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FUNCTIONAL SIGNIFICANCE OF FLEXIBILITY IN PROTEINS

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Abstract - The structural basis and the functional implications of large-scale flexibility are discussed for three systems: trypsin-trypsinogen, immunoglobulins, and citrate synthase. The trypsin-trypsinogen system provides an example in which an order-disorder transition is used as a means to regulate enzymatic activity. Immunoglobulins demonstrate how flexibly linked domains may be used to allow the binding of ligands with diverse arrangements. In citrate synthase, domain motion forms an active site that is shielded from solvent. Analogous large-scale flexibility has been observed in a number of other systems.

INTRODUCTION

The static picture of protein molecules derived from X-ray diffraction analysis is deceptive. Protein molecules are flexible but often differently so in different parts. Amino acid side chains may be flexible, peptide segments may be mobile or structural domains may move as rigid bodies relative to other parts of the molecule (Fig. 1).

Evidence for mobility within proteins comes from numerous physical methods: single-crystal X-ray or neutron diffraction, electron microscopy, spectroscopic techniques such as NMR, ESR, fluorescence depolarization, perturbed angular correlation spectroscopy, Moessbauer spectroscopy, time resolved absorption spectroscopy and hydrogen exchange studies. Theoretical approaches such as potential energy minimization and molecular dynamics calculations may also be used to study flexibility. Of these methods, single-crystal diffration has the unique advantage of spatial resolution in the characterization of flexibility but has very limited time or energy resolution. The latter are the domain of the spectroscopic techniques. It is clear that a combination of methods is required to localize the flexible segments of a protein and to characterize their dynamic properties.

Even when we restrict our attention to examples of protein flexibility in which motion of large parts of the molecule occurs, the functional roles of flexibility are quite diverse (Fig. 2):

(a) Flexibility might serve regulatory properties by controlling substrate binding. Interaction with a rigid, stereochemically complementary surface would be stronger than binding to a flexible segment which must be stabilized before it can provide optimal noncovalent interactions. Binding to a flexible segment thus requires the reduction of its conformational entropy at the expense of association energy. Substrate binding could be regulated by allosteric linkage of the transition between flexible and rigid states to other environmental factors.

(b) Some proteins are composed of domains which are structurally separated but covalently linked by segments that allow them to move and function independently. The covalent linkage localizes the functions in space without imposing stereochemical restrictions that would result from a rigid arrangement of the functional sites.

(c) In some enzymes motion of domains relative to one another occurs during the catalytic cycle. These enzymes occur in an open form, which presumably allows substrate binding and product release, and in a closed form, in which the substrate is shielded from solution and properly aligned in relation to catalytic groups so that catalysis can proceed.

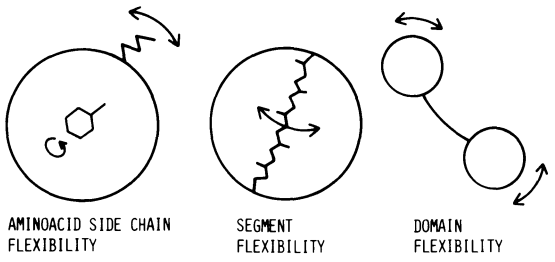


Fig. 1

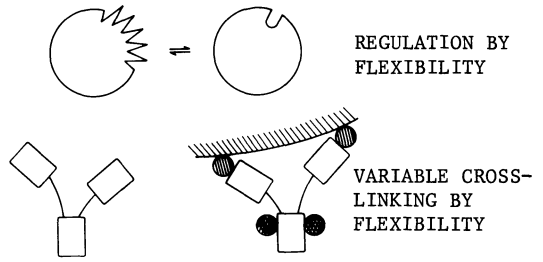


Fig. 2 →

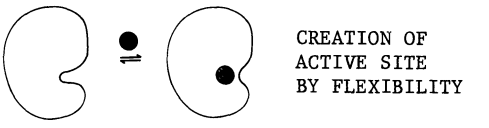


Fig. 1. Scheme illustrating three levels of flexibility in proteins: motion of amino acids, peptide segments and domains.

