Multistable proteins are defined as those that have available more than one most stable conformation. This capability requires a reversal of the most stable and metastable designations dependent upon environmental conditions. The concept of multistability is discussed with regard to enzyme maturation, hybrid vigor, metabolic temperature compensation, developmental gradients, membrane transport and the classical theory of allostery.

1. Introduction

One of the great triumphs of molecular biology has been the elucidation of exact protein tertiary structures by X-ray crystallography (Kendrew, 1963). Despite this achievement, it is still commonly felt that in solution these proteins exhibit varying amounts of flexibility essential for their proper functioning. The concept of conformational change is crucial to the allosteric theory of cellular control mechanisms (Monod, Changeux & Jacob, 1963), as well as to the cooperative binding utilized in oxygen transport, for instance, by various hemoglobins and hemocyanins (DePhillips, Nickerson, Johnson & Van Holde, 1969). In this paper I shall show how many higher order regulatory and adaptive processes can be understood if the number of stable protein conformations available and the requirements for their interconversion are considered.

Our knowledge of a protein's tertiary structure in solution is immense when compared with the meager information available on how and why the protein chose to fold the way it did. There is excellent evidence that the final structure achieved is determined by the primary sequence of that protein (Anfinsen, 1966), but we can only assume that a precise pathway of folding is followed until a deep potential well has been reached. If this kinetically trapped structure is the only stable conformation, the dogma
that primary determines tertiary is simply confirmed. However, many times more than one stable state exists, and between these there must necessarily be intervening energy barriers. It is conceptually sufficient to consider only the simplest example with two stable conformations.

2. Metastability

The four possible relative energies that these two conformations can possess are shown in Figs 1–4. These figures represent stability in the absence of any ligands or effectors. It is inappropriate to refer to these alternative states as isozymes since they are of identical primary sequence. Kitto, Wassarman & Kaplan (1966) referred to them as conformers, whereas Hotchkiss (1964) called them configurational isomers. The term metastable was used by Epstein & Schechter (1968), Levinthal (1966) and Nickerson & Day (1969) to describe the less stable alternative conformations, i.e. state $A$ in Fig. 3. Precisely because a potential well is metastable, given enough time each occupant of that well will migrate to the other more stable conformation.

Metastability, as originally defined by Nickerson & Day (1969), was irreversible. Because each state was still regarded as part of a folding pathway, only a defined sequence of stable states was considered to be available. Once
a protein migrated from state 3A to 3B, it was stuck there. Only new protein synthesis would suffice to regain any activity uniquely present in the metastable 3A state. As such, metastability represented an evolutionary obstacle to be overcome, and indeed, the examples cited—obligate psychrophily and additional nutritional requirements at elevated temperatures—were generally restrictive and deleterious examples of naturally occurring temperature sensitive proteins.

3. Multistability

Figure 3 represents the relative energies of two conformations under a single defined set of static environmental conditions. The key to the evolutionary advantage of multistable proteins is that relative stabilities may be reversed under a new set of conditions. What had been metastable is now most stable and vice versa. This reversal would set up an equilibrium between Fig. 1 and Fig. 3 depending upon environmental conditions. If stabilities are altered when a new situation is encountered, a suitable migration will occur to adjust the populations of each conformation. This adjustment can be advantageous when each conformation has evolved so that the efficiency with which it carries out its designated function is maximal under precisely the same conditions that favor preferential population of that conformation. For example, a new conformation populated in response to a shift to lower temperatures may have a smaller $K_m$ value, and by this compensatory mechanism, overall enzymic activity is maintained despite the decreased temperature.

Essentially a multistable protein has two different most stable conformations reversibly at its disposal; consequently, one enzyme can do the work of two. When a new environment is encountered, there is no need for additional genetic information and protein-synthesizing capacity to be used to meet it. In a cyclic environment, such as all intertidal areas, this capability could be of immense selective value. Shifting the equilibrium between Fig. 1 and Fig. 3 on exposure to new conditions is more efficient than the continual presence of both conformations under all conditions, regardless of whether these are in such rapid equilibrium as to constitute effectively a single population (Fig. 2) or are kinetically separated as in Fig. 4.

4. Maturation

So far no effort has been made to distinguish the multistable states of monomers from those of oligomeric proteins. Formally, the ideas are completely transposable. Because in practice most of the systems that exhibit
multistability turn out to be polymeric, the concept of maturation is especially pertinent here. A variety of enzymes—including phosphofructokinase (Alpers, Paulus & Bazylewicz, 1971), threonine deaminase (Hatfield & Burns, 1970) and β-galactosidase—initially form an inactive oligomer when the requisite monomers first associate. Each subunit still retains the conformation it possessed as a monomer. By following the appearance of enzymic activity kinetically, Alpers & Paulus (1971) saw that the complex needs to mature, to overcome an energy barrier before the active conformation is assumed. This maturation takes time and is temperature-dependent. The inactive oligomer formed first is a perfect example of a metastable conformation. Mutations that give cold-sensitive, incomplete ribosome assembly (Nashimoto, Held, Kaltschmidt & Nomura, 1971) would also fall in this class. The role of metastable conformations in cold-sensitive enzymes has been discussed previously (Nickerson & Day, 1969).

