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Review

Enzymes and the supramolecular organization of the living cell. Information transfer within supramolecular edifices and imprinting effects

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Abstract. Simple considerations of statistical mechanics show that the association of an enzyme with another protein or with an 'inert' surface results in a decrease of its information content and thus that it receives an 'instruction' from this protein or from the surface. As a consequence, the free energy stored in the enzyme increases, and this energy may be used to alter the intrinsic catalytic properties of the enzyme. This may imply, for instance, that an enzyme which is devoid of activity may have its activity enhanced when bound to another protein or to a membrane. A possible consequence of this communication between proteins is that, upon dissociation of the complex, one of these enzymes may transitorily retain an imprinting of the other protein and this imprinting may in turn alter the properties of the enzyme. Different enzyme systems may illustrate this view. A particular emphasis has been put on the study of a phosphoribulokinase-glyceraldehyde phosphate dehydrogenase complex from *Chlamydomonas* chloroplasts. Whereas the isolated oxidized phosphoribulokinase is almost completely inactive, it becomes active when bound to glyceraldehyde phosphate dehydrogenase. Moreover, upon dissociation of the complex, the phosphoribulokinase retains for a while an imprinting exerted by glyceraldehyde phosphate dehydrogenase. It then displays properties that are markedly different from those of the free stable enzyme. Thermodynamics allows us to calculate the amount of energy stored in this enzyme and used to facilitate substrate binding and catalysis. There is thus little doubt that information and instructions are transferred from protein to protein within enzyme complexes that result in a complete change of their biological function.

Key words. Information transfer; imprinting effects in proteins; multienzyme complexes.

Introduction

It is now obvious, from numerous experimental studies, that in eukaryotic cells most enzymes are associated with cell organelles and with other proteins [1–3]. It is indeed tempting to consider that this type of fuzzy supramolecular organization may represent a functional advantage for the economy of the cell, and thus it is legitimate to search for the nature of this advantage. In the case of multienzyme complexes for instance, the traditional answer to this important question is to assume that channelling from active site to active site takes place within the multienzyme complex [4–13]. Let us consider for instance a metabolic network

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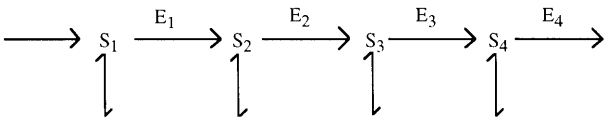

that takes place in a 'soluble' medium. The enzymes E_1, E_2, E_3, ... are considered as physically distinct entities Each step of the process is catalysed by a specific enzyme. The occurrence of this multienzyme process requires that each reaction intermediate, released from the catalytic site of the corresponding enzyme, randomly collides with the active site of the enzyme that comes next in the reaction sequence. The probability of this event will then depend upon the concentration of this reaction intermediate and therefore upon the volume of the cell compartment where the reaction sequence takes place. If this volume is small, as occurs with bacteria, the efficiency of the metabolic network will be good, and its time response to a perturbation of concentration will be short. But if the volume of the cell compartment is much larger, as occurs with eukaryotes, the dilution of the reaction intermediates should increase, and therefore the efficiency of the whole sequence should fall off. In order to avoid this unfavourable situation, one may speculate that a selection pressure has been exerted on living systems as to avoid the dilution of the reaction intermediates of a metabolic process in the cell milieu. If the enzymes that catalyse consecutive reactions are aggregated as a complex, and if their active sites are oriented in a favourable manner, one may conceive that the reaction intermediates are channelled from site to site without diffusing in the cell milieu. One may therefore expect the efficiency of the overall process to be considerably increased owing to the supramolecular organization of these enzymes. There exists a vast, and often conflicting, literature about the existence and role of these channelling effects [4–13].

An obvious implication of these ideas is the absolute requirement for the existence, in any multienzyme complex, of enzymes that catalyse consecutive reactions of the same metabolic sequence. Although there is no doubt that a significant number of multienzyme complexes catalyse consecutive reactions, others do not. The most significant example of this situation is that of aminoacyl-transfer RNA (tRNA) synthetases. Whereas in prokaryotic cells these enzymes often appear as physically distinct entities, they occur as multienzyme complexes in eukaryotes [14–17].

If, as is usually believed, it is function that is the driving force of evolution and that explains the structure of biological systems, it becomes evident that, at least in these cases, channelling cannot represent the prima ratio of these complexes. Therefore, if there is a functional advantage in the physical association of different enzymes, this advantage should be an alteration of the intrinsic properties of the enzymes of the complex. This means that information, or rather instructions, are transferred from protein to protein within the multi-enzyme complex.

As already mentioned, many enzymes may be associated with cell organelles, and in particular with biomembranes. It is therefore sensible to wonder whether some specific properties may emerge from this association. Thus the general question raised by the existence of multienzyme complexes and of membrane-bound enzymes is to know whether there is some alteration of the expression of the information coded in a gene by the supramolecular organization of the cell. A likely consequence of instruction and information transfer in a multienzyme complex is the occurrence of imprinting effects. That is, upon disruption of this complex, one of the enzymes may retain, or recall, for a while a conformation that is not the stable one but that has functional properties different from those of the stable free enzyme.

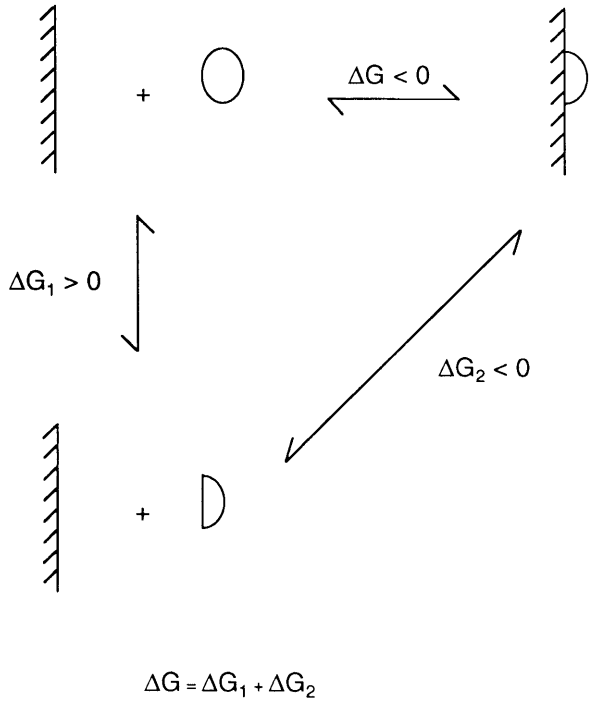

Figure 1. Thermodynamic scheme showing that the binding of a protein to an inert surface results in the storage of energy in the protein. The real binding process, associated with a negative ΔG value, is split into two ideal contributions, ΔG_1 and ΔG_2. ΔG_1 represents the free energy required to change the conformation of the protein ($\Delta G_1 > 0$), and ΔG_2 is the free energy of assocation of this modified conformation on the surface ($\Delta G_2 < 0$).

Statistical mechanics and instruction transfer within a supramolecular edifice

From a structural viewpoint, the information content, I_σ, of a protein requires the prior knowledge of the number of complexions, Ω_σ, that may be derived from the total number of amino acids, N, and from the number, $n_1, n_2, \ldots$, of amino acids belonging to the different species present in the sequence. This complexion number is [18–20]

$$\Omega_\sigma = \frac{N!}{n_1! n_2! \ldots} \tag{1}$$

Thus Ω_σ represents the number of messages that may be conveyed through sequences of N amino acids of different nature. Simple inspection of this expression shows that this number of messages increases with the total number of amino acids and with the number of different species of amino acids involved in the sequence. The information content of the amino acid sequence may thus be defined by

$$I_\sigma = \log_2 \Omega_\sigma \tag{2}$$

For the enzymologist this classical definition of information is of little interest. Even if it is true that the sequence of a protein is sufficient to define its specific folding in diluted solutions [21–25], there is also little doubt that many enzymes have different sequences but the same activity. Moreover, the same enzyme may exist under different interconvertible conformations that have different activities. Thus, to investigate the function of an enzyme, one has to leave out this static definition of information.

It is therefore mandatory to define the information content of an enzyme in a different way, directly related to its function. This approach consists in analysing the distribution of the population of enzyme molecules over all possible discrete energy states and defining an information content, I_ε, based on this energy distribution. As a matter of fact, a folded polypeptide chain in solution is not a rigid entity, for it may adopt slightly different conformation states. The population of enzyme molecules is distributed over all the energy states associated with these conformations according to the Boltzmann law [18], namely

$$v_i = v_0 \, \mathrm{e}^{-(\varepsilon_i - \varepsilon_0)/k_\mathrm{B}T} \tag{3}$$

where v_0 is the number of molecules in the fundamental state, v_i the number of molecules in the ith state, ε_0 and ε_i the energies of these states. During an enzyme reaction, the free enzyme, the enzyme-substrate(s) and the enzyme-product(s) complexes have to overcome the energy barriers pertaining to the transition states. This implies that the enzyme is able to supply the substrate(s) and the product(s) the amount of energy required for the conversion of these ligands into the corresponding transition states. If these events are carried out by the ith state of the enzyme, the whole process will be best achieved if the difference $\varepsilon_i - \varepsilon_0$ is as small as possible and such that the corresponding v_i value is large. The immediate consequence of this statement is that the energy level of the fundamental state has to be relatively large. This is in good agreement with the classical view that the biologically active conformation is the fundamental state [25], or a state close to this fundamental state, that can be reached without overcoming too high an energy barrier [26, 27].