Fig. 2. Scheme showing the structural basis of protein regulation by a transition between flexible and rigid states, the coupling of protein functions by flexible linkage of domains, and the formation of an active site protected from solvent by domain motion.

Other functional roles of large-scale flexibility are known (in the proteins which form the capsids of icosahedral viruses or in those involved in contractile systems, for example), and more will probably be discovered. We will concentrate in this article on examples of the classes outlined above which illustrate the structural and functional aspects of flexibility in detail.

TRYPSIN-TRYPSINOGEN

The trypsin-trypsinogen system demonstrates how segmental flexibility can be used to regulate enzymatic activity. Trypsinogen is the catalytically inactive precursor of the protease trypsin. Activation occurs upon cleavage and release of the N-terminal hexapeptide of the zymogen. Trypsinogen cannot bind peptide substrates, but the basic pancreatic trypsin inhibitor (PTI) is bound with considerable strength. This binary complex binds exogenous peptides related to Ile-Val (the N-terminal residues of trypsin) with high affinity, showing that trypsinogen in the trypsinogen-PTI complex assumes a trypsin-like conformation in solution, as was found to be the case for the crystalline trypsinogen-PTI complex (6).

Figure 3 summarizes a large body of experimental data on the trypsin-trypsinogen system. Several of the structures represented schematically in the figure (A, B, C, D, E and free PTI) have been determined by crystallographic methods; other structures shown are hypothetical intermediates. Thermodynamic constants and some kinetic parameters of the various reactions have been determined (for a review, see Ref. 1-3). In trypsinogen (A), a part of the molecule referred to as the activation domain is disordered and not visible in the electron density map. This domain is represented by the sawtooth surface in the figure. The N-terminus of trypsinogen is also flexible. In trypsin (B), the activation domain is ordered. The activation domain participates in the formation of the substrate binding site and a closely associated binding site for the first two residues of trypsin, Ile 16-Val 17.

PTI binds to trypsin very strongly to form the complex E. It also binds to trypsinogen but seven orders of magnitude less tightly to form the binary complex C. This association is only possible when trypsinogen is in a trypsin-like conformation with the activation domain rigid. The Ile-Val binding site is also formed but remains empty as Ile 16 is blocked by the activation hexapeptide in the zymogen. Trypsinogen in state C is similar to trypsin in structure, but not identical. The binary complex (C) binds exogenous Ile-Val peptide strongly and forms the ternary complex D,which is extremely similar to E, as shown by the similarity of their NMR spectra (4). The reduced affinity of trypsinogen for PTI is a consequence of the energy required to order the activation domain without the N-terminus binding in its specific pocket (2).

