1a and 1b. This behaviour is formally identical to Bi-Bi Ping-Pong mechanisms (17). However, since we are dealing with a Bi-Uni reaction, equations 1a and 1b must be the result of a negligible $\frac{K}{SER \cdot IND}$ - term. The kinetic data (Fig. 3) are best explained by the strictly ordered addition mechanisms presented in scheme 2, top line.

In this mechanism, serine adds first and a step (k_3) after serine binding and before indole binding is largely irreversible

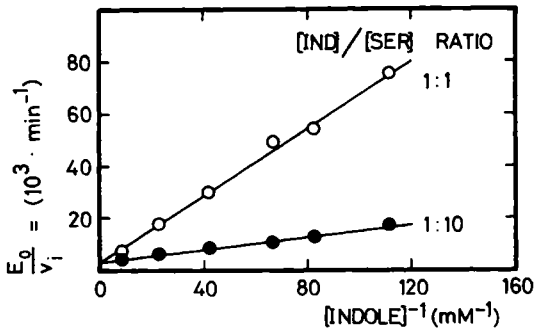
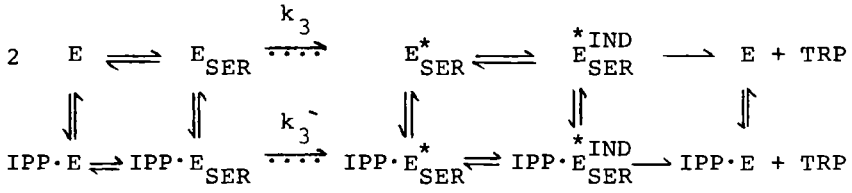


Fig. 3. Initial velocity pattern for the synthesis of tryptophan from indole and serine catalyzed by the $\alpha_2\beta_2$ - complex. Indole and serine were varied simultaneously, keeping constant ratios as indicated in the figure. Buffer as in Fig. 2. Temperature: 25°C. Enzyme concentration: 10^{-7} M β-equivalents, 4 x 10^{-7} M α-equivalents.

Scheme 2



and rate determining. k_3 probably characterizes the formation of the aminoacrylate Schiff's base intermediate (13).

The mechanism of reaction 3 remains qualitatively the same in presence of saturating concentrations of indolepropanol-P, although there are quantitative changes. The dependence of the three steady-state kinetic parameters of eq. 1a on the concentration of indolepropanol-P are shown in Fig. 4. V_{max} decreases by a factor of 3 but the apparent inhibition constant of indolepropanol-P (K_I = 1 μM) is approximately tenfold smaller than the dissociation constant measured directly (7). This means that serine bound to the $\alpha_2\beta_2$ complex enhances the affinity of indolepropanol-P and vice versa. This conclusion was confirmed by direct measurement of binding of indolepropanol-P

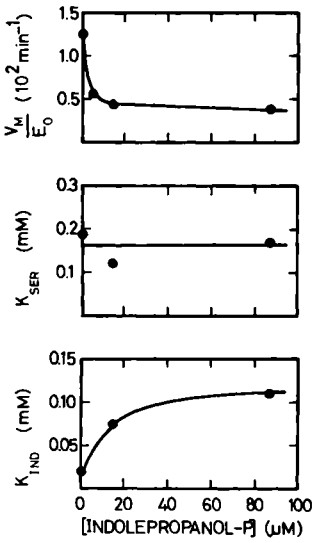


Fig. 4. Non-competitive inhibition of tryptophan synthesis by indolepropanol-P (cf eq. 1a). Conditions as in Fig. 3.

in presence of serine.

These findings are interesting with regard to the postulated composite active site (8,9), since indolepropanol-P is known to bind to the site catalyzing the cleavage of indoleglycerol-P. In a general way they reflect both positive and negative interactions between two different catalytic sites contributed by two different subunits. This is certainly compatible with the idea that the catalytic sites are juxtaposed, but gives no direct information on the fate of indole during the course of reaction 1 (6,9).