5. Hybrid Vigor

Multistable behavior can be observed when a tetramer is composed of identical subunit chains, but purely symmetry considerations would lead one to expect multistability to be more prevalent when the protein is composed of dissimilar chains. The dissimilarity causing this synergistic multistability can be either allelic (the producing organism is heterozygous for that locus) or simply that the protein in question is biquaternary, and thus consists of chains coded by two separate gene loci. When an organism is heterozygous, hybrid vigor is often observed to result from that heterosis (Manwell & Baker, 1970). Heterosis can be positively correlated with adaptation to changing environmental conditions (Powell, 1971), species diversity of habitat and resistance to habitat modification, e.g. pollution. Many attempts have been made to explain these observations on a molecular level. These have generally been in terms of protein quaternary structure since that provides the best explanation for much of the protein polymorphism observed by gel electrophoresis to result from heterosis. Aspects of quaternary structure that have already been proposed as the molecular basis of hybrid vigor (Manwell & Baker, 1970) include enhanced solubility properties; a change in complementarity (i.e. a tighter structure would be more resistant to denaturing pollutant conditions while a looser structure might be more flexible and allow a higher turnover rate); and for plasma proteins, increased resistance to bacterial invasion. Each of these suggestions attempts to discover an advantage under a single set of defined conditions. However, the advantage may merely be in the existence of two or more stable conformations, in which neither one need be any better than
the single conformation available to the non-hybrid parent. Multistability would allow continued near-optimum performance under conditions previously inaccessible to either parent.

This interpretation of heterosis in terms of multistability is easily seen to include negative heterosis if, for instance, on exposure to altered conditions an enzyme reverted to its inactive immature form, or simply denatured. In either event, that activity would be lost.

6. Temperature Compensation

In order for this explanation of hybrid vigor to be correct, all interconvertible forms of an enzyme must be active, unlike maturation wherein only one form is active. Such a situation has been shown by Somero (1969a,b) and Somero & Hochachka (1969) to be the basis of the metabolic temperature compensation exhibited by many aquatic poikilotherms. For example, they found that Alaskan king crab leg-muscle pyruvate kinase exists in two interconvertible forms, a cold-variant and a warm-variant whose respective minimum $K_m$ values for phosphoenolpyruvate are at 5° and 12°C. They recognized that since the king crab is subjected to large seasonal variations in surrounding temperature (4-12°C), a mechanism to maintain its metabolic rate at decreased temperatures by a concomitant decrease in $K_m$ would be of adaptive value. Conversion of the warm-variant pyruvate kinase to a conformation with a smaller $K_m$ value serves such a function. Similar immediate compensation has been observed for rainbow trout lactic dehydrogenase and pyruvate kinase.

The further interpretation of poikilotherm temperature compensation in terms of multistable interconversion is complicated because an organism may require an acclimation period of several weeks or longer (Somero, 1969b), as opposed to the immediate metabolic compensation discussed above. Now, the possibly slow conversion of a pre-existing multistable enzyme must be weighed against de novo biosynthesis of another isozyme with properties optimized for the newly encountered conditions. In general, no such distinction has been made nor attempted. Since the multistable conformations may differ in isoelectric point, as well as in a host of other potentially adaptive ways, such methods as electrophoresis and isoelectric focusing do not suffice to prove new synthesis the result of acclimation. Indeed, published comparisons of citrate synthase (Hochachka & Lewis, 1970) and isocitrate dehydrogenase (Moon & Hochachka, 1971) from rainbow trout acclimated at 2° and 18°C give every indication that the warm variant has been partially converted to the cold variant because the isoelectric focusing required an extended period at 4°C. With this danger in mind, it is
hoped that future comparisons might include more definitive techniques, such as peptide mapping.

7. Membrane Transport

Another situation wherein two conformations are thought to possess different substrate affinities occurs in membrane transport (Pardee, 1968; Kalckar, 1971). If the permease oscillates between the inside and outside of the cell membrane, conversion to a conformation of lowered affinity would accomplish substrate release. The galactose-binding protein isolated by Boos & Gordon (1971) has been found to possess the requisite conformations of differing affinity. Two things are presently unclear: how the interconversions of a multistable permease are coupled to the energy requirement of active transport, and if an environmental variable acts as a signal to trigger such a conversion. In the latter regard, we should consider the electrostatic forces generated by the membrane resting potential as a possible stimulus for the conversion of permease from high to low substrate affinity.