From the distribution of the enzyme molecules over the energy states, one may derive the corresponding number of complexions Ω_ε

$$\Omega_\varepsilon = \frac{N!}{v_0! v_1! v_2! \ldots} \tag{4}$$

where N is now the total number of enzyme molecules, $v_0, v_1, \ldots$ the number (or the frequency) of molecules having the different energy states $\varepsilon_0, \varepsilon_1, \ldots$. The information content of these energy levels will be

$$I_\varepsilon = \log_2 \Omega_\varepsilon \tag{5}$$

Ω_ε represents the number of messages that may be conveyed by a population of the same protein molecules. If this value is very large, it does not offer any kind of 'instruction', for the molecules are distributed over a large spectrum of energy levels. If the same number of molecules is partitioned over a much smaller number of energy states, the value of Ω_ε decreases. One may also describe the energy distribution of enzyme molecules thanks to a function of statistical mechanics called partition function [18–20]. If the same enzyme exists in a free and in a bound state, and if the energies are counted from their lowest levels, these partition functions are defined as

$$\begin{aligned} \Pi^\mathrm{f} &= \sum_i \exp\{-(\varepsilon_i^\mathrm{f} - \varepsilon_0^\mathrm{f})/k_\mathrm{B}T\} \\ \Pi^\mathrm{b} &= \sum_j \exp\{-(\varepsilon_j^\mathrm{b} - \varepsilon_0^\mathrm{b})/k_\mathrm{B}T\} \end{aligned} \tag{6}$$

Indeed Π^f and Π^b are the partition functions for the free and bound enzyme, respectively. Inspection of equations (6) shows that this type of partition function will have a large value if the number of energy levels allowed is large and if these various energy levels do not have very different values. These conditions are precisely those that generate a large number of complexions Ω_ε. Therefore both the number of complexions and the partition function express the degree of 'organization', or of 'disorganization', in the distribution of the enzyme molecules over the allowed energy levels. If, as assumed previously, an enzyme exists in a free and in a bound state,

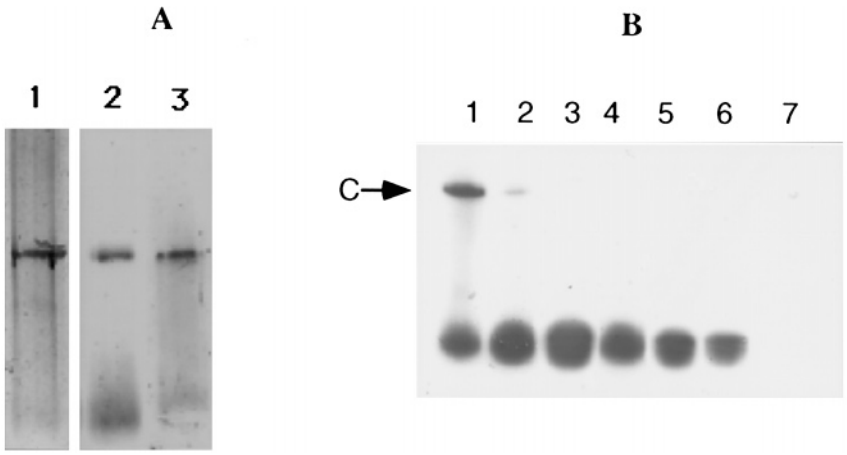

Figure 2. Electrophoretic behaviour of the PRK-GraPDH complex. (*A*) Native polyacrylamide gel electrophoresis and western immunoblotting analysis of the complex probed with antisera raised against either PRK (lane 2) and GraPDH (lane 3). The native complex was silver-stained (lane 1). (*B*) Abilities of some recombinant PRKs to form a complex with GraPDH. Lane 1: formation of the complex (labelled C) in the presence of the wild-type recombinant PRK; lane 2: slight formation of the complex in the presence of [K64] PRK; lane 3: lack of complex formation in the presence of [A64] PRK; lanes 4–6: electrophoretic behaviour of the free PRKs used in the previous experiments; lane 7: GraPDH not recognized by the anti-PRK antibody. Reprinted with permission from: Avilan L., Gontero B., Lebreton S. and Ricard J. (1997) Information transfer in multienzyme complexes. II. The role of Arg64 of *Chlamydomonas reinhardtii* phosphoribulokinase in the information transfer between glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase. Eur. J. Biochem. **250:** 296–302, © 1998 Federation of European Biochemical Societies, Cambridge.

$$\Pi^{\mathrm{f}} > \Pi^{\mathrm{b}} \tag{7}$$

Moreover, if the same number N of enzyme molecules exists in the free and in the bound state,

$$N = \sum_i v_i^{\mathrm{f}} = \sum_j v_j^{\mathrm{b}} \tag{8}$$

and therefore

$$v_0^{\mathrm{f}} \Pi^{\mathrm{f}} = v_0^{\mathrm{b}} \Pi^{\mathrm{b}} \tag{9}$$

Comparing this equation with expression (7) leads to

$$v_0^{\mathrm{b}} > v_0^{\mathrm{f}} \tag{10}$$

This relationship means that the fundamental energy level is more populated for the bound than for the free enzyme.

If Ω_ε and the corresponding partition function Π are large, the information content of the population of enzyme molecules is large. Thus the 'instruction content' of this population is small. Alternatively, if Ω_ε and Π are small, the information content of the population of molecules is smaller, a number of a priori possible messages have been deleted and the corresponding instruction content is increased.

There is an obvious similarity between this information content and the entropy, S, of the population of molecules, for the entropy assumes the form

$$S = k_{\mathrm{B}} \ln \Omega_\varepsilon \tag{11}$$

where k_{B} is now the Boltzmann constant. Simple inspection of equation (4) shows that the larger the number of populated energy levels, the larger the information content and the entropy may expected to be. It thus appears that there is a close similarity between both the entropy and the information content on the one hand, and the organization (or negentropy) and the 'instruction' content on the other hand.

As an enzyme in the eukaryotic cell may be associated with a cell organelle or another enzyme, one may wonder whether the association of an enzyme with a 'rigid body' or another 'flexible body' results in a change of its entropy or of its instruction content (fig. 1). In fact this association leads to entropy loss, for the enzyme loses translational and rotational degrees of freedom, and the mobility of the polypeptide chain in the vicinity of the zone of contact between the two partners is decreased as well. The number of complexions of the bound enzyme molecules is now

$$\Omega_\varepsilon^{\mathrm{b}} = \frac{N!}{\prod_j v_j^{\mathrm{b}}!} \tag{12}$$

whereas that of the free enzyme is

$$\Omega_\varepsilon^{\mathrm{f}} = \frac{N!}{\prod_i v_i^{\mathrm{f}}!} \tag{13}$$

As the number of energy states of the bound enzyme has been reduced owing to its association with the rigid body,

$$\Omega_{\varepsilon}^{\mathrm{f}} > \Omega_{\varepsilon}^{\mathrm{b}} \tag{14}$$

This difference in the number of complexions explains the loss of entropy and of the information content of the bound enzyme. It is easy to evaluate this entropy loss. One has

$$S^{\mathrm{b}} - S^{\mathrm{f}} = k_{\mathrm{B}} \ln \frac{\Omega_{\varepsilon}^{\mathrm{b}}}{\Omega_{j}^{\mathrm{b}}} \tag{15}$$

A somewhat simpler form of this expression can be derived, since

$$\ln \frac{\Omega_{\varepsilon}^{\mathrm{b}}}{\Omega_{\varepsilon}^{\mathrm{f}}} = \ln \frac{\prod_{i} v_i^{\mathrm{f}}!}{\prod_{j} v_j^{\mathrm{b}}!} \tag{16}$$

which may be rewritten as

$$\ln \frac{\Omega_{\varepsilon}^{\mathrm{b}}}{\Omega_{\varepsilon}^{\mathrm{f}}} = \sum_{i} \ln v_i^{\mathrm{f}}! - \sum_{j} \ln v_j^{\mathrm{b}}! \tag{17}$$

Making use of the Stirling formula leads to

$$\ln \frac{\Omega_{\varepsilon}^{\mathrm{b}}}{\Omega_{\varepsilon}^{\mathrm{f}}} = \sum_{i} v_i^{\mathrm{f}} \ln v_i^{\mathrm{f}} - \sum_{j} v_j^{\mathrm{b}} \ln v_j^{\mathrm{b}} \tag{18}$$

As $v_i^{\mathrm{f}} = p_i^{\mathrm{f}} N$ and $v_i^{\mathrm{b}} = p_i^{\mathrm{b}} N$ (where p is now the probability), this expression assumes the simple form

$$\ln \frac{\Omega_{\varepsilon}^{\mathrm{b}}}{\Omega_{\varepsilon}^{\mathrm{f}}} = N\left(\sum_{i} p_i^{\mathrm{f}} \ln p_i^{\mathrm{f}} - \sum_{j} p_j^{\mathrm{b}} \ln p_j^{\mathrm{b}}\right) \tag{19}$$

Either of these two terms in parentheses represents a new expression of information content, that is the number of all the possible messages of the protein in the free and bound states, respectively. A decrease of this number of messages when going from the free to the bound state, is equivalent to a 'choice' of information among all the messages available in the free protein. It therefore corresponds to an instruction given to the bound protein.