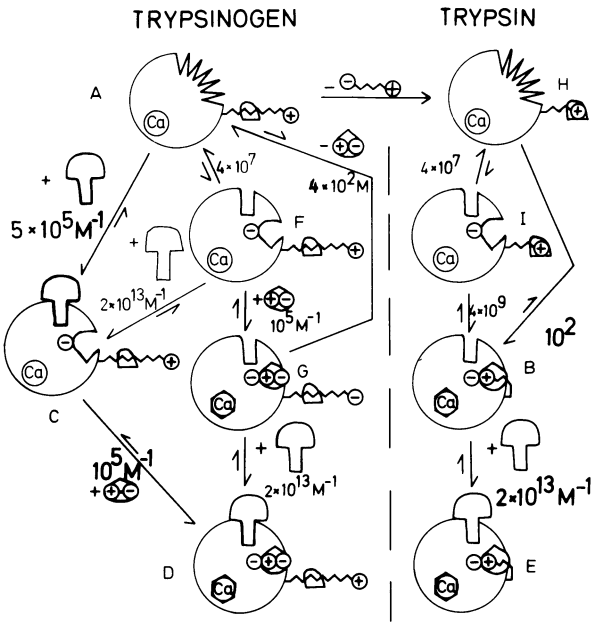


Fig. 3

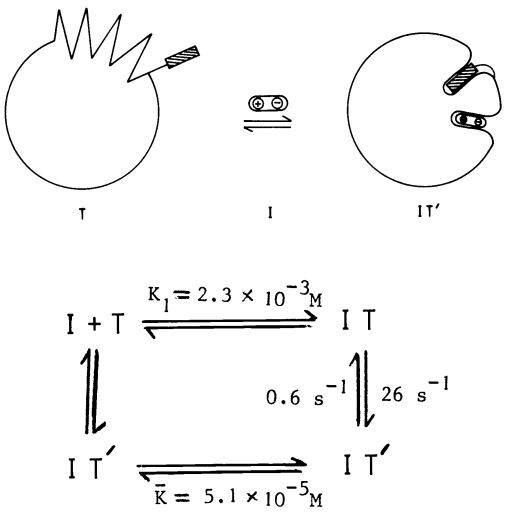


Fig. 4

Fig. 3. Scheme showing structures and thermodynamic parameters for the trypsinogen/PTI/Ile-Val system (left) and the trypsin/PTI system (right); ~~~ denotes flexible segments, and (+ ⌒ −) is the Ile-Val dipeptide. Structures A, B, C, D and E have been observed crystallographically; others are postulated intermediates. Binding constants denoted with large symbols have been measured; others have been inferred on the assumption of identical equilibria of species with the same structural features (2).

Fig. 4. Structural scheme of Ile-Val binding to p-guanidinobenzoate trypsinogen. ▨ , p-guanidinobenzoate; (+ ⌒ −), Ile-Val (3).

The transition of trypsinogen into a trypsin-like state bears some resemblance to a folding process, in the sense that the activation domain becomes rigidly structured during the transition. This makes the trypsin-trypsinogen system an interesting subject for fast kinetic studies. Nolte and Neumann (3) have studied trypsinogen with p-guanidinobenzoate bound to Ser 195. This complex undergoes the transition to a trypsin-like state in the presence of the effector dipeptide Ile-Val; i.e., the small inhibitor p-guanidinobenzoate causes the same ordering of trypsinogen as PTI does, but only with the aid of the binding energy of Ile-Val (5). The transition was studied by temperature-jump measurements, which showed that the reaction can be described as an induced-fit process initiated by the formation of a weak complex (IT) between the Ile-Val dipeptide (I) and p-guanidinobenzoate trypsinogen (T). The IT complex isomerizes in a slow step to the stable complex IT'. T presumably is similar to trypsinogen in structure, while T' has been shown by CD measurements to be in a trypsin-like conformation (5). The alternative binding pathway (to the left in Fig. 4) is not kinetically significant.

The schemes in Fig. 3 & 4 assume a two-state model for the trypsin-trypsinogen system, with the activation domain disordered and flexible in trypsinogen but ordered and fixed in trypsin. This model is not strictly correct, as shown in solution by NMR (4) and crystallographically by the observation of somewhat higher temperature factors in the autolysis loop of trypsinogen (residues 140-150) in state C as compared to state D (6).

The recent crystal structure analysis of the complex between trypsinogen and pancreatic secretory trypsin inhibitor (PSTI) shows quite clearly the variability of the trypsin-like structure induced in trypsinogen by ligand binding. The trypsinogen-PSTI complex is similar to state C, except that PSTI is the ligand instead of PTI. PSTI is not structurally related to PTI except in the segment that interacts with the protease (7,8). In Fig. 5 the autolysis

loop of trypsinogen in the trypsinogen-PTI complex is superimposed with the same segment from the trypsinogen-PSTI complex. The large difference in the conformation of the two trypsinogen structures in this region is evident. Further differences are observed in other parts of the molecule, mainly in segments of the activation domain. Obviously there are a number of trypsin-like conformations quite similar to trypsin but with distinct local variations that depend on the ligand used to induce the trypsin-like state.