8. Ligand Promotion

Thermal energy is a well-known, but often slow, method of overcoming an intervening energy barrier. This obstacle can often be reduced by a sort of tunneling phenomenon; ligand binding can act catalytically, with the protein as substrate, to enhance the rate of transition from one conformation to the other. Alpers & Paulus (1971) demonstrated that inactive, immature enzymes do not accumulate because their conversion is enhanced by many so-called maturation-promoting ligands. Since these often include the effector ligands and substrate of the mature enzyme, there is little delay. The significant point is that once the conversion has been accomplished, the continued presence of the promoting ligands is no longer needed.

Enzymes susceptible to immediate temperature compensation would then either possess an energy barrier low enough that it could be easily surmounted by the thermal energy of ambient temperatures or require the presence of an analogous acclimation-promoting ligand. Similar requirements may exist for multistable hybrid vigor but by analogy with known maturation-promoting ligands, any necessary ligands may be readily available within the average cell.

9. Multistable vs. Allosteric

Binding of a promoting ligand serves only to achieve population of the competing conformations in accordance with their intrinsic thermodynamic
stabilities. As soon as metabolite binding is allowed to reverse the stabilities themselves, in which instance the continued presence of that metabolite would be necessary to maintain such a reversal, a situation is reached which is identical to the classical theory of allostery. All allosteric proteins are multistable but not vice versa. Allostery is a subclass of multistability, distinguished by the continued presence of the effector metabolite. The generalized theory of multistability does not require effector metabolites, and if they should be present to promote conversion, it does not require their continued presence. For this reason, it is not concerned with the controversy between pre-existing allosteric conformations and newly created induced fit conformations (Koshland & Neet, 1968). The multistable theory is more closely related to the theory of allostery because it is presently framed in terms of just two pre-existing conformations. This has been done for convenience and in no way precludes additional intermediate forms.

The theory of allostery in itself is already so powerful that Monod et al. (1963) warned about its most serious objection, “it could be used to explain away almost any mysterious physiological mechanism”. This warning applies with equal justification to all the non-allosteric portions of the multistable concept.

10. Non-metabolite Ligands

Non-metabolite constituents of the cellular milieu should also be considered candidates for both allosteric and promotional ligands. Let us examine first the many unusual compounds produced by fungi and bacteria on the cessation of logarithmic growth. Woodruff (1966) considered this secondary metabolism to be due to enzymic ambiguity resulting from extremely large concentrations of certain substrate analogs. Alternatively, Weinberg (1970) proposed that critical concentrations of Mn$^{2+}$ and Fe$^{2+}$ are necessary for both the initiation of secondary metabolism and the unique characteristics of the biosynthetic end products. The ability of metal ions to modify protein structure is well known (Vallee & Wacker, 1970). It may be that Mn$^{2+}$ binding causes a transition in a multistable enzyme such that the new conformation is of altered biosynthetic specificity, resulting in the observed, somewhat permuted, end products.

Limited proteolysis, as for example in chymotrypsinogen and insulin, might be thought of in terms of multistability, but it is decidedly irreversible. I should like to suggest, however, that this drawback does not necessarily apply to all covalent modifications. The prevalence of amino acid side-chain modification by methylation, acetylation, hydroxylation, phosphorylation or adenylation has recently been documented (Paik & Kim, 1971; Holzer &
Duntze, 1971). These modifications could easily serve to interconvert alternative multistable conformations of vastly different function.

11. Fields, Gradients and Polarity

Of the myriad potential functions of side-chain modification-induced transitions, I think it will suffice here to point out only their potential usefulness in development and differentiation. Gradients are ubiquitous in embryology (Child, 1941) but despite intensive efforts not one of them has yet been elucidated on a biochemical level. It is reasonable to expect that a gradient might exert its influence by interacting with a multistable protein and, at a critical concentration, cause that protein to switch to another conformation of altered function. Such an effect could be generated by the localized production of a modifying enzyme, e.g. a methylating enzyme. Crick (1970) has calculated that for a small protein, diffusion would then suffice to maintain the gradient. One characteristic of developmental gradients is the common existence of two competing, diametrically opposed gradients. In the so-called animal and vegetable poles in a developing embryo, this hypothesis would entail the localized diffusion of a methylating enzyme from one pole countered by the localized diffusion of the corresponding demethylating enzyme from the other. Naturally, any other modifying enzyme would work equally well, but when attempting to detect such modifications, one would have to avoid the acid hydrolysis so commonly used in amino acid analyses.

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REFERENCES

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