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Protein Dynamics Studied by NMR

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Abstract. The results of NMR studies using several nuclei indicate that proteins have considerable internal mobility. The most obvious is the mobility of side-chains. This mobility is general on the exterior surfaces but extends internally in a differential way. The functional value of surface mobility concerns both on and off rates of ligand binding (e.g. metal ions and parts of substrates) and protein/protein interactions. The mobility, which indicates that recognition is more in the hand-in-glove class than in the lock-in-key class, makes for a modified view of the specificity of protein interactions. Thus, fast on/off systems cannot be as selective as slower systems. Segmental mobility of proteins is considered in the context of protein secondary structure. The least mobile segments are the β-sheet and the tight β-turn. Mobility is always possible for, but not within, rod-like helices and in loose turns. Many examples are given and the importance of mobility in molecular machines is described. Finally, examples are given of virtually random-coil proteins, segments, and linker regions between domains and the functional value of such extremely dynamic regions of proteins is discussed.

Key words: NMR – β-sheets – α-helices – dynamics – protein-actions

Introduction

In this article I wish to explain my attitudes to the study of proteins by NMR. While I believe that the ability to derive structures in solution is very important and adds to the information on structures provided by X-ray diffraction in crystals (Wüthrich, 1986), I consider that it is the further information which NMR gives about the nature of binding sites, their mobility and exchange rates, and the mobility of side-chains and segments within proteins which is of the greatest value. Biological "structures" are designed not as rigid shapes but as units of flow, parts of molecular machines. To a first approximation the interesting phases in which they are found are liquids, water and fatty acid membranes. They are *not* in solid and certainly not in ordered crystalline phases. A central feature of the aqueous compartments limited by the membrane phases is that there is feedback communication within and between them, so as to gain homeostatic and development control of cells. Such communication must have a flow or message system which interacts with its functional protein units. It is only possible to create such a system if the series of liquid solutions are linked to dynamic polymers. Both material and energy must be distributed in this system and questions arise not about particle flow alone but about communicating mechanical action in the polymers. It is these time-related properties which NMR can tackle.

Protein surfaces

A biological system is in liquid water (not ice) and there is no way in which we can handle the properties of water free from the study of statistical dynamics. Life is a product of the existence of water. Rates of translation of atoms and small molecules in water are characterized by exchange rates $> 10^9\ s^{-1}$. For this reason it is necessary to consider the dynamics of the surfaces of proteins, since they have but small structural restraint due to the protein backbone. Virtually all NMR data show that many of the groups after the β-CH_2 of the surface amino acids fluctuate and probably rotate at rates approaching $10^9\ s^{-1}$. Certainly this rate is often found for the side-chains of lysine, arginine, glutamic acid and glutamine. Even some valines and leucines show free motion though they may appear static by some experimental criteria since they are asymmetric tops. Relaxation of aromatics, fast flipping rates, $> 10^5\ s^{-1}$ is very common (Wüthrich 1986) as is oscillating behaviour of tryptophan (Cassels et al. 1978).

* This work is based on a lecture at the EBSA Symposium, organised by the Italian Biophysical Society (S.I.B.P.A.), Tabiano Terme, September 1992

Table 1. Some residues with slow flip rates ($< 10^3\ s^{-1}$)

Protein	Residues
Cytochrome *c*	Tyr67, Phe10, Phe94, Tyr46, Phe48
FUR (Ferric Uptake Regulatory Protein)	Tyr55
Trypsin Inhibitor	Tyr35, Phe45
Osteocalcin	Tyr42

Note: The rate of exchange of water molecules from inside proteins is similar to the flip-rates of aromatic rings (Wüthrich, K., personal communication). Most examples found so far are in proteins not classified as enzymes

Table 2. Water exchange rates

Fast $> 10^6\ s^{-1}$	Intermediate	Slow $< 10^3\ s^{-1}$
Na^+, K^+, Cu^+, H^+	–	–
Ca^{2+}, Cu^{2+}, Mn^{2+}	Mg^{2+}, Ni^{2+}	–
	Y^{3+}	Al^{3+}, Fe^{3+}

Note: The exchange rate is taken to be a first order dissociative process

Table 3. Dynamic-dependent biological functions of metal ions

Function	Metal ion	Mobility Requirement	Rate (s^{-1})
Carrying Current	H^+, Na^+, K^+, Ca^{2+}	Fast	$> 10^8$
Triggering	Ca^{2+}, Zn^{2+}, H^+	Relatively Fast	$> 10^2$
Catalysis	Zn^{2+}, Cu^{2+} (in enzymes)	Slow	$\ll 10^{-4}$
Allosteric switch in O_2 carriers	Fe^{2+}, Cu^+	Intermediate	$\sim 10^2$
Copper/Zinc	Cu^+ or Zn^{2+}	Intermediate	$< 10^{-3}$

Note: The table shows the wide range of time scales for biological processes whose rates are determined by the dynamic properties of the metal ions as shown. These rates relate to the exchange properties of the ions between the bound and free state, controlled by the dissociation rate constant. The slower rates imply that a stable metallo-protein can be readily isolated, whereas metallo-proteins in the intermediate range would tend to suffer a partial loss of metal ion, and those in the fast range a complete loss of metal ion, under conditions of isolation