It is surprising that neither of the two sites catalyzing condensation reactions with indole are capable of binding indole alone in a productive fashion. This raises the question whether lack of indole binding can be demonstrated directly by equilibrium dialysis.

5a

5b

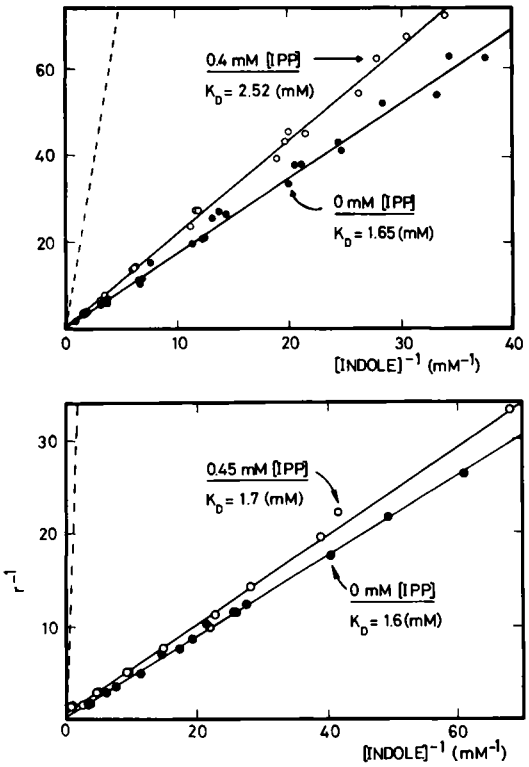


Fig. 5. Binding of indole to α-subunit (a) and the $\alpha_2\beta_2$ - complex (b) in presence and absence of indolepropanol-P. Double reciprocal representation of equilibrium dialysis with 14 C-indole. Dashed curves have been calculated for competition by indolepropanol-P with dissociation constants listed in Table 1 (K_{IPP}). Buffer and temperature: same as in Fig. 1. Enzyme concentrations: a) 0.40 mM and b) 0.1 mM α-subunit equivalents.

The data (most conveniently presented in double reciprocal form) confirm earlier reports (11) that weak binding occurs to the α- subunit (Fig. 5a) and the complex (Fig. 5b). Moreover, the isolated β_2 - subunit binds indole very unspecifically ($K_{IND} = 10^{-2}$M). The data further show that saturation of the enzymes with indolepropanol-P has only a minor and non-competitive influence on the indole saturation curves. (The dashed curves have been calculated for strict competition between the two ligands).

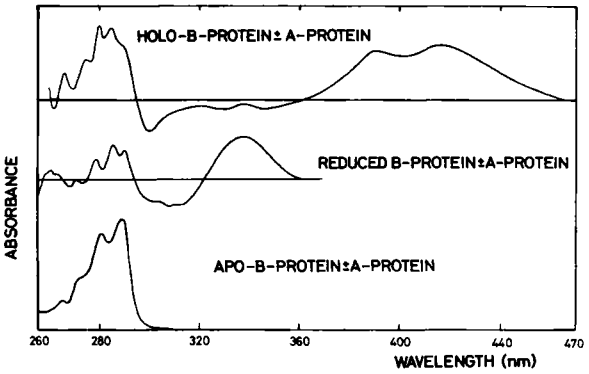


Fig. 6. Difference spectra of α-subunit forming a complex with holo- and apo-β_2 subunit and with holo-β_2 subunit reduced by $NaBH_4$. (0.1 M K-phosphate buffer pH 7.6, 25°C). Concentration of proteins: 0.1 mM β-equivalents, 0.2 mM α-equivalents

The results are in accord with the steady-state kinetics because if indole were capable of productive binding in absence of glyceraldehyde-3-P, strict competition by indolepropanol-P with regard to indole should have been observed.

KINETICS OF ASSEMBLY

One explanation for the mutual activation experienced by the α- and β_2 - subunits upon assembly is based on conformational changes. Assembly might induce or stabilize active conformations of each subunit (7). The application of rapid reaction techniques has led to direct evidence that both the β_2 - (14) and the α- subunits (7) are indeed flexible proteins. When the β_2 - subunit is complexed with α- subunit, the rapid isomerization (indicative of a conformational equilibrium in the β_2 - subunit) disappears (12).