During the binding process the free energy content of the bound enzyme can be shown to increase relative to that of the free state. For the free enzyme

$$G^{\mathrm{f}} = H^{\mathrm{f}} - TS^{\mathrm{f}} \tag{20}$$

where G, H, T and S represent Gibbs free energy, enthalpy, absolute temperature and entropy, respectively. Similarly, for the bound enzyme,

$$G^{\mathrm{b}} = H^{\mathrm{b}} - TS^{\mathrm{b}} \tag{21}$$

The change of free energy of the enzyme during the binding process is thus

$$\Delta G = \Delta H - k_{\mathrm{B}} T \ln \frac{\Omega_{\varepsilon}^{\mathrm{b}}}{\Omega_{\varepsilon}^{\mathrm{f}}} \tag{22}$$

where

$$\Delta G = G^{\mathrm{b}} - G^{\mathrm{f}}$$

$$\Delta H = H^{\mathrm{b}} - H^{\mathrm{f}} \tag{23}$$

As ΔH is very close to 0 for nearly constant conditions of temperature and volume, ΔG can be expected to be positive.

It is worth stressing that the expression of ΔG above (equation 22) does not represent the free energy of association of the enzyme with the rigid body, but the free energy that has been stored in the enzyme upon its association with this rigid body. The ideal thermodynamic scheme of figure 1 may be used to clarify this point. The free energy of binding of an enzyme on a surface and the associated conformational change is assumed to be negative ($\Delta G < 0$). This overall free energy may be ideally split into two contributions: the first one corresponds to the energy required to change the enzyme conformation and is endergonic ($\Delta G_1 > 0$); the second one pertains to the binding of this modified conformation on the surface and is exergonic ($\Delta G_2 < 0$). The ΔG which appears in equation (22) pertains to ΔG_1 only and represents the free energy stored in the enzyme.

These simple considerations of physical chemistry have some important implications that may be submitted to experimental control. Owing to its association with the cell organelle or with another protein, the information content of an enzyme can be expected to be different from that of the corresponding structural gene. It can also be expected that the free energy stored in the bound enzyme will be used, at least in part, to catalyse a chemical process or to facilitate the binding of a ligand to a protein. Moreover, the above reasoning shows that the association of a relatively compact and rigid protein with an enzyme having a rather loose structure may result in a 'discrimination', a choice of information or an instruction given to the enzyme as well as in a free energy gain of this enzyme. This may be viewed as an energy and information transfer from protein to protein.

Instructions given by chaperones and prion proteins

The aim of this section is certainly not to present a review on chaperones and prion proteins. It is rather to discuss, on the basis of our knowledge of these two important types of macromolecules, whether the association of polypeptide chains having different or identical primary structure but different conformations may result in instruction transfer between proteins.

Chaperones

Since the pioneer work of Anfinsen [21, 22], numerous authors have shown that in vitro, in highly dilute solutions, the renaturation of unfolded polypeptide chains

results in an active protein. It was therefore inferred that the 'correct' folding of the polypeptide chain was solely defined by the amino acid sequence. However, the discovery of chaperones and chaperonins has led to the conclusion that these proteins play an important role in the correct folding of polypeptide chains in vivo. It has sometimes been claimed that these chaperones are in a way catalysts that accelerate the folding process without altering its nature. If this were so, the idea that the conformation of the protein is under the sole control of its primary structure would imply that the correct folding process spontaneously takes place both in vitro and in vivo.

In order to know whether chaperone proteins instruct polypeptide chains about the correct conformation to assume, it is important to recall very briefly some of the properties of these macromolecules. Class I chaperones bind to the nascent polypeptide chain and prevent their spontaneous folding, thus allowing transport of the unfolded chains in various organelles such as mitochondria [28–32]. In fact, most of the mature protein found in mitochondria and chloroplasts have been synthesized in the cytoplasm and transported through membranes. It is therefore obvious that these proteins cannot be transported in a globular state. Class I chaperones bind to the hydrophobic regions of the nascent polypeptide chain, preventing aggregation processes. Once in the organelle, a class II chaperone directs the correct folding of the protein. Some class II chaperones or chaperonins are large molecular edifices which display a cleft that may accommodate one or two polypeptide chains in the molten globule state [33]. This association directs the correct folding of the polypeptide chain thanks to the numerous bonds that have been formed with the chaperone. The hydrolysis of adenosine triphosphate (ATP) is thus required to break these bonds. Some chaperones may, depending on the conditions, prevent or direct the folding of polypeptide chains [32]. There is also a relationship between heat shock and chaperones. Under conditions of stress, many cellular proteins tend to unfold, thus leading to aggregation. Under in vivo conditions this process is prevented by stimulation of chaperone synthesis, which results in the correct refolding of aggregated, or misfolded, polypeptides [34–36].

On the basis of this very brief survey of the properties of chaperones, one may thus wonder whether these chaperones may be considered as the molecular agents of an instruction process. There is no reason to believe that the principles that govern protein folding in vivo are any different from those that apply to in vitro situations. Therefore it seems reasonable to conclude that the information required for the correct folding of the active, isolated protein in dilute media is indeed present in the corresponding structural gene. However, owing to the molecular crowding of the living cell, the probability of aggregation of polypeptide chains and misfolding is very high, and chaperones are mandatory for correct protein folding in vivo. The role played by chaperonins to overcome this molecular crowding is illustrated by some of these molecular edifices which acts as an Anfinsen cage [37] by preventing the collision of polypeptide chains and their aggregation. Thus, although the physical processes involved in protein folding are basically the same in vivo and in vitro, the nascent polypeptide chains need the presence of chaperones in order to fold properly. Moreover, there is little doubt that many denatured enzymes have never been renatured in vitro. This may be so either because the spontaneous renaturation is thermodynamically impossible or because the optimal conditions for this renaturation have not yet been found [38]. If the spontaneous renaturation is, in some cases at least, thermodynamically impossible, one must conclude that these enzymes, which exist in vivo in their correctly folded conformation, have received some information or instruction from the chaperones.

Some proteins require a specific region of their primary sequence to fold correctly. During the folding process this sequence is removed by an autolytic or exogenous proteolytic cleavage. The fragment of the polypeptide chain which is removed is called an intramolecular chaperone. Subtilisin [39–44], α-lytic protease [45], aqualysin [46] and carboxypeptidase Y [47] are some examples of these types of proteins. At low concentrations, and in the absence of the intramolecular chaperone, subtilisin and α-lytic protease may fold up to the molten globule state [48–50]. Addition of the intramolecular chaperone domain allows the correct refolding up to the final active state [49, 50]. It may thus be tentatively concluded that the intramolecular chaperone helps overcome the free energy barriers that occur during the late stages of the folding process [48, 51]. A mutation in the intramolecular chaperone domain may result into two subtilisin molecules that indeed have the same sequence but different conformations and activities [44]. The question whether chaperones instruct proteins of their active conformation is thus a matter of debate.

Prions

Although it is difficult to conclude at the moment whether instruction transfer takes place between a chaperone and a protein, it appears very likely that this instruction transfer does indeed occur between prion proteins. Scrapie and other diseases have been shown to be due to a protein called prion protein (PrPc). This protein does not appear to be associated with any nucleic acid [52–55]. Surprisingly, however, this protein has been shown to be present in healthy organisms [53]. Thus the origin in molecular terms and how the disease is propagated have long been a puzzle [54]. The struc-

ture of this PrPc protein has been established, and its gene has been identified and cloned. In animals displaying the scrapie disease, this gene is unaffected as well as the primary structure of the corresponding prion protein. Several experimental results, however, strongly suggest that it is the conformation of the protein that is altered [52]. The 'pathogenic' prion protein is called PrPsc, and the propagation of the disease is thought to occur through an interaction between PrPc and PrPsc resulting in the conversion of PrPc into PrPsc [56]. Although this interpretation has been the subject of a controversy, it appears today very likely [54].

In the context of this theory of the molecular nature of the prion disease, the pathogenic protein, PrPsc, gives a 'nonpathogenic' protein, PrPc, the instruction to become pathogenic. As soon as these views were presented, they were considered incompatible with the most widely accepted ideas of classical molecular biology. Still, the considerations of statistical mechanics, which have been presented above, allow one to predict that an instruction may possibly be transferred from protein to protein thanks to their association. The cardinal idea of the present review article is precisely to show that this instruction transfer is most probably the rule in multienzyme complexes rather than the exception.