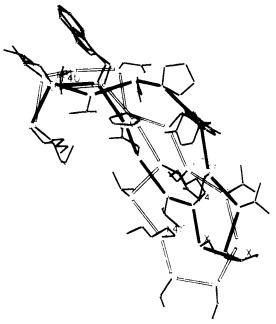


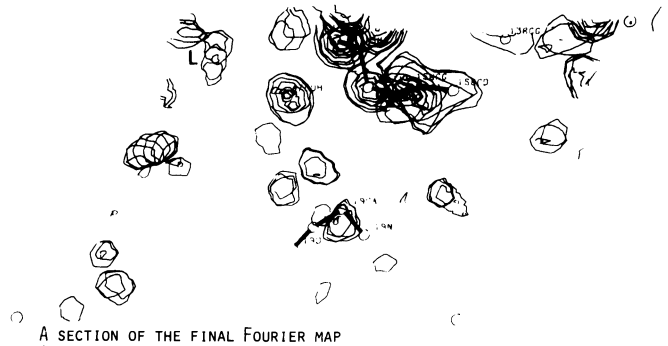
Fig. 5

The autolysis loop in trypsinogen-PSTI (===) superimposed on that of trypsin-PTI (---). From Ref. 8.

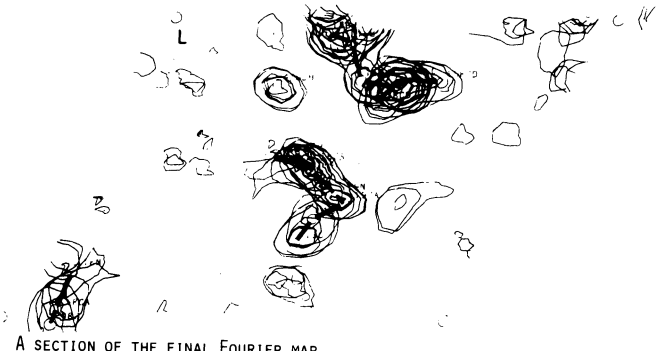
The disorder of the activation domain in the trypsinogen state appears also to involve several conformations, as shown by the following experiments. It is well known that a crystallographic experiment at a single temperature cannot distinguish between dynamic disorder (harmonic vibration around a single equilibrium configuration) and static disorder (many close ly related but different conformers). By lowering the temperature, however, thermal vibration can be discerned from static disorder, as only the former is reduced. The low temperature X-ray diffraction analysis of free trypsinogen at 173° and 108° K (9) showed a substantial reduction in temperature factor (thermal vibration) for the ordered parts of the molecule when compared with the structure determined at room temperature. Interestingly, the activation domain did not show noticably increased order, even though a few residues at the N-terminus did. Figure 6 shows that Val 17 and Gly 18 display increasingly ordered electron density upon temperature reduction. This experiment suggests that predominantly static disorder occurs in the activation domain and substantial dynamic disorder at the N-terminus.

It is important to recognize, however, that the X-ray studies just mentioned show only that the activation domain of trypsinogen exists in a number of distinct conformations on the time scale of an X-ray diffraction experiment (several days in this instance). Perturbed angular γ-correlation spectroscopy, which can detect motion on the ns time scale, shows that there is interconversion among the conformers of the activation domain. A derivative of trypsinogen with an appropriate Hg isotope inserted between the Cys 192-220 disulfide bond to form the group-Cys 191-^{199m}Hg-Cys 220- was used in these experiments. Figure 7 compares the spectrum of trypsinogen with that of the ternary complex between trypsinogen, PTI, and Ile-Val. In contrast to the ternary complex, the anisotropy vanishes in free trypsinogen. This observation indicates that intramolecular reorientation of the γ-emitter occurs in the free molecule with a correlation time of 11 ns and that no dynamics occur in this time range in the trypsin like-state. Motion in this time range is consistent with the interconversion of different conformers (10).