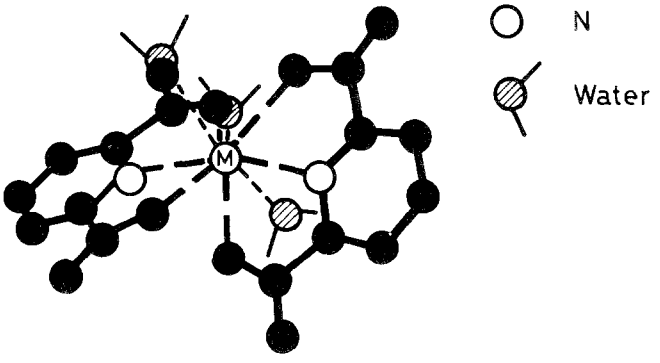

Fig. 1. The structure of lanthanide (dipicolinate)$_2$ complexes. The ligand rotates on the metal (Alsaadi et al. 1980) at a rate dependent of the size of the metal ion, M

Of course the exact environment of a side-chain has an effect upon its mobility, see Table 1.

The conclusion is that a protein plus a binding unit does not present a lock and key fitting system. It is closer to a hand-in-glove fit in which motion is retained even after binding. This fitting allows fast on/off reactions as both partners relax even within the bound state. There must be a great variety of motions but, in general, flowing surfaces assist fast recognition or activation best. At the same time the general truth applies: the less rigid the system the poorer the selectivity, and vice versa, the more rigid system the better the selectivity but the slower the on/off rates. We now know that some proteins are much more rigid than others for good functional reasons (Williams 1989; Cheetham et al. 1991 and see below).

Complementary metal ion dynamics

Just as proteins can be divided into groups on the basis of their dynamics so can metal ions. There is the general rule that dynamics of the unit, ML_n where M is the metal and L the ligand which can be water, are faster for cations of low charge and large ionic radius. The simplest case is shown by the data on water exchange, Table 2 (Eigen 1960).

Equally important is the fluctional rate of ligands, L in ML_n. Here we showed that ion size was important by studying the fluctional motion of the dipicolinate anion on the surface of the metal ion for the series of lanthanide cations. Fig. 1, (Alsaadi et al. 1980). The fluctional rate varied over many orders of magnitude, 10^4 to $10^8\ s^{-1}$, from the largest to the smallest cation. These internal fluctional motions are the equivalent of internal aromatic ring flipping in proteins. The results show that very small change of ionic radius (of the order of 0.1 Å) alter rates of rotation by factors of ten or more. The dynamics of ML_n have to be seen in the context of the value of the size of M in a variety of motion dependent processes in proteins. Some of these are listed in Table 3.

We turn next to the mobility of the secondary structure of proteins which, by mechanical action, can allow long-range communication where this is desirable.

The classification of secondary structure

β-sheets

Proteins fall into two extreme classes according to their basic secondary structure elements, with many intermediate examples especially in the multidomain proteins. The first class is the simple β-barrel seen, for example, in the copper blue proteins, copper enzymes, and superoxide dismutases. Here the metal ions are held in a strained fixed state, the entatic state (Williams 1953, Vallee and Williams 1968). The description applies to many metallo enzymes and electron transfer metallo-proteins which have β-sheet structures in large part. This now includes the iron/sulphur, Fe_3S_4 proteins where the thermodynamically favoured small model molecule Fe_3S_4 *linear*

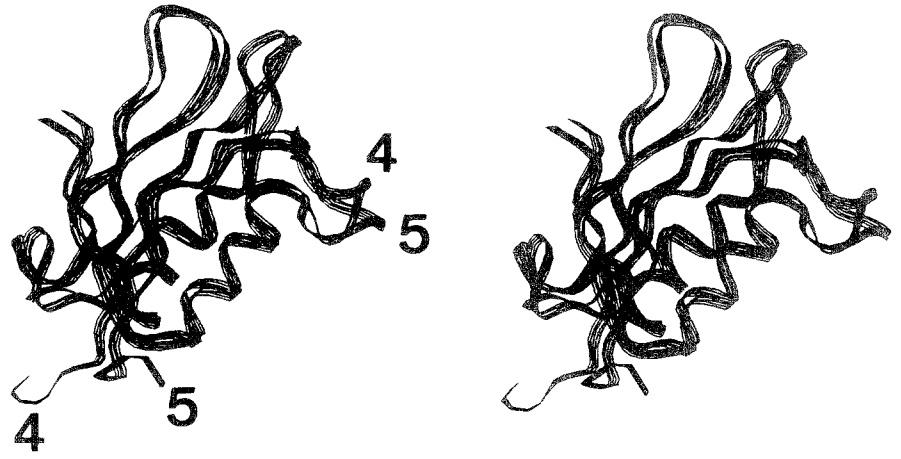

Fig. 2. The NMR derived structure of acylphosphatase (Pastore et al. 1992). The numbers 4 and 5 refer to two alternate structures determined using identical data, indicating that at terminii and some loops, the NMR method leaves the structure ill-defined