We have used stopped-flow techniques in studying the kinetics of assembly of the multienzyme complex. Fig. 6 presents the difference spectra between the complex and the separated sub-

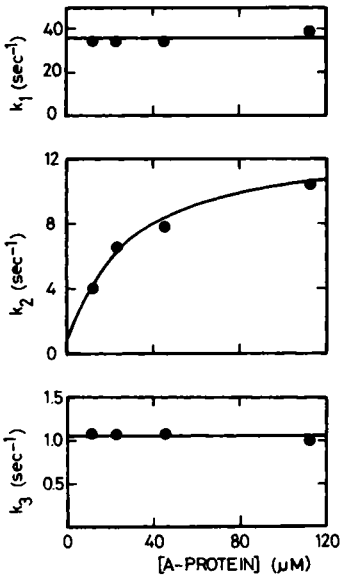


Fig. 7. Kinetics of assembly of $\alpha_2\beta_2$ - complex. Holo-β_2-subunit (0.02 mM β-equivalents) was mixed with excess α-subunit (concentrations indicated in the figure) in a Durrum stopped-flow spectrometer. The rate constants of the three superimposed exponential decay processes were obtained from the progress curve of absorbance at 405 nm vs. time. Buffer and temperature same as in Fig. 2.

units. Apparently the environment of both the pyridoxal-P bound to the β_2 - subunit and the aromatic aminoacid side chains of one or both of the subunits are perturbed in the complex. Similar difference spectra can be obtained using the reduced or the apo- β_2 subunit.

Using light of either 288, 334 or 405 nm for detection, rapid mixing of β_2 - subunit with excess α- subunit leads to complex progress curves. These can be easily interpreted in terms of at least three exponential processes well separated on the time axis. The dependence of the three time constants on the concentration of excess α - subunit are shown in Fig. 7. The time constant of the intermediate process (k_2) increases towards a plateau value. This behaviour is indicative of an

isomerization step following a more rapid binding process by which the first complex between α- and β_2-subunits is formed:

eq. 3 $$\alpha + \beta \underset{\text{rapid}}{\overset{k_o}{\rightleftharpoons}} \alpha\,\beta \underset{\text{slow}}{\overset{k_2}{\rightleftharpoons}} (\alpha\,\beta)$$

Eq. 3 is formulated for a β- monomer. This appears to be justified because there is no evidence that the α- binding sites on the β_2 - subunit are non-identical or interact (15).

k_o is probably too rapid for resolution by stopped-flow experiments. The unequivocal interpretation of k_1 and k_3 in terms of a more complicated mechanism must also await further experiments. However, since the latter processes are first order with respect to β_2 - subunit, they must correspond to further isomerization steps. Reaction 1 (indoleglycerol-P→tryptophan) can now be used to answer the interesting question, at which stage during the dynamics of assembly the catalytic activity appears. Once again, the difference spectrum between tryptophan and indoleglycerol-P at 297 nm can be used to follow the turnover of substrate spectrophotometrically. Fig. 8 shows the progress curves obtained for two rapid mixing experiments in which the order of addition differs. In the control, the preformed complex is mixed with the substrates. Zero-order kinetics are established after a short transient period. However, when α- subunit and serine is mixed with β_2 - subunit (10 μM β- monomers) and indoleglycerol-P, a lag-time of 1.2 sec is observed before the steady-state is reached. This proves that the initial complex formed (cf. eq. 3) is enzymically inert.

The step responsible for the lag period can be identified using the assumption that it corresponds to about 10 half-lives of a firstorder process. With $t(\frac{1}{2}) = \frac{\ln 2}{k} = 0.12$ sec, $k = \frac{0.7}{0.12} = 6\ \text{sec}^{-1}$, in good agreement with the value of k_2 at the concentration of α- subunit (20 μM) used in the experiment