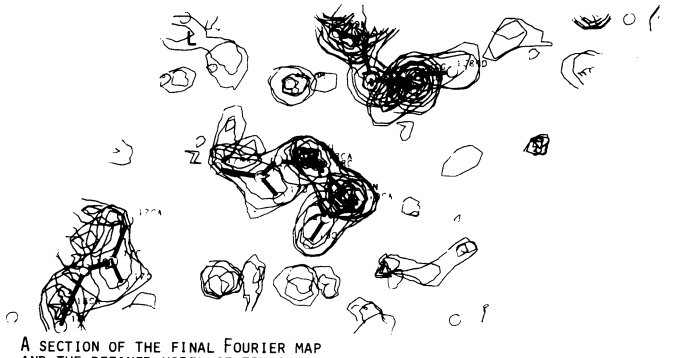
The manifestation of disorder in crystalline proteins is strongly influenced by lattice packing. The intermolecular crystal forces may stabilize a particular conformation out of the ensemble of conformations accessible in solution, giving the impression of a single, rigid conformation. There are several well documented examples of chemically but not crystallographically identical molecules which differ significantly in certain regions with respect to segmental disorder. In general, tight crystal packing tends to increase order. The influence of crystal packing on order will be illustrated with the Fc fragment later in this article. Other examples are ovomucoid (7,11) and kallikrein (12,46). Stabilization of a certain conformation may explain why chymotrypsinogen (13) and trypsinogen crystallized under conditions different from those of the structure described above (14) show less disorder in the crystalline state.



A SECTION OF THE FINAL FOURIER MAP AND THE REFINED MODEL OF TRYPSINOGEN AROUND GLY 19 AT ROOM TEMPERATURE

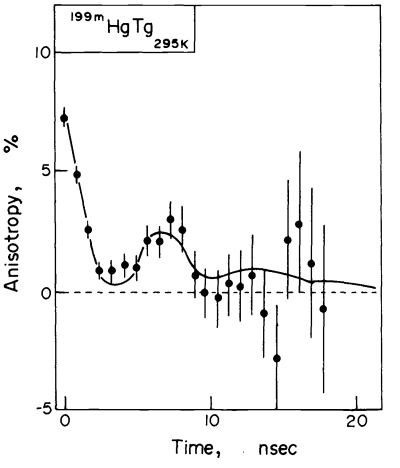


A SECTION OF THE FINAL FOURIER MAP AND THE REFINED MODEL OF TRYPSINOGEN AROUND GLY 19 AT 173 K



A SECTION OF THE FINAL FOURIER MAP AND THE REFINED MODEL OF TRYPSINOGEN AROUND GLY 19 AT 103 K

Fig. 6



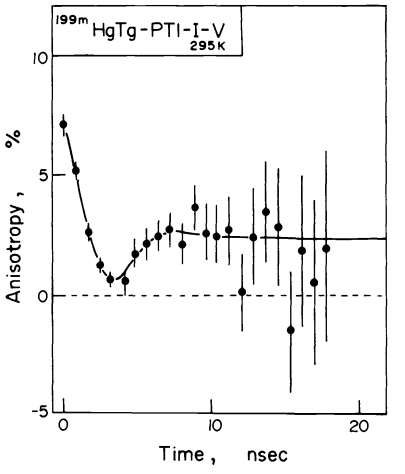


Fig. 7

Fig. 6. Comparison of the Fourier maps of trypsinogen at room temperature (a), 170° K (b), and 100° K (c) in the vicinity of the N-terminus (9).

Fig. 7. Plots of γ-correlation anisotropy versus time for mercury trypsinogen (HgTg) and HgTg-PTI-Ile-Val (10).

An order-disorder transition similar to that in trypsinogen has been observed in tobacco mosaic virus (TMV) protein, where a stable fold of the RNA binding segment exists only in the presence of RNA (15,16). A similar phenomenon appears to occur in pancreatic prophospholipase (17) and might account for the disordered regions of the tyrosyl tRNA synthetase structure (18), although the distinction between static and dynamic disorder cannot be made in the latter examples.

The structural features of segmental flexibility are reflected to some extent in the amino acid sequence: the disordered domains in trypsinogen and TMV lack aromatic residues. The transition (hinge) region is rich in glycine residues. These observations are also valid for the hinge segment in molecules with domain motion discussed below, in those instances where comparison is possible.