Multienzyme complexes, instruction and energy transfer

The enzyme complexes that catalyse nonconsecutive reactions represent good candidates for studying instruction and energy transfer from protein to protein. Three examples of this transfer within bienzyme complexes will now be discussed: the plasminogen streptokinase, the phosphoribulokinase-glyceraldehyde-3-phosphate dehydrogenase (PRK-GraPDH) systems and the Ras-GTPase activating protein (Ras-GAP). The properties of the first type of complex will be discussed only briefly, for very little is known about the physical chemistry of this instruction transfer.

The plasminogen-streptokinase system

The conversion of plasminogen into the active enzyme plasmin requires a specific proteolytic cleavage which can be effected thanks to various serine proteases such as urokinase. Moreover, a single-chain polypeptide of 414 amino acids derived from different strains of *Streptococcus* and called streptokinase can also generate plasminogen activation and is thus widely used in humans to dissolve the thrombi that cause myocardial infarctions. Surprisingly, this polypeptide is totally devoid of proteolytic activity. It binds to plasminogen and forms a 1:1 molar complex with this protein [57–60]. Although this complex is not a real bienzyme complex, for streptokinase is not a true enzyme, it may be viewed as a simple model system that displays instruction transfer between a polypeptide chain and a protein. The productive binding process must involve a conformational change that results in the appearance of an accessible site in the zymogen, without any proteolytic cleavage. From the functional properties of the plasminogen-streptokinase complex, one may infer that its active region is a Ser active site that seems similar although not identical to that of the plasmin [61, 62].

Recombinant streptokinase binds to plasminogen in a similar fashion. Recombinant streptokinase fragments have also been tested for their ability to bind to plasminogen and to induce its activation [63]. Recombinant streptokinase fragments spanning amino acids 1–352, 120–352, 244–414 and 244–352 bind tightly to plasminogen and competitively inhibit the attachment of streptokinase. Other recombinant streptokinase fragments spanning amino acids 1–127 or 1–253 do not bind to plasminogen. Moreover, none of the fragments that specifically bind to plasminogen is able to induce its specific activity [63]. It thus appears likely that other regions of the streptokinase polypeptide chain are involved in correct binding to plasminogen and in inducing of the conformational change involved in expression of functional activity.

The inescapable conclusion of these results is that plasminogen receives an instruction from streptokinase through the 1:1 molar complex.

The PRK-GraPDH complex

Several multienzyme complexes have been isolated from chloroplasts [64–81]. The results described below concern a bienzyme complex from *Chlamydomonas reinhardtii*.

Properties of the PRK-GraPDH complex. This complex is made up of phosphoribulokinase and glyceraldehyde-3-phosphate dehydrogenase. Both enzymes located in the chloroplast take part in the Benson-Calvin cycle of fixation and reduction of carbon dioxide. This bienzyme complex may be isolated as a relatively stable supramolecular entity which has a molecular mass of 460 kDa and is made up of eight GraPDH and four PRK chains. As the chloroplast GraPDH is tetrameric and the phosphoribulokinase dimeric, this means that the complex is made up of two molecules of GraPDH and two molecules of PRK. Under nondenaturing conditions this complex may be labelled with antibodies raised against either PRK or GraPDH [70] (fig. 2A).

Chloroplast PRK may also be obtained in a free stable state, not associated with GraPDH. Under these conditions, chloroplast PRK displays significant activity only if it is reduced. The bienzyme complex PRK-GraPDH

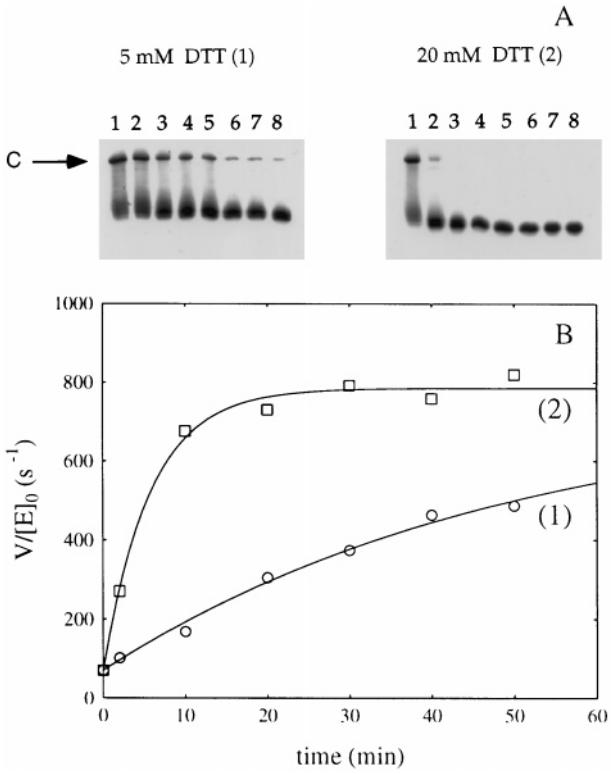

Figure 3. Activation of PRK by dithiothreitol. (*A*) The PRK-GraPDH complex dissociates when submitted, for different time periods, to different concentrations (5 and 20 mM) of dithothreitol. Both the complex and the free PRK are labelled with antisera raised against PRK. (*B*) The PRK activity of the PRK-GaPDH complex in the presence of either 5 mM dithiothreitol (curve 1) or 20 mM dithiothreitol (curve 2) is followed as a function of time. Reprinted with premission from: Avilan L., Gontero B., Lebreton S. and Ricard J. (1997) Memory and imprinting effects in multi-enzyme complexes. I. Isolation, dissociation and reassociation of a phosphoribulokinase-glyceraldehyde-3-phosphate dehydrogenase complex from *Chlamydomonas reinhardtii* chloroplasts. Eur. J. Biochem. **246:** 78–84. © 1998 Federation of European Biochemical Societies, Cambridge.

spontaneously dissociates in the presence of either physiological or nonphysiological reducing agents such as thioredoxin or dithiothreitol. In parallel with the dissociation of the complex, the PRK becomes more and more active [70] (fig. 3). Under oxidizing conditions, this dissociation process is reversible: PRK and GraPDH spontaneously associate to form the complex [71].

The gene coding for chloroplast PRK has been isolated, cloned and expressed in *Escherichia coli*. The recombinant protein may form, with GraPDH, a complex which is apparently indistinguishable from that extracted from *Chlamydomonas* cells. A PRK has been isolated from the 12-2B mutant of the same organism. In this mutant, the Arg 64 residue has been replaced by cysteine, and the bienzyme complex does not exist. These results suggest that this arginine residue plays a key role in the formation of the complex. This conclusion may be experimentally demonstrated by site-directed mutagenesis. Arg 64 has been replaced by alanine [A64], lysine [K64] and glutamic acid [E64]. Whereas the [A64] and [E64] PRKs are unable to form a complex with GraPDH, the [K64] mutant does, although slightly [72] (fig. 2B).

Control of the activity of PRK in the complex: instruction transfer and imprinting effects. Although isolated oxidized PRK is nearly totally devoid of activity, significant activity may be detected when the complex and the substrates are introduced in the same reaction mixture. The enzyme reaction takes place even in the presence of glutathione, that is under oxidizing conditions. Under these conditions, the progress curve of PRK reaction displays a lag. This lag is related to the dissociation of the complex, for if this complex is incubated 15 min in the same reaction mixture but lacking PRK substrate no lag is observed when the reaction is started [71] (fig. 4A).

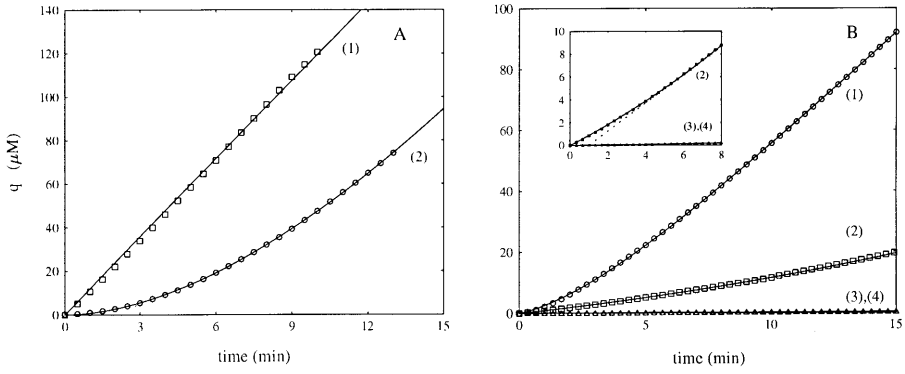

Figure 4. Progress curve of PRK activity within the PRK-GraPDH complex. (*A*) The oxidized bienzyme complex is preincubated for 15 min in a suitable medium but in the absence of the substrates. The reaction is initiated by adding the substrates in the reaction medium. No lag is observed (curve 1). The reaction is initiated by adding the complex to the reaction medium containing the substrates (curve 2). From ref. 71. (*B*) Progress curves obtained by adding to the reaction medium containing the substrates a mixture of recombinant PRK and GraPDH. Curve 1: wild-type recombinant PRK and GraPDH. Curve 2: [K64] PRK and GraPDH (main figure and insert). Curves 3 and 4: wild-type and [K64] PRK alone (main figure and insert). Reprinted with permission from: Avilan L., Gontero B., Lebreton S. and Ricard J. (1997) Information transfer in multienzyme complexes. II. The role of Arg64 of *Chlamydomonas reinhardtii* phosphoribulokinase in the information transfer between glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase. Eur. J. Biochem. **250:** 296–302, © 1998 Federation of European Biochemical Societies, Cambridge.