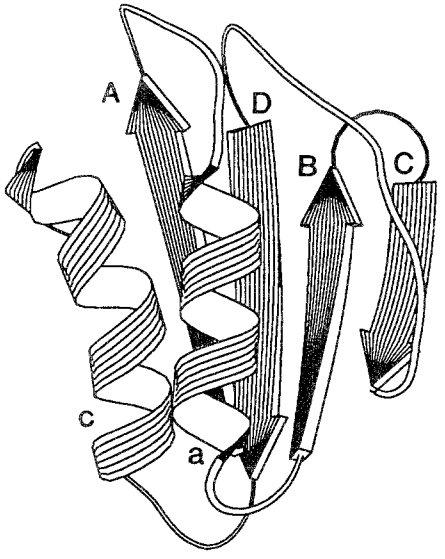

Fig. 3. An example of a protein fold, see Table 4 for the proteins concerned, in which some segements, including helices, can move on a platform made from a β-sheet. The a, c region is particularly mobile

Table 4. Proteins with the acyl-phosphatase fold (two helices on *one* side of a β-sheet)

Protein	Function
Acyl Phosphatase*	Enzyme
Histocompatibility Protein	Antigen Binding
Phosphocarrier Protein	Phosphoenol pyruvate: sugar transport sugar
RNA-binding Proteins (a) U1 sn RNP A (b) Hn RNP C	Binding to RNA in Ribosomes

Note the simplicity of the reaction of the only enzyme in this group
* Perhaps these proteins are "designed" for their mechanical properties. See Wittekind et al. (1992) for a discussion of these structures.

structure is not found but instead, a structure with the strained three corners of a cube is observed (Holm, R., personal communication).

The description of β-sheet proteins by NMR is very straightforward. Figure 2 shows the NMR derived structure of acylphosphatase (Pastore et al. 1992). The backbone of the structure is relatively rigid. All the tests for its rigidity have been confirmatory. (1) The definition of structures itself is excellent. (2) The relaxation rates of ^{1}H, ^{13}C and ^{15}N are those expected for whole protein body rotation (3) the internal NH exchange rates are extremely slow (4) the chemical shifts are temperature independent. By way of contrast there is quite fast motion of some side-chains, even inside a β-structure (Pastore et al. 1992), as shown by the fast flipping. $> 10^{4}\ s^{-1}$, of many aromatic residues. It is also possible for motion of molecules to occur on the surfaces of sheets, but the individual strands can be seen as supports of an effectively rigid platform. It is this feature which allows the protein to induce strain in the site of a bound metal ion as described above. Individual sheets thus generate very valuable surfaces for recognition and activation but by themselves they can hardly be parts of mechanical devices, though sheets can move on other sheets, e.g. β-barrels on one another. Enzyme recognition sites are nearly always based on sheets since they have the least variation in structure with time. Sheets can also act as a platform on which other secondary structural units can move, see Fig. 3, (Garret et al. 1989, Madden et al. 1992, Wittekind et al. 1992). In this structure the two helices lie on one side of a four-stranded β-sheet and parts of the structure move relative to the sheet. Several examples of this construction are now known, Table 4 (Wittekind et al. 1992), and the question arises as to whether it is "designed" as a dynamic mechanical device where specific side chains of the helices grip a bound molecule.

Helices

There is no *a priori* reason to suppose that a single helix cannot be as rigid as a single strand of a β-sheet. Again N-H bonds and relaxation measurements in helices often show all the properties of rigid bodies. However, helices are not always well located relative to other helices and their ends tend to be less regular. Thus helices can slide and/or rotate as rigid rods, Fig. 4, i.e. (1) with respect to the angle they subtend to another helix, (2) with respect to

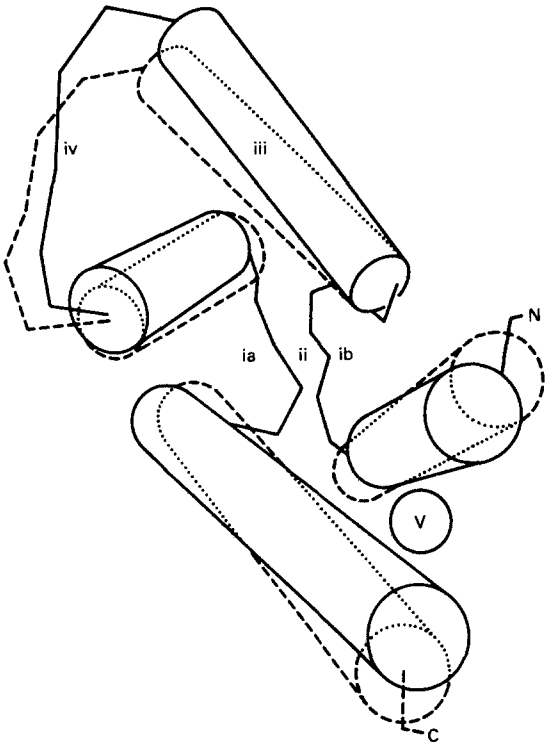

Fig. 4. The structural change derived for calmodulin on binding calcium (Williams 1989). i (a) and i (b) are two calcium binding sites linked by a very small β-structure (ii), (iii) is a helix, (iv) a long mobile loop and (v) is the binding surface for drugs and proteins, e.g. kinases