IMMUNOGLOBULINS

We now focus on a different type of flexibility, in which independent rigid domains move relative to one another with considerable freedom. Very little conformational change in the connecting segment occurs in such motions. This seems to be a quite common phenomenon but has been studied thoroughly only in the example of the immunoglobulins. A folding scheme based on repeated structural domains was originally proposed for immunoglobulins on the basis of the amino acid sequence and is depicted in Fig. 8 for the IgG class. An IgG molecule is formed from two heavy chains with a molecular weight of 50,000 and two light chains with a molecular weight of 25,000. The heavy and light chains are subdivided into four and two domains, respectively, which are linked by short, extended polypeptide strands. A wealth of structural data on IgG molecules is available (for recent reviews, see Ref. 19 & 20).

The first direct observation of domain flexibility in protein crystals came from a study of the IgG Kol crystals (21,22). In these crystals the Fab parts are well ordered, but the Fc part is disordered and does not contribute to the electron density (23). The crystal packing is solely determined by the Fab arms with the Fc parts occupying a central channel (Fig. 9).

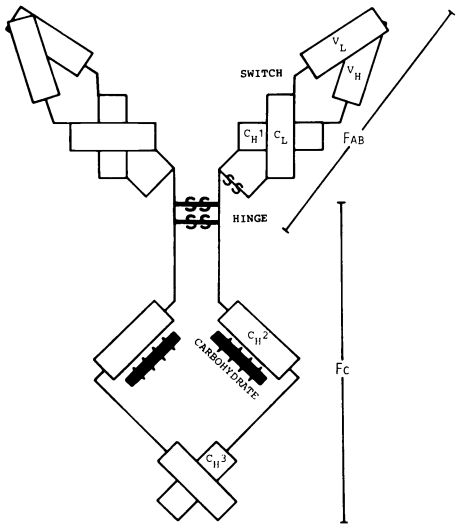


Fig. 8

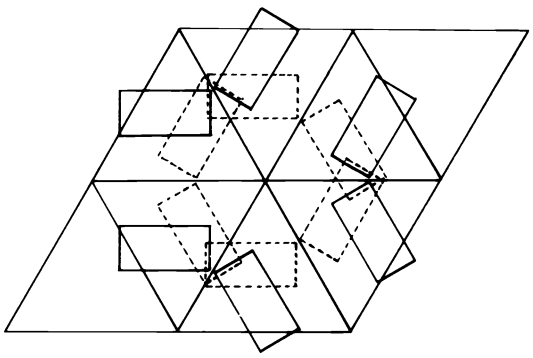


Fig. 9

Fig. 8. Schematic diagram of an IgG molecule. The heavy chain consists of four domains, the light chain of two. The N-terminal tip of the Fab arms is the antigen binding site. The domains aggregate in the indicated fashion; the CH2 domains, to which carbohydrate is bound, exist as single domains.

Fig. 9. Crystal packing of Kol IgG projected down the 3_2 axis. The three solid pairs of rectangles are the $(Fab)_2$ components; the Fc components are disordered in the space around the central 3_2 axis. The open rectangles are related to the solid ones by unit-cell translations (21).

The $(Fab)_2$ fragment of the Kol molecule, a pepsin degradation product cleaved C-terminal to the hinge segment, crystallizes isomorphously to the intact molecule. This was a particularly fortunate coincidence, as comparative studies of the same or closely related molecules are of particular interest in the context of flexibility. Comparison of the diffraction intensities of the $(Fab)_2$ fragment with those of the intact molecule allowed a quantitative study of the effect of the Fc portion on the diffraction pattern (23). If the Fc region were vibrating harmonically around a single equilibrium position in the crystals of the intact molecule, we would expect to see substantial intensity differences at least in the innermost region of the pattern. On the other hand, if the Fc segment were distributed among several different sites, the intensity differences would be small at all resolutions. The analysis clearly shows the latter to be the case. As with the activation domain in trypsinogen crystals, static disorder prevails for the Fc portion in Kol crystals.