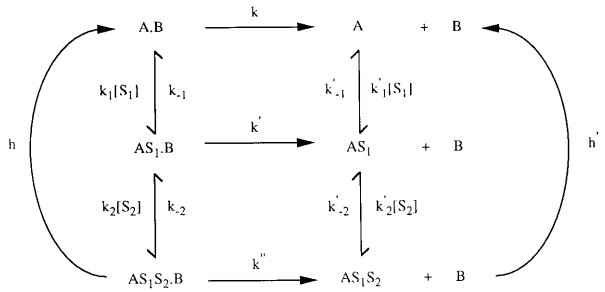

Figure 5. Theoretical model for the dissociation of a PRK-GraPDH complex. k, k' and k'' are the rate constants of dissociation, h and h' the apparent catalytic constants. A and B represent one, or several, enzyme molecule(s). Both the isolated enzyme, A, and the complex, AB, are assumed to display a catalytic activity. Reprinted with permission from: Lebreton S., Gontero B., Avilan L. and Ricard J. (1997) Memory and imprinting effects in multienzyme complexes. II. Kinetics of the bi-enzyme complex from *Chlamydomonas reinhardtii* and hysteretic activation of chloroplast oxidized phosphoribulokinase. Eur. J. Biochem. **246:** 85–91, © 1998 Federation of European Biochemical Societies, Cambridge.

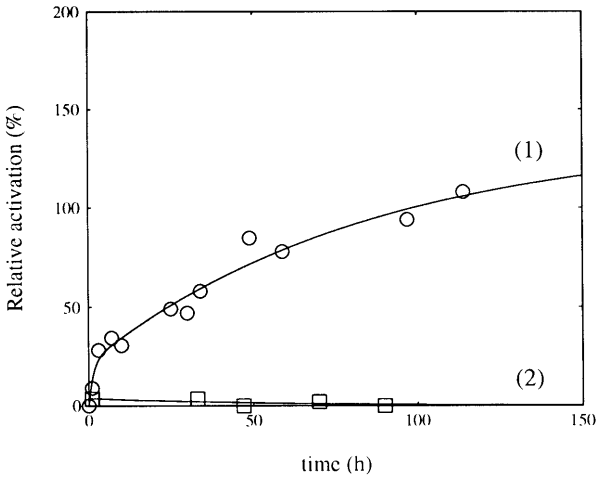

Figure 6. Appearance of PRK activity during the reconstitution of the PRK-GraPDH complex. Activation of oxidized stable PRK by GraPDH (curve 1). The activity is paralleled by formation of the complex (not shown). Curve 2 shows the lack of PRK activity when this enzyme is mixed with bovine serum albumin. Reprinted with permission from: Lebreton S., Gontero B., Avilan L. and Ricard J. (1997) Memory and imprinting effects in multienzyme complexes. II. Kinetics of the bi-enzyme complex from *Chlamydomonas reinhardtii* and hysteretic activation of chloroplast oxidized phosphoribulokinase. Eur. J. Biochem. **246:** 85–91, © 1998 Federation of European Biochemical Societies, Cambridge.

The recombinant PRK associated with GraPDH displays the same type of kinetic behaviour as the wild-type enzyme in the complex (fig. 4B). Whereas the [K64] mutant of PRK displays a slight enzyme activity in the presence of GraPDH, the other mutants [A64] and [E64] do not [72] (fig. 4B). A simple model that represents the dissociation of the complex together with PRK activity is shown in figure 5. This model takes account of the ordered binding of the substrates, ribulose-5-phosphate (Ru5P) being bound first and ATP afterwards. The equation that may be derived from the model fits the experimental results best only if one assumes (as postulated in the model) that both the free PRK released upon dissociation of the complex and the bound PRK of this complex are active. If only the free PRK is assumed to be active, the fit is biased. Thus the reaction rate catalysed by the bound PRK of the complex can be measured by monitoring the reaction rate immediately after mixing this complex with its substrates in a suitable reaction medium. The active oxidized form of PRK, which is released upon dissociation of the complex, is not stable, for it slowly loses its activity and becomes identical to the stable and almost inactive form. It is a metastable conformation of the enzyme that may be characterized by fluorescence spectroscopy [73]. Thus,

Table 1. Kinetic parameters of the three forms of PRK.

State of oxidized phosphoribulokinase	K_m (Ru5P) (μM)	K_m (ATP) (μM)	k_{cat} (s^{-1}/site)
As part of the complex	30 ± 2.3	46 ± 4.6	3.25 ± 0.1
Free 'metastable state'	59 ± 2.5	48 ± 0.9	56.5 ± 1.1
Free 'stable state'	115 ± 5	89 ± 9	0.06 ± 0.02

Table 2. Thermodynamic parameters associated with the PRK reaction in the PRK-GraPDH complex.

	Ru5P	ATP
$\overline{K}^*$ (mM^{-1})	8.69 ± 1.4	11.23 ± 1.22
$\overline{K}$ (mM^{-1})	31.29 ± 2.23	20.37 ± 1.91
k^* (s^{-1}/site)	0.062 ± 0.02	0.065 ± 0.025
k (s^{-1}/site)	3.52 ± 0.065	3.3 ± 0.097
λ (kJ/mol)	-3.05 ± 0.09	-1.4 ± 0.11
U_γ^σ (kJ/mol)	-12.76 ± 0.07	-10.84 ± 0.09
$\overline{\Delta G}^*$ (kJ/mol)	-21.77 ± 0.4	-22.38 ± 0.26
$\overline{\Delta G}$ (kJ/mol)	-24.84 ± 0.17	-23.81 ± 0.22
$\Delta G^{\neq *}$ (kJ/mol)	77.33 ± 0.36	77.22 ± 0.46
$\Delta G^{\neq}$ (kJ/mol)	67.65 ± 0.04	67.79 ± 0.07

The significance of λ and U_γ^σ is given in the text. $\overline{\Delta G}^*$ and $\overline{\Delta G}$ represent the apparent binding energies of either substrate to the isolated enzyme and to the enzyme inserted in the bienzyme complex. $\Delta G^{\neq *}$ and $\Delta G^{\neq}$ are the free energies of activation associated with the catalytic step for the free enzyme and for the enzyme in the complex, respectively.

although there is only one struc-tural gene coding for chloroplast PRK, there are three different forms that indeed have the same amino acid sequence but different conformations and activities: the stable enzyme form, the enzyme form bound to GraPDH and the metastable free enzyme form. These three forms differ in their K_m and k_{cat} values (table 1).

From these data it is therefore clear that GraPDH has given PRK an instruction and that the instruction has resulted in an increase of activity of this enzyme. This view may be confirmed by mixing the stable, oxidized and almost inactive PRK with GraPDH. The bienzyme complex is thus formed, and the catalytic activity of PRK increases (fig. 6).

Moreover, as mentioned above, the free PRK which is released after dissociation of the complex is in a metastable state that slowly relapses into the stable and almost inactive state. This metastable state is very active and retains an imprinting exerted on PRK by GraPDH. Why is this isolated form of PRK in a metastable state more active than the same enzyme bound to GraPDH? The reason is probably that the catalytic activity requires significant mobility of the enzyme molecule, which is favoured if this molecule is in a free state.

Thermodynamics of instruction transfer and imprinting effects. Statistical mechanics offers the possibility of quantitatively expressing the extent of this instruction and energy transfer and how the energy is used to alter the kinetic parameters of the reaction. If k represents the rate constant of a given chemical process carried out by an enzyme which is part of a multienzyme complex, and k^* the rate constant of the same process carried out by the same enzyme in a free state, thermodynamics dictates that

$$k = k^* \exp\{-(U_\gamma - U_\tau)/RT\} \tag{24}$$

where U_γ and U_τ represent the stabilization-destabilization energies of the ground and of the transition states exerted by protein-protein association. R and T have their usual significance. The difference $(U_\gamma - U_\tau)$ thus represents the fraction of energy transferred in the complex and used to increase or decrease the rate constant. In the case of a multimeric enzyme embedded in a multimolecular edifice, stabilization-destabilization energies are generated by the spatial arrangements (α) and the conformational constraints (σ) that may exist between identical or different polypeptide chains. Thus, if $U_\gamma^{\alpha,\sigma}$ represents the stabilization-destabilization energy of the ground state of a given process carried out by the active site embedded in a supramolecular edifice,

$$U_\gamma^{\alpha,\sigma} = U_\gamma^\alpha + U_\gamma^\sigma \tag{25}$$

where U_γ^α and U_γ^σ represent the stabilization-destabilization energies exerted by the arrangement of polypeptide chains (U_γ^α) and their conformational constraints (U_γ^σ), respectively. Current transition state theory as applied to polymeric enzyme and enzyme complex implies that

$$U_\tau^{\alpha,\sigma} = U_\gamma^\alpha \tag{26}$$

or put in another way, the conformational constraints between polypeptide chains must vanish in the transition states. If the enzyme in the complex is multimeric and follows Michaelis-Menten kinetics, this means that the stabilization-destabilization energies in the ground and the transition states vary linearly along the reaction coordinate and that this variation is the same for the ground and the transition states. Thus one has

$$\frac{\partial U_\gamma^{\alpha,\sigma}}{\partial \xi} = \frac{\partial U_\gamma^\alpha}{\partial \xi} = \lambda \tag{27}$$

where ξ is the advancement of the reaction. In the language of nonequilibrium thermodynamics, λ is equivalent to the affinity of a chemical process. It will therefore be termed the affinity of the stabilization-destabilization process. Equation (27) above implies that

$$\frac{\partial U_\gamma^\sigma}{\partial \xi} = 0 \tag{28}$$

and therefore that U_γ^σ is constant and represents the stabilization-destabilization energy exerted through conformational constraints between identical or different polypeptide chains.

Free phosphoribulokinase in its oxidized and stable state is a dimer and follows Michaelis-Menten kinetics, and its subunits do not appear to display any significant interaction. Thus, if the steady-state rate is plotted relative to the concentration of either substrate, when the nonvaried substrate is saturating,

$$\frac{v}{2k^*[E]_0} = \frac{\bar{K}^*[S]}{1 + \bar{K}^*[S]} \tag{29}$$

where S is the varied substrate (the other substrate being kept at a saturating level), $\bar{K}^*$ the apparent affinity constant of the varied substrate for the enzyme, k^* the catalytic constant and $[E]_0$ the total enzyme concentration. If the same PRK is associated with GraPDH, it still exhibits Michaelis-Menten behaviour, and the corresponding steady-state equation relative to either substrate (when the other is saturating) is now

$$\frac{v}{4k^*[E]_0} = \frac{\bar{K}^*[S]\exp(-U_\gamma^\sigma/RT)}{1 + \bar{K}^*[S]\exp(-\lambda/RT)} \tag{30}$$

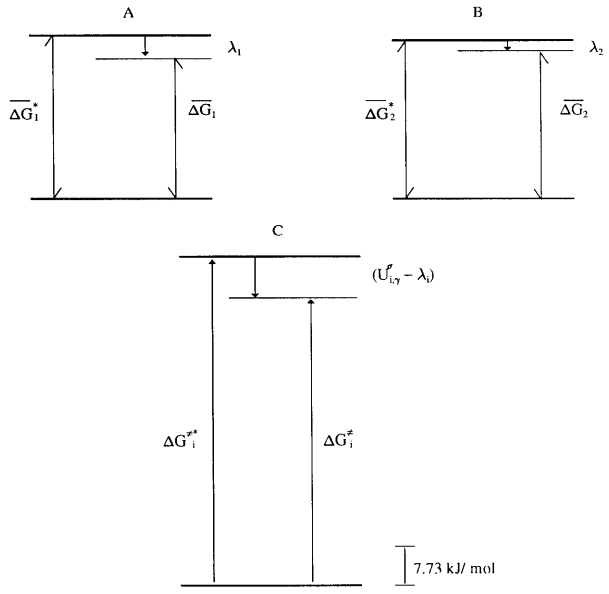

Figure 7. Instruction transfer within the PRK-GraPDH complex. (*A*) The instruction transfer between GraPDH and PRK results in a decrease of the exergonic character of the Ru5P binding to the enzyme in the complex. (*B*) The same kind of effect is observed for the ATP binding, but its amplitude is decreased relative to the previous one. (*C*) The instruction transfer between the two enzymes results in a marked decrease of the height of the energy barrier associated with the catalytic constant. The index *i* may adopt values 1 (Ru5P) or 2 (ATP). Reprinted with permission from: Lebreton S., Gontero B., Avilan L. and Ricard J. (1997) Information transfer in multienzyme complexes. I. Thermodynamics of conformational constraints and memory effects in the bienzyme glyceraldehyde-3-phosphate dehydrogenase-phosphoribulokinase complex of *Chlamydomonas reinhardtii* chloroplasts. Eur. J. Biochem. **250:** 286–295, © 1998 Federation of European Biochemical Societies, Cambridge.

Comparison of equations (29) and (30) shows how the spatial organization of the supramolecular edifice and conformational constraints between the polypeptide

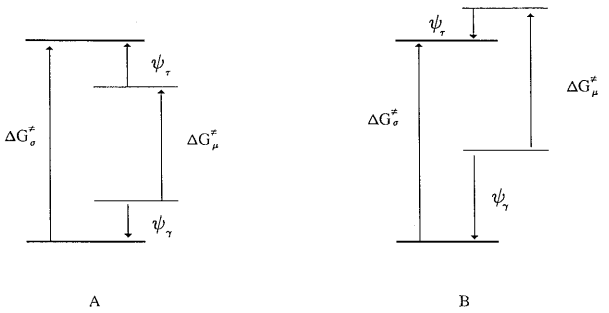

Figure 8. Imprinting of an instruction in an enzyme. Upon dissociation of the complex, the activity of PRK is transitorily enhanced. This implies that the difference $\psi_\gamma - \psi_\tau$ is of necessity negative. The situations A and B in the figure are compatible with this requirement.

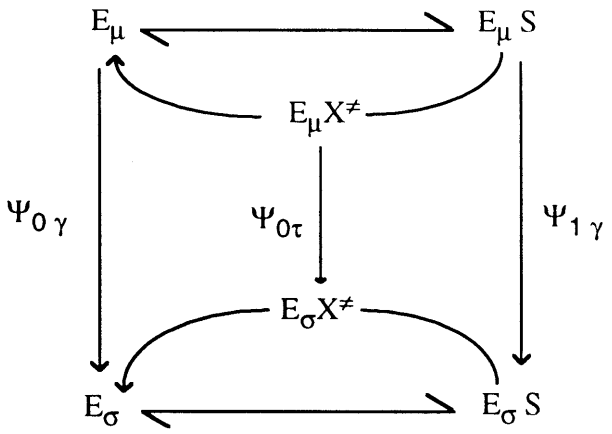

Figure 9. Significance of ψ_γ and ψ_τ involved in an enzyme reaction. The enzyme is assumed to exist in a stable (σ) and a metastable (μ) enzyme form. $\psi_{0\gamma}$ and $\psi_{1\gamma}$ represent the free energy differences between the mestastable and the stable forms of the free enzyme and of the enzyme-substrate complex in their ground states. $\psi_{0\tau}$ is the corresponding free energy difference between the two transition states.

chains have altered the rate equation of PRK. The communication and energy transfer between the two enzymes is thus exerted thanks to the two parameters λ and U_γ^σ. This effect may be exerted on both the apparent substrate affinity constant and on the catalytic constant. It can thus easily be shown that

$$\bar{K} = \bar{K}^* \exp(-\lambda/RT)$$
$$k = k^* \exp\{-(U_\gamma^\sigma - \lambda)/RT\} \quad (31)$$

where $\bar{K}$ represents the apparent affinity constant of either substrate for the enzyme within the complex and k the corresponding catalytic constant. $\bar{K}^*$ and k^* still have the significance they had in equation (29).

All the thermodynamic parameters associated with the transfer of energy from protein to protein can thus be determined (table 2). These parameters are the following: two values of λ and U_γ^σ (for either ATP or Ru5P saturating); two apparent binding energies, $\overline{\Delta G}^*$, of the substrates on the free enzyme; two apparent binding energies, $\overline{\Delta G}$, of the substrates on the GraPDH-bound enzyme; the free energy of catalysis, $\Delta G^{\neq *}$, for the free enzyme; the free energy of catalysis, $\Delta G^{\neq}$, for the enzyme in the complex.

The affinity of stabilization-destabilization, λ, favours binding of Ru5P and ATP on the associated PRK by about 3 kJ/mol and 1.5 kJ/mol, respectively. The effect on the association of the polypeptide chains and of their respective constraints on substrate binding to the PRK in the complex is thus weak, but it is very large on the

Table 3. Thermodynamic parameters associated with the imprinting of GraPDH on PRK.

	Ru5P	ATP
$\bar{K}_\sigma$ (mM^{-1})	8.69 ± 1.4	11.23 ± 1.22
$\bar{K}_\mu$ (mM^{-1})	16.89 ± 0.7	20.84 ± 0.4
k_σ (s^{-1}/site)	0.062 ± 0.02	0.065 ± 0.025
k_μ (s^{-1}/site)	57 ± 0.71	56.5 ± 0.34
$\bar{K}_\sigma k_\sigma$ (mM^{-1} s^{-1}/site)	0.54 ± 0.15	0.72 ± 0.2
$\bar{K}_\mu k_\mu$ (mM^{-1} s^{-1}/site)	962 ± 30	1177 ± 17
Transferred energy used to alter $\bar{K}$ $\psi_{0\gamma} - \psi_{1\gamma}$ (kJ/mol)	-1.59 ± 0.01	-1.48 ± 0.06
Transferred energy used to alter k $\psi_{1\gamma} - \psi_{0\tau}$ (kJ/mol)	-16.31 ± 0.14	-16.25 ± 0.26
Transferred energy used to alter k $\bar{K}$ $\psi_{0\gamma} - \psi_{0\tau}$ (kJ/mol)	-17.9 ± 0.01	-17.73 ± 0.01
$\overline{\Delta G}_\sigma$ (kJ/mol)	-21.77 ± 0.4	-22.38 ± 0.26
ΔG_μ (kJ/mol)	-23.35 ± 0.01	-23.86 ± 0.05
$\Delta G_\sigma^{\neq}$ (kJ/mol)	77.33 ± 0.36	77.22 ± 0.46
$\Delta G_\mu^{\neq}$ (kJ/mol)	61.61 ± 0.03	61.69 ± 0.02

The difference between ψ_s is already defined in the text. $\overline{\Delta G}_\sigma$ and $\overline{\Delta G}_\mu$ represent the apparent binding energies of either substrate on the stable and metastable enzyme forms. $\Delta G_\sigma^{\neq}$ and $\Delta G_\mu^{\neq}$ are the free energies of activation for the catalytic step of the stable and the metastable enzyme forms.

catalytic process itself. The height of the energy barrier associated with catalysis is decreased by almost 10 kJ/mol. Within the complex, GraPDH must therefore have given PRK an instruction. This process is indeed accompanied by a transfer of energy from GraPDH to PRK, and the energy is mostly used to facilitate catalysis. This instruction and energy transfer from protein to protein may be depicted by the thermodynamic boxes of figure 7.

PRK activity is enhanced after dissociation of the bi-enzyme complex. This oxidized PRK is not in a stable state, for it slowly relapses to the stable and almost inactive conformation. This metastable form thus keeps an imprinting of GraPDH. Thermodynamics allows us to understand how the energy stored in the metastable enzyme form may be used to alter its intrinsic kinetic properties. Let us consider a chemical process carried out at different rates by the metastable and stable enzyme forms. If k'_μ and k'_σ are the rate constants of the same process carried out by the metastable (μ) and the stable (σ) enzyme forms, according to the laws of thermodynamics

$$k'_\mu = k'_\sigma \exp\{-(\psi_\gamma - \psi_\tau)/RT\} \tag{32}$$

The difference $(\psi_\gamma - \psi_\tau)$ represents the fraction of energy transferred from protein to protein, transiently stored in the PRK and used to alter the rate constant. As the metastable form is more active than the stable one, the difference $(\psi_\gamma - \psi_\tau)$ has to be negative. The thermodynamic diagrams of figure 8 illustrate this property.

The experimental results considered above show that the metastable form of PRK follows Michaelis-Menten kinetics. If $\bar{K}_\sigma$ is the apparent substrate affinity constant (at saturating concentration of the other substrate) and k'_σ the corresponding catalytic constant of the stable enzyme form, the steady-state rate of the metastable enzyme form may be written as

$$\frac{v}{2k'_\sigma[E]_0} = \frac{\bar{K}_\sigma[S]\exp\{-(\psi_{0\gamma} - \psi_{0\tau})/RT\}}{1 + \bar{K}_\sigma[S]\exp\{-(\psi_{0\gamma} - \psi_{1\gamma})/RT\}} \tag{33}$$

where S is, as previously, the varied substrate (the other substrate being saturating). The significance of the energy terms Ψ is made clear in figure 9. For a Michaelis-Menten process, $\psi_{0\gamma}$ and $\psi_{1\gamma}$ refer to the free enzyme and to the enzyme-substrate complex, respectively, and $\psi_{0\tau}$ to the corresponding transition state.

From equation (33) above we can derive the expressions of the apparent substrate-binding constant for the metastable form as well as that of the corresponding catalytic constant. One finds

$$\bar{K}_\mu = \bar{K}_\sigma \exp\{-(\psi_{0\gamma} - \psi_{1\gamma})/RT\}$$

$$k'_\mu = k'_\sigma \exp\{-(\psi_{1\gamma} - \psi_{0\tau})/RT\} \tag{34}$$

where $\bar{K}_\sigma$ and k'_σ are the apparent substrate-binding constant for the stable form and the corresponding catalytic constant, respectively. The energy differences $(\psi_{0\gamma} - \psi_{1\gamma})$ and $(\psi_{1\gamma} - \psi_{0\tau})$ represent the fraction of energy stored in the metastable state of PRK and used to alter the apparent substrate-binding constant and the catalytic constant, respectively. These energies are shown in table 3.

The metastable conformation of PRK facilitates the binding of either substrate to the enzyme by about the

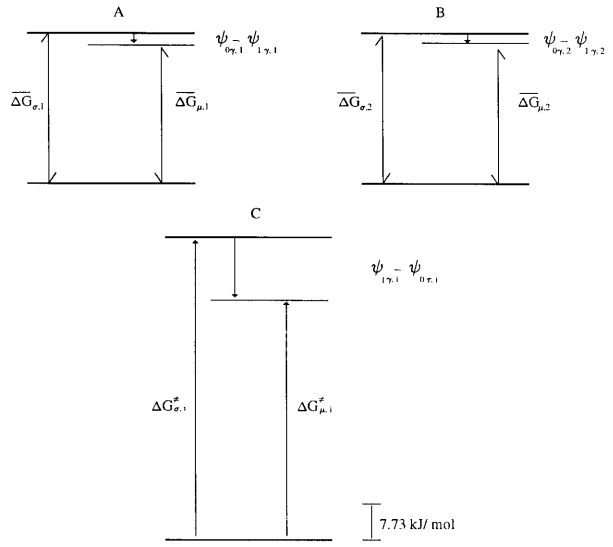

Figure 10. Imprinting effects after dissociation of the PRK-GraPDH complex. (*A* and *B*) The imprinting exerted by GraPDH on PRK is used to slightly decrease the exergonic character of Ru5P and ATP binding on the enzyme. (*C*) The main effect of imprinting is to decrease the height of the energy barrier associated with the catalytic constant. The index *i* adopts the value 1 (Ru5P) and 2 (ATP). Reprinted with permission from: Lebreton S., Gontero B., Avilan L. and Ricard J. (1997) Information transfer in multienzyme complexes. I. Thermodynamics of conformational constraints and memory effects in the bienzyme glyceraldehyde-3-phosphate dehydrogenase-phosphoribulokinase complex of *Chlamydomonas reinhardtii* chloroplasts. Eur. J. Biochem. **250:** 286–295, © 1998 Federation of European Biochemical Societies, Cambridge.

same value (1.5 kJ/mol) but decreases the height of the energy barrier associated with catalysis by about 16 kJ/mol (fig. 10).

The Ras-GAP complex

The guanosine triphosphate (GTP)-binding protein Ras plays the role of a regulator of signal transduction and switches between an inactive guanosine diphosphate (GDP)-bound and an active GTP-bound conformation [82–84]. During this switch, GTP is hydrolysed into GDP plus phosphate. This process, however, is extremely slow, and the apparent catalytic constant of hydrolysis is enhanced in the presence of a variety of proteins, including guanine-nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP). This enhancement may be considerable. Thus, at saturating concentrations of GAP, the catalytic constant of GTP hydrolysis by Ras is multiplied by a factor of 10^5 [85, 86]. Different hypotheses have been proposed [87–90] to explain the rationale for this activation process, but the most likely mechanism is the so-called arginine finger hypothesis [89–91]. This hypothesis has received strong experimental support and can be considered today to be firmly established. The central idea of this proposed mechanism is that GAP is drawn to the active site of Ras and stabilizes the transition state of the reaction through its positive charges [92, 93]. Several experimental findings strongly support this view.

The crystal structure of the catalytic domain of a GAP has been solved by X-ray crystallography [94]. This structure reveals the presence of several arginine residues in the presumed active site of the protein.

In single-turnover stopped-flow experiments performed with GAP-active neurofibromin and Ras [91, 92], a sudden rise is observed, followed by a decline of fluorescence. This may be explained by the binding of GAP on Ras and the subsequent hydrolysis of GTP into GDP and phosphate. Then GAP dissociates from Ras.

The arginine residues that constitute the arginine finger are involved in the catalytic process. In the case of the GAP-active neurofibromin, if these residues are replaced by another uncharged residue, the catalytic activity is nearly totally abolished. In stopped-flow experiments, a rise of fluorescence is observed that is not followed by a decay. This implies that GAP binds to Ras, but that this process is not followed by a significant catalytic process. Last but not least, if lysine residues are introduced by site-directed mutagenesis, significant catalytic activity may be recovered [92].

As mentioned previously, the inescapable conclusion of these results is to assume that the positive charges of arginine residues in the finger of GAP (or lysine residues) neutralize the γ-phosphate oxygen and thus stabilize the transition state [92, 93].

Proteins at the lipid-water interface and instruction transfer to a protein

The above theoretical considerations suggest that binding of an enzyme at the interface between lipids and aqueous medium may give this enzyme an instruction that may alter its intrinsic properties. This is precisely the case for a number of enzymes that may exist either in free solution or bound to a biomembrane. In the course of the binding process, a dramatic increase of the enzyme activity can be observed. For instance, this increase in activity has been observed with protein kinase C and pancreatic lipase. The main properties of these two enzymes will be briefly considered below.

Protein kinase C

Protein kinases C are involved in signal transduction and may phosphorylate glycogen synthase and phosphorylase kinase. Protein kinases appear to be present in the

cytoplasm of the cell but may also be translocated and bound to lipid membranes. The structure of conventional kinase C is complex and reveals four domains [95–101]: the C1 domain with a cysteine-rich motif and that bears an ester-binding site; this domain is immediately preceded by a sequence that plays the part of a pseudo-substrate [96]; the C2 domain contains the site of recognition for acidic lipids and calcium ions [95]; the C3 and C4 domains form the ATP- and substrate-binding sites of the kinase [96].

The so-called pseudo-substrate sequence occupies the active site of the C4 domain and therefore prevents the substrate from entering the site. Thus the enzyme is inactive. Full activity of this enzyme requires first, in the cytoplasm, a maturation process that takes place in three successive steps, the phosphorylation of Thr 500, Thr 641 and Ser 660, all these residues being located in C4 domain. Translocation and binding to the membrane result in the removal of the pseudo-substrate from the active site and therefore promote activation of protein kinase C [102, 103]. Binding of the enzyme to the diacylglycerol and phosphatidylserine of the membrane takes place on the C1 and C2 domains of the protein, respectively [104, 105]. This process is still enhanced in the presence of calcium ions.

The interesting conclusion of this complex process is that the free enzyme becomes active only if it binds to a membrane. It is therefore obvious that the functional activity of the enzyme is not entirely encoded in the corresponding gene.

Pancreatic lipase

In 1958 Sarda and Desnuelle made the important observation that pancreatic lipase becomes strongly activated by lipid-water interfaces [106, 107]. In the absence of these interfaces, the enzyme is almost totally devoid of activity with respect to soluble substrates. Desnuelle and co-workers thus proposed that the enzyme binds at the lipid-water interface and undergoes a conformational change that markedly increases its catalytic activity [107]. The phenomenon was termed interfacial activation. This interfacial activation was subsequently observed with many other lipases and was considered a basic property that distinguishes lipases from esterases [108–110].

Resolution of the three-dimensional structure of several lipases by crystallography [111–113] has indeed confirmed the existence of this conformational change that initiates catalytic activity. The active site is composed of a Ser-His-Asp/Glu catalytic triad reminiscent of the Ser proteases [114]. However, this triad is not exposed on the protein surface and is not directly accessible to the substrate. The active site is in fact covered by surface loops that constitute a lid [115–117]. Several lipases show large rearrangements relative to the free enzyme structure of one or several of the surface loops when inhibitors [115] or phospholipids are bound to these enzymes. These conformational changes result in increased accessibility of the active site of the lipase to its substrate.

Specific experimental conditions may favour the prevalence of open or closed conformations of lipases. Thus, for instance, polyethylene glycol promotes the crystallization of the closed form, whereas alcohols or detergents lead to crystallization of the open state. Comparison of the open conformation with the corresponding closed conformation reveals strikingly that hydrophobic regions of the protein become exposed whereas some of the hydrophylic surface becomes buried, thus leading to an overall increase of the hydrophobic surface.

Communication between proteins and between membranes and proteins

Although it is well known that the eukaryotic cell is highly organized, classical molecular biology, in its mechanistic approach to molecular phenomena, implicitly adopts the view that enzymes act as separate physical entities that catalyse reactions taking place in dilute solution. Although this attitude has proved useful in deciphering the basic chemistry and genetics of many biological processes, it is essentially incorrect. Thus, for example, phosphofructokinase is usually considered to be a main site of regulation of glycolysis on the basis of its properties in vitro. In free solution, this enzyme is allosteric and is inhibited by ATP and citrate, which can be both considered as the end products of glycolytic flux. This classical view, which can be found in most biochemistry textbooks, appears to be wrong in most cases [118]. As a matter of fact, within the cell most phosphofructokinase molecules are bound to membranes and lose their allosteric character. Moreover, in many cells, for instance in muscle cells, the ATP and citrate levels are so high that the enzyme should be completely inhibited if its properties were the same in vitro and in vivo.

Even if it is true that the functional properties of an isolated enzyme are fully encoded in the corresponding structural gene, this does not hold any longer if the enzyme is associated with another protein or with the cell organelles. The discovery that pathogenic prion proteins can transfer their pathogenicity to nonpathogenic particles has sometimes been considered a major twist to some of the time-honoured principles of molecular biology. This is indeed so, for the prion protein that turns pathogenic, receives an instruction from another protein.

Communication between proteins does not appear to be limited to prior diseases since elementary considerations of statistical mechanics show that this association of an enzyme with another protein, or even with a rigid structure, results in a decrease of its entropy, an increase

in free energy and an instruction given to the enzyme. The free energy stored in the enzyme may thus be used to alter its functional properties.

This appears precisely to be the case with the three enzyme complexes already considered in this review. Plasminogen is normally converted into an active enzyme, plasmin, thanks to a specific proteolytic attack exerted by Ser proteases. However, a polypeptide completely devoid of protease activity, streptokinase, can bind to plasminogen, inducing a conformational change that generates enzyme activity. It thus appears obvious that enzyme function is not entirely encoded in the corresponding structural gene but also requires the binding of streptokinase.

The bienzyme complex made up of GraPDH and PRK of *Chlamydomonas* chloroplasts is an obvious illustration of these ideas. Free oxidized PRK is nearly totally inactive. However, it becomes active when bound to GraPDH. Dilution of the complex results in its dissociation. The PRK released under these conditions keeps for several hours an imprinting of GraPDH and is then extremely active. The dissociation process is reversible, and PRK regains its activity as it forms a complex with GraPDH. The communication and the free energy transfer that take place between GraPDH and PRK are used to alter the K_m of the latter enzyme for Ru5P and ATP, as well as its catalytic constant. Although the binding free energies of the substrates for their corresponding subsites are not very much altered by the energy transfer, most of this energy is used to decrease the height of the free energy barrier associated with catalysis.

A somewhat similar situation has been observed with Ras-GAP. Although Ras alone can catalyse the hydrolysis of GTP at a slow rate, the catalytic process is strongly enhanced if the protein is bound to another protein, GAP. This increase of GTP hydrolysis is promoted by the arginine residues of an arginine finger of GAP. The positive charges of these arginine residues tend to stabilize the transition state of the reaction. Information is transferred from GAP to Ras and allows an unlikely reaction to occur.

The most striking properties to be expected from the association of proteins in a complex are those of imprinting and memory. These properties, which exist and probably play a functional role in the living cell, may be mimicked and amplified with organic molecules and enzymes in organic solvents. Thus, when metacrylic acid and ethylene glycol dimetacrylate are copolymerized in the presence of theophylline and the latter is then removed, the resultant cross-linked copolymer can bind this drug. If the same copolymer is synthesized in the absence of theophylline, it is unable to bind the ligand. The binding of theophylline is thus explained by assuming that the copolymer has kept the imprint, or the memory, of the ligand [119].

The studies of Klibanov and co-workers [120, 121] and Yennawar and co-workers [122] on enzymes in organic solvents have led to a similar conclusion. Thus, if subtilisin in aqueous solution and in the presence of competitive inhibitors is lyophilized and if the inhibitor is then washed out, the enzyme appears about 100-fold more active in anhydric solvents than it would normally be if it had never been in contact with the inhibitor [121]. According to Klibanov and co-workers [121], the best way to obtain a stable imprinted enzyme is first to incubate the enzyme in water with a nonreactive substrate analogue to exert an imprinting effect under conditions of high mobility of the protein. The enzyme is then lyophilized, the substrate analogue is washed out and the protein is transferred to an organic solvent that will tend to freeze the imprinted conformation [121]. Klibanov hypothesizes that imprinted enzymes in organic solvents hold special promise in biotechnology [123, 124]. Whether this hope is justified or not, there is little doubt that Mother Nature already does what biotechnologists only try to imitate . . . . Thus, it has been known for a long time [125–133] that some enzymes retain, or recall, the conformation stabilized by the last product of the reaction sequence for a while before relapsing to the initial conformation. Under steady-state conditions, the coexistence of two free enzyme forms generates a kinetic cooperativity even for one-sited monomeric enzymes. Indeed, this cooperativity vanishes in equilibrium conditions. What the recent findings considered above have shown is the existence of imprinting effects exerted between proteins.

Beyond communication between proteins, there also exists communication between biomembranes and enzymes. This may be illustrated by the study of a few simple models such as protein kinase C, pancreatic lipase and a lipid-water interface. Whereas these enzymes are nearly totally inactive when they are in free solution, they become very active after binding at a lipid-water interface. In both cases, binding to the interface generates a conformational change that makes the active site accessible to the substrate. These two enzymes probably represent the simplest model system that illustrates the concept of communication between a membrane and an enzyme.

It thus appears obvious to the authors of the present review that the study of enzyme action in the living cell has in recent years undergone a paradigm shift [134]. It is no longer sufficient to study the structure-function relationships of enzymes using the techniques of structural biology and enzymology. It is also essential to know whether these enzymes communicate with other proteins, which instruction is sent out to these proteins or received from them and how the instructions received modulate enzyme activity.

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