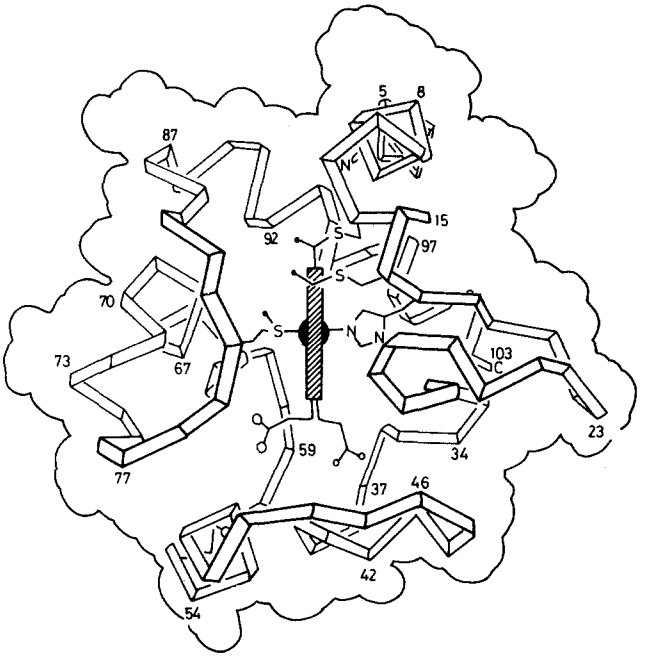

Fig. 5. The structure of cytochrome *c*. Helices 1–13 and 87–103 do not move on change of oxidation state. Much of the structure on the left of the S-atom is somewhat adjusted, so coupling the electron transfer with a conformation change and the making and breaking of many H-bonds. This makes the protein an ideal model for coupling (Gao et al. 1992) but a poor model for electron transfer itself (Gray and Malmstrom 1989)

Table 5. Highly helical proteins

Protein	Function
Hemoglobin	Dioxygen Carrier
Hemerythrin	Dioxygen Carrier
Hemocyanin	Dioxygen Carrier
Calmodulin	Calcium Trigger
Calbindin	Calcium Carrier?
FUR	Iron Uptake Regulation
Colicin	Channel Protein
Histones	DNA-binding (Reversible)
Viral Coat Proteins	Viral NA Binding (Reversible)
Ferritin	Iron Storage Protein
Acyl Carrier Protein	Acyl-transfer Protein
Membrane Proteins	Channels in Membranes
Myosin Light Chains	Mechanical Transmission
Cytochrome *c*	CO Binding Protein
Interleukin	Signalling Protein
Cytochrome *c*	Electron Transfer Protein
Insulin	Hormone

Note: None of these proteins are classified as enzymes

the cross-over position with another body and (3) by a rotational motion on a frame e.g. a β-sheet or another helix, Fig. 3. Combination of all the motions is possible when force is applied. A good example of the rotational/sliding is provided by the variety of motions revealed by very detailed studies of calmodulin. The rearrangements occur in 10^{-3} s. (Williams 1989; Ikura et al. 1992). Changes in α-helical structures which require these motions have been seen by X-ray structure determination (Derewenda et al. 1991).

However, it would be erroneous to suggest that all helical proteins must be segmentally very flexible, see examples in Table 5. Fluctuations in individual proteins must be analysed today using relaxation data (^{1}H, ^{13}C, ^{15}N), N*H* exchange, or by studies of chemical shift and its temperature dependence, especially in such cases as ring flipping of aromatic side-chains. In some helical proteins there is already sufficient evidence to show that only certain regions are mobile. A good example is provided by the properties of cytochrome *c*, (Wand et al. 1986, Gao et al. 1991, Timkovich et al. 1992). The range of ring flip rates is from 10^1 to $> 10^5$ s^{-1} and the range of peptide N*H* exchange from seconds to weeks. Here it has been shown that the N- and C-terminal helices are fixed relative to one another, Fig. 5. By way of contrast those helices and other regions which are to the S-side of the heme are more flexible, Fig. 5. Again in calbindin the helices are less mobile relative to one another than in calmodulin, (Akke et al. 1991). There are several ways of placing constraints on motions of helices, including steric hindrance, -S-S- cross-links and H-bonds e.g. in phospholipase A_2. (Aguair et al. 1979). However, it remains the case that rod-like motions of helices mean that sequence runs from *n* to $n + x$ can move relative sequence runs *m* to $m + y$ relatively easily when the major interactions across these helical strands is through apolar hydrophobic contacts. Motion may seem less likely in some helical membrane proteins where the hydrophobic surfaces are externalised but motion within lipids will generally be less constrained than in water, since lipids themselves are less constrained. In fact, many membrane proteins are helical and it is

thought that their helices are able to move relative to one another e.g. in pumping activities (Orekhar et al. 1992). As NMR data accumulate it will be of interest to uncover these segmental constraints on motion in greater detail.

These considerations make it clear that helices are not the best design unit for enzymes where high selectivity in recognition is required at the binding site. However if the enzymes has allosteric properties requiring long range effects, or if it has an ordered mechanism, then a mechanical switch is needed. In this case, helices are most valuable. Example are phosphorylase (allosteric enzyme) and cytochrome P-450 and citrate synthetase (largely helical, required order enzymes).

Within helical bundles side-chain motion may be extremely fast e.g. ring flipping in calmodulin (Williams 1989). An extreme example is the rotation of the huge heme co-factor within myoglobin (Neya et al. 1992).

Turns

We have recently studied acyl-phosphatase in great detail by proton NMR and discovered that there was a variety of motion in turns, which themselves have a great structural variety, (Pastore et al. 1992). A tight β-turn of 4 or 5 residues which has a central backbone H-bond is relatively rigid. Turns having more extended sequence runs are frequently very mobile as illustrated by all the diverse NMR methods in this and several other proteins. More detailed study by Ikura et al. (1992) of calmodulin and by Smith et al. (1992) of interleukin using ^{15}N and ^{13}C relaxation data have confirmed these observations. The longer turns or connecting loops away from the calcium sites of calmodulin, see (iv) in Fig. 4, are the more mobile. It is relatively easy to understand why the representation of such regions of proteins in X-ray crystal structures may give a false impression of rigidity (see Rini et al. 1992 for discussion).

General comments on structural definition by NMR and X-ray diffraction

The detection of mobility by NMR, especially in long bends or at the termini of folds, cannot rely on NOE data alone, nor can the "structures" computed from such data be called reliable. NOE data are always a confusion of atom-atom distance and relaxation data i.e. dynamics. Moreover there may well be insufficient NOE constraints to define such regions. All in all, while there is no reason to suppose that an NMR description of a well ordered, relatively rigid structure, e.g. a β-sheet, is less accurate than in X-ray crystallographic description, the analysis of NMR data of looser structure is full of problems. Intrinsically, the X-ray method is better for looser parts of the fold simply because the crystal lattice constrains the mobility, but of course these structures are not always relevant in solution. There is always the danger that demands for a single "structure" especially based in part on energy minimized refinement, no matter what the method, NOE or X-ray, will hide mobility. Yet mobility is of the essence of function. Note that it is the *anisotropic* motion which is of the greatest interest, which includes the vectorial motion of helices. Other NMR methods often reveal changes in structural parameters which will be missed by NOE studies owing to mobility problems. One such procedure to which we turn below is the analysis of chemical shift data over a temperature range. At the same time as we examine NMR data critically we should look at the ways in which structural interpretation of X-ray structures interpretation could go astray.

Frequently in the past, little attempt was made to analyse X-ray diffraction in terms of more than one conformation in a crystal. We demonstrated the error this can lead to in interpretation in a very early NMR study of a chemical modification of a tryptophan residue (Cassels et al. 1978). Here two states are equally populated in slow exchange as proven by NMR, but the possibility of such exchange was not used as a contributing factor in the interpretation of the original X-ray data, which gave one position from a somewhat nebulous intensity map. No matter what the exchange process is in a protein in a crystal it is difficult to describe it by X-ray diffraction. Here great care is needed in the interpretation of B-factors which cover vibronic motion (relative to one energy minimum), exchange (between two or more minima), and static disorder. It is my opinion that X-ray data are often over interpreted for long turns, where little constraints by H-bonds can be seen, and yet single structures are shown. Notice that short turns emerging from β-sheets in convoluted parts of structure often have low B-factors and are also sites of enzyme action. Undoubtedly some degree of modest flexibility at the active site is required and is achieved by this constructional motif.

NMR shift data

Simple use of NMR chemical shift data to define rigid structures is becoming more valuable but as yet no really good procedure exists. Here the diamagnetic shift can be used to follow changes in structure. It is always the case that NMR shift data will give better knowledge of changes and their time dependence than any other procedure. Typical examples are changes in protonation states and isomerisation of proline. Of very considerable importance is the temperature dependence, since a continuous change or shift with temperature may well reveal that a protein has many structures effectively in equilibrium and this could include the denatured or random coil. In such a case the use of NOE data is not really very helpful.

Paramagnetic shifts are very valuable in the verification in solution of protein crystal structures. The combination of ^{13}C and ^{1}H NMR data can be most telling since diamagnetic shifts are very different while paramagnetic shifts are identical for the two nuclei. Where an H-bond is broken from one state of a protein to another the movement of atoms may be insignificant relative to a paramagnetic probe so that the change of paramagnetic shift is very small but the diamagnetic shifts of the H-atom concerned and adjacent atoms show characteristic and sometimes large changes. (Turner and Williams 1992). The

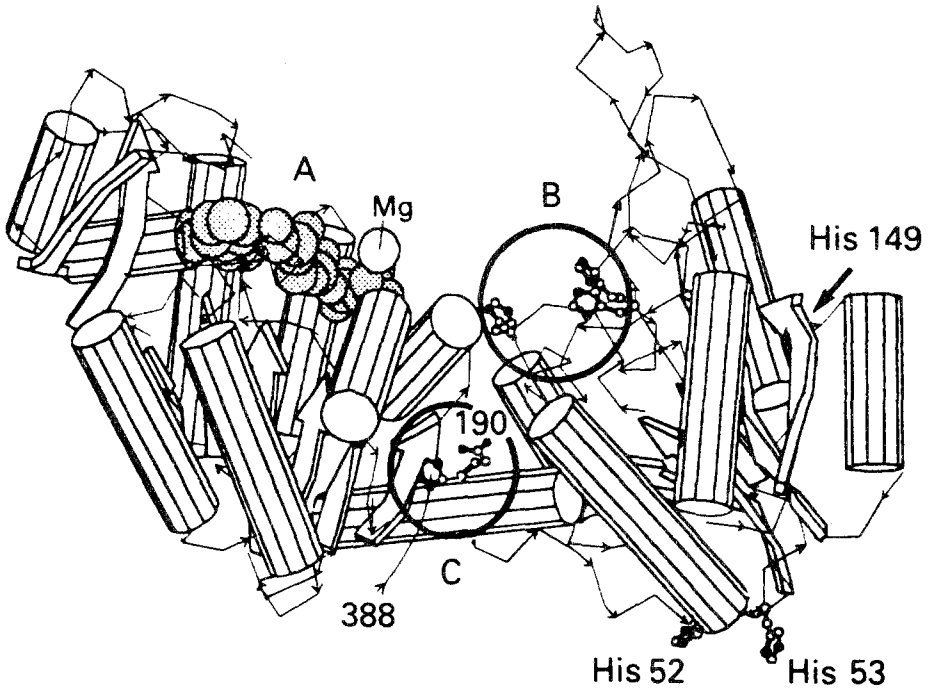

Fig. 6. The structure of phosphoglycerate kinase showing the two relatively rigid β-cores of the domains surrounded by helices, the hinge region between, and the long connected helices e.g. down to His 52 and 53. A is the binding site of ATP, B that for phosphoglycerate, and C is a region where mutation affects activity from a distance

combination of the shift data with NOE data is sometimes indispensible in uncovering features of mobility as opposed to structural changes (Gao et al. 1992). In the case of the study of a cytochrome *c* mutant, Gly 92 Phe, we found that NOE data in the region of the Gly substitution were lost but the paramagnetic shifts or resonances were unaltered. The clear interpretation is that motion had increased relaxation of signals but that the average structures were unaffected.

"Hinges" in proteins

We can next consider how a combination of all methods may help to resolve a problem, that of relative movement of multi-domain proteins, see Table 6. The earliest X-ray studies of phosphoglycerate kinase (PGK), Fig. 6, showed that the binding site of ATP of the C-domain was far too distant from the 3-phosphoglycerate (3-PG) site on the N-domain (B) to allow phosphate transfer and the formation of 1,3 diphosphoglycerate, 1,3 DPG. The situation was confirmed even in the substrate bound state of the enzyme in crystals, Harlos et al. (1992). It is true that some movement of the two binding domains, both β-sheet based, relative to one another occurs on substrate binding, especially of 3-PG in the crystalline state. The conformational change has been called a hinge-bending by X-ray crystallographers. Many proteins and enzymes are now described in this way.

The many different NMR studies of PGK (Tanswell et al. 1976), including those involving binding and single site mutations, show that there is much more to it than this (Joao et al. 1992). A hinge by itself, as on a normal door, allows open and closed states to equilibrate with no energy difference. In proteins this motion would become simple movement of domains about friction-less points, for example both X-ray and NMR studies agree that any

Table 6. Examples of "compression-hinge" motion

Protein	Motion
1. Phosphoglycerate Kinase (PGK) 2. Transferrin 3. Iron/Sulphur Protein of Nitrogenase (ATP-ase)	Two αβ domains close together about a fulcrum while driving helices in selected directions and increasing the internal energy of the protein
4. Phosphorylase	One helix is extended by phosphate binding which in turn adjust the active site
5. Ion Pumps 6. ATP-synthetase	The ATP binding zone is comparable to PGK. The "hinge" motion is connected to the motion of helices in the membrane which form the ion channel. The last motion is suggested to pump the ions.

Note: The "hinge" description appears to apply to a considerable number of proteins including kinases, transferrin and the sulphate binding protein

motion is about the cross-over fulcrum of helices in the inter-domain region as in Fig. 6. (Note this use of helices). There is a real hinge but this mechanical analogy does not indicate that there is any energy input to the protein structure on hinge bending. The NMR studies show that movement actually extends to the ends of some helices, across the N-terminal β-sheet domain, to His 52 and His 53 for example, Fig. 6. This means that the motion at the fulcrum, the hinge, is connected to a piston like drive of the helices over the surface of the sheet, see Figs. 3, 4 and 6. Consider again the analogy with a door. The motion of a simple hinge is around an axis parallel to the door. We can also design a door which, after opening,

closes automatically by attaching a compression cylinder to the door such that energy is expended in opening the door against motion of a piston into the compression cylinder. The door is forced to close on its own by release of the energy in the compression cylinder. (An alternative device is a "rising hinge"). Returning to PGK, energy is supplied by binding, that is the α-helices move over the β-domains somewhat. In this condition thermal motion of the domains is presumably sufficient to allow collision of substrates. The final condition of the enzyme after exchange of phosphate is that the leaving of 1,3 DPG, the rate determining step of enzyme action, is driven by relaxation of the enzyme from its energised fold at a rate of around $10^2\ s^{-1}$, which is seen to be in the NMR slow exchange range. This rate determining step is equivalent to the decompression in the cylinder of the mechanism of the automatic door. The helices move so as to contain the energy of binding but also to direct the swing of the domains so as to allow correct substrate alignment. Now this principle is general when stated in the form that substrate binding drives transitions between the conformational states of those proteins which are loosely assembled, e.g. calmodulins, cytochrome P-450, citrate synthetase, ATP synthetase. There is therefore a mobility factor to add to Pauling's statement that enzymes act by driving their substrates toward transition states. This only applies where the enzyme is relatively rigid. In other cases, the mobility of protein domains makes an essential, and possibly dominant, contribution.

Disorder/order transformations

We must now distinguish two cases
(1) Disordered → Ordered
(2) Order A → Order B

In phosphoglycerate kinase we were looking at case (2) where the energy supplied creates a *vectorial transmission* so that the energy is not dissipated in an isotropic manner. The question then arises as to the type of protein structure which will allow case (2) to occur. As stressed above, combinations of helices are ideal structures for this transmission where one helix motion is guided by movement on another helix, on a set of helices, or on a sheet. The next question is which type of protein structure allows case (1) to occur? NMR studies have shown that quite a number of proteins and protein segments are effectively disordered at room temperature even *in vivo*, see Table 7, but they fold on binding to a partner (Weiss et al. 1990) i.e. these proteins fold only on binding to particular surfaces or molecular units. Their function may be just to allow the incorporation of a complex co-factor within the interior structure of a protein, e.g. haem into cytochrome *c* which is disordered in the apo-form. However a more interesting case is that of metallothionein. This protein, again disordered in the apo-form, folds quickly (minutes) in different ways when it incorporates copper, cadmium or zinc so that different metal ions may control their own homeostasis based on feedback recognition of the different shapes of different metallothioneins but based on the same protein. These observations allow us to summarize, through the study by NMR of many metal/protein interactions, three extreme types of mobility which without doubt also apply to protein/organic molecule interactions.

Table 7. Protein segments with virtually random coil conformations

Protein	Cofactor	Function
Osteocalcin	Ca^{2+}	Binding to Calcium Phosphate (Bone)
Cytochrome *c*	Heme	Electron Transport
Zinc Fingers	Zn^{2+}	Binding to DNA
Metallothionein	Zn^{2+}, Cu^{+}	Homeostasis of Metal Ions
Peptide A	Ca^{2+}	Gla Control Sequence (e.g. prothrombin)
Chromagranin A	Ca^{2+}	Signal Peptide Storage
Calsequestrin	Ca^{2+}	Calcium Storage
Domain of GCN4	DNA	Transcriptional Activation
Glycophorin	Lipids	Cell Surface Protein

Note: A number of regulatory DNA-binding proteins appear to be of low structural definition, compare regions of histones

Classes of mobility of metal-proteins

It is relatively easy to divide metallo-proteins into different mobility classes. The first class is that of the β-sheet; the almost rigid metallo-enzyme and superoxide dismutase. A quite different description applies to proteins in the second class where the binding of the metal ion adjusts the structure of the protein although it has an initial apostructure, usually helical. They are conformationally variable. Here the protein is usually not an enzyme but is often part of signalling or of a mechanical device. Similarly, a compulsory-order-mechanism enzyme has to be a mechanical device and therefore must be largely helical. The third class contains typically the random coil protein which folds into a defined secondary structure in the presence of a limited number of cofactors. This classification applies to single domain proteins.

Many extracellular proteins appear to be formed from series of domains with linker regions which allow the domains to move relative to one another. (Esnouf et al. 1985). Typical are the kringle and the EGF domains (including calcium binding forms) which appear to have disordered connecting linkers. The domains are functional in binding, to receptors for example, when appropriately oriented but there is no suggestion that they themselves change conformation. Many of them are β-sheet domains.

A feature of some of these random-coil segments and connecting links in extracellular proteins which have many domains is that they have calcium-binding properties. The proteins are synthesised in the Golgi and are then exported to the extracellular fluids. In the former the calcium ion concentration is low so that the proteins remain in the apo-form and extremely flexible. This is clearly shown by our NMR studies. On entering the extracellular fluids these flexible regions are given structure, or at

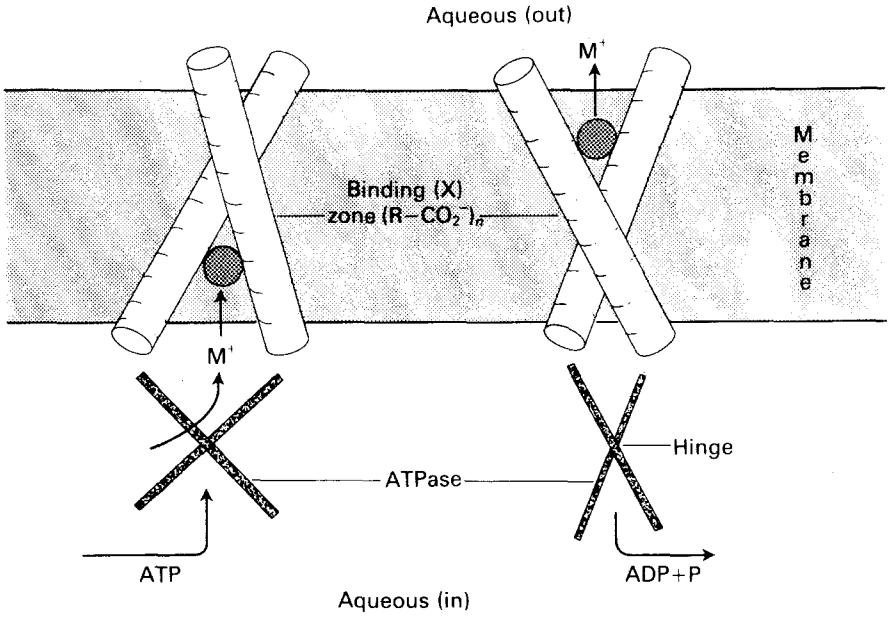

Fig. 7. A simple way in which mechanical transmission can occur between an ATP hydrolysis site and an ion channel i.e. pumps or ATP-synthetases. The ATP zone is to be pictured in more detail by the phosphoglyceratekinase structure, Fig. 6. The channel is more like the helix-bundle of calmodulin, Fig. 4. The connections are made by helix/helix lateral movement (Norwood et al. 1992), and rotation generating a synthetic (hydrolysis) site for ATP reaction steps coupled to the gated energised movement of ions past a barrier. The connection between the enzyme and the membrane domains is thought to be by long helices (see PGK, Fig. 6). The binding zone (x) is the carboxylate-rich region along the helices where the cation is held before it passes the cross-over point of those helices

least reduced in flexibility, by binding calcium. The structure of one segment, the Gla domain of prothrombin, has been determined by X-ray diffraction studies of crystals, (Soriano-Garcia et al. 1992) and that of another, osteocalcin, by NMR (Atkinson 1990). In another case it has been shown that many EGF domains have a calcium binding site in the sequence region which links it to an accompanying domain, (Handford et al. 1991, Mayhew et al. 1992). The suggestion is that the structure of these multidomain proteins is calcium-dependent and in this bound, less mobile, form they recognise their targets. The calcium binding is selective against magnesium and is of such strength that the domains and linkers are always calcium bound in the extracellular fluids.

It is clear that similar principles often apply to intracellular multidomain proteins as to extracellular proteins. Frequently domains will have linker regions with loose structure, so that the domains can be adjusted relative to one another. We have mentioned some limited mobility of this kind in kinases but there are now many examples in proteins such as the GTP binding elongation factor EF. Tu, the multi zinc finger proteins, and the cell surface binding proteins which have immunoglobular domains linked to one another. Inside the vesicles of cells there are some virtually completely unfolded structures such as chromagranin A in the chromaffin granule (Daniels et al. 1978) and calsequestrin in the calcizomes of many cells.

Mechanics and mobility

An important part of living systems depends upon mechanical action at the molecular level. The fact that biochemistry in the past has depended on organic chemistry not physics for its ideas about activity has led to the concentration on electronic factors in enzyme mechanism. Biophysics has been differently and closely involved with two topics – electrolytic function (ion movements) and mechanical functions. The connections to the two features of biophysics are everywhere apparent where there is controlled flow of particles or energy. Thus kinases and pump ATP-ases are mechanical devices and the mechanism of any kinase e.g. that described here, PGK, is immediately relevant to ATP-pumps and ATP-synthetases. They too are mechanical devices in which the movement of helices driven with energy transfer in the body of the enzyme (e.g. by ATP hydrolysis) can be coupled to gating and pumping of ions, (Frausto da Silva and Williams 1991). The ion to be transported enters the pump from the inside of cell at a site with a high binding constant within a series of roughly parallel flexible helices in a membrane, Fig. 7 (Norwood et al. 1992). This set of helices is connected to the enzyme ATP-ase. The *energised* "hinge" action of this enzyme (described above in the case of phosphoglycerate kinase as being due to binding and activity) acts on the membrane helices so as to displace outwards the site of binding of the cation while lowering the binding constant. This is a forced long range movement due to connections from energised helices to the "hinge". The ion leaves the membrane and the pump, after releasing ADP + P, returns to its initial state.

Thus a biological system can have not only local static strain enabling it to generate the desired *states*, e.g. the entatic state, but it can have appropriate *dynamics*, allowing it to generate the desired motion and hence controlled action at a distance from the energy input. An allosteric protein is a specific example of one such dynamic machine: the forced motion of filaments constitutes a general mechanism based, in large part, on movements of helices, (Williams 1989). The transmission of mechanical energy and its relaxation are central to cellular flow i.e. dynamics homeostasis, (Williams 1987). Most enzymes have mechanical as well as electronic features, and it is for this reason that a detailed knowledge of protein dynamics is essential. I believe NMR has the potential, slowly being realised, to generate the required information about dynamic processes involved in the mechanism of action of a wide variety of proteins.

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