The polypeptide segment linking Fab and Fc has a unique conformation consisting of a short polyproline double helix with disulfide crosslinks flanked on the N-terminal side by an open turn of helix (23). Flexibility in Kol crystals starts abruptly at the C-terminus of the polyproline double helix -- i.e., after the last P of the -C-P-P-C-P- segment (Fig. 10). The open turn of helix formed by residues 522 to 526 lacks stabilizing intramolecular contacts and may allow Fab arm movement in solution. Indeed, electron microscopic and ultracentrifuge studies of the complexes between IgG antibody and bivalent haptens show a large range of different angles of the Fab arms around the hinge and suggest that the energy differences in these angular positions is small (24). The abrupt order-disorder transition seen in Kol is comparable to those seen in the previous examples of trypsinogen and TMV. Disorder begins within a single peptide, which is often a glycine residue, suggesting motion around a well defined hinge.

This aspect of flexibility is particularly clear in the elbow motion of the Fab segment. In Fig. 11 we compare the Fab conformations in intact Kol crystals, Kol Fab fragment and McPc 603 Fab fragment (23,25). They differ in elbow angle by up to 60°. A closed elbow, as in McPc 603, is also found in the other Fab or Fab-like molecules whose structures are known (26,27). Figure 12 compares hinge conformations of Kol (open elbow, Ref. 23) and New (closed elbow, Ref. 26) in detail. The difference in conformation is very small and essentially confined to residues Val 107 to Gly 109 of the light chain and Val 416 to Ser 418 of the heavy chain, which define the hinge.

A similar but much smaller conformational difference is observed in the crystalline Fc fragment with respect to the relative arrangement of the two chemically identical but crystallographically independent CH3 and CH2 domains. An angular difference of about 7° was observed (28). This difference in CH2-CH3 arrangement must be a consequence of crystal packing. In addition CH2 shows asymmetry in the degree of disorder of some segments at one end of the domain. These segments are oredered where they are involved in crystal packing contacts but disordered if they face the solvent. Electron microscopy has indicated much larger flexibility in Fc, although the effects of sample preparation are difficult to evaluate in view of the sensitivity of the CH2 structure to its environment.

The dynamic aspects of immunoglobulin flexibility were studied as early as in 1970 by nanosecond fluorescence spectroscopy (29). Although intramolecular motion was detected, the nature of this motion was (and is) debated. It now seems likely that the larger of the two correlation times observed for the decay of anisotropy in the fluorescence of bound hapten is due to Fab arm motion. The faster correlation time stems from elbow motion within the Fab arm (29-32). The time constants observed are consistent with the proposal that domain motion in immunoglobulin is controlled by rotational diffusion (33). In immunoglobulin crystals, certain conformations are evidently frozen by lattice forces. In some favourable cases, such as the crystals of intact IgG Kol, the crystal packing allows multiple conformations to occur in the crystalline state and thus allows flexibility to be "observed" in the form of disorder.

Flexibility in the immunoglobulin molecule clearly allows the molecule to adapt to the variable disposition of antigenic sites on cell surfaces. In addition the extreme arrangements of closed and open elbow in Fab, corresponding to the existence or absence of contacts between variable and constant domains, may reflect different functional states of the immunoglobulin molecule -- i.e., unliganded and liganded states. The extremely open elbow conformation in the Kol molecule, for example, could well be an example

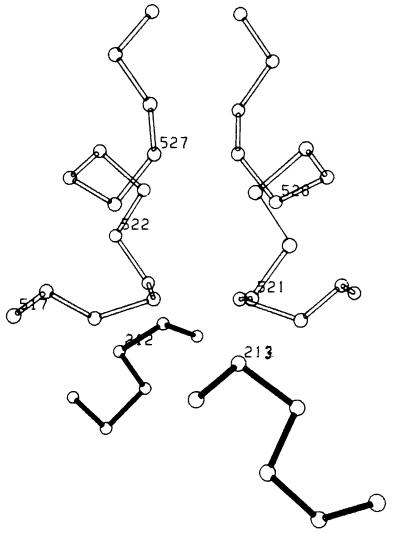


Fig. 10

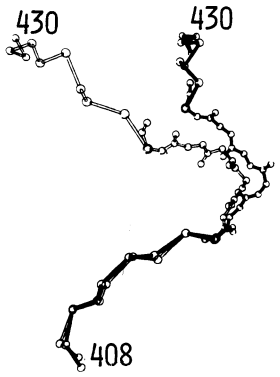


Fig. 12

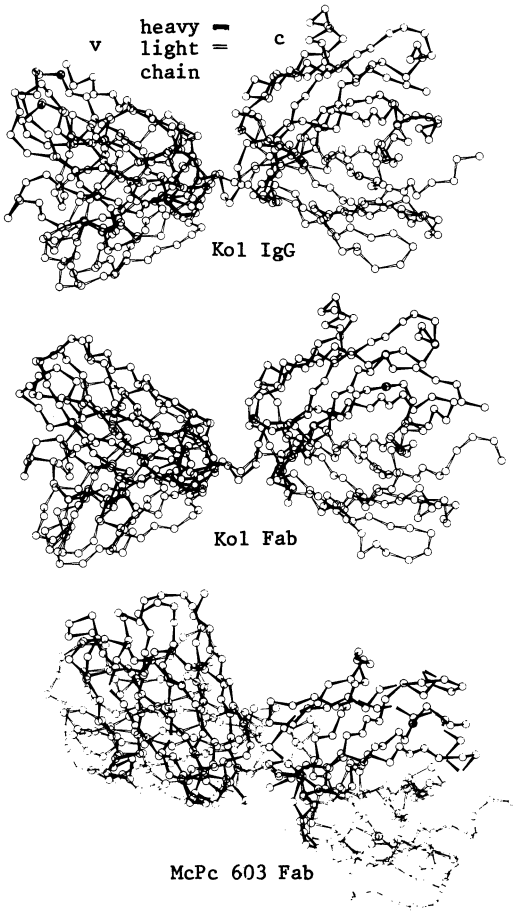


Fig. 11

Fig. 10. The hinge segment in Kol IgG. Filled bonds, light chains; open bonds, heavy chain. The amino acid sequence of the heavy chain segment is Cys 527-Pro 528-Pro 529-Cys 530. Residues 522 to 526 form an open turn of helix (23).

Fig. 11. The Fab parts of Kol IgG, Kol Fab and McPc 603 Fab looking along an axis through the switch peptides. The Kol molecules are characterized by an open elbow angle, while McPc 603 forms a closed structure (22).

Fig. 12. Comparison of the switch peptides of the heavy chains of intact Kol and Fab New (23). Filled bonds; open bonds, New.

of a liganded state, as the crystal-packing contacts of this molecule resemble antigen-antibody interactions in several respects (23). However, the fluorescence depolarization data discussed above clearly demonstrate flexible domain arrangement in hapten-antibody complexes; whatever the exact nature of the motion, this observation would seem to indicate that the energy barriers between different domain arrangements in immunoglobulins are small.

CITRATE SYNTHETASE

The relatively unhindered domain motion in immunoglobulins may be contrasted with the domain motion observed in some enzymes which we now wish to consider as our final example of protein flexibility. In these enzymes, the domain motion occurs upon substrate binding during the catalytic cycle. The phenomenon has been observed and established by crystal structure analyses of the different forms of yeast hexokinase (41,34), liver alcohol dehydrogenase (35) and citrate synthase (36), and appears to occur in glyceraldehyde-3-phosphate dehydrogenase (47) and in aspartate aminotransferase (H. Jansonius, private communication).

The observation that kinases generally have a bilobal structure roughly similar to that found in hexokinase has led to the suggestion that analogous domain motions are a common feature of this class of enzymes (37). Structural evidence consistent with this proposal has been presented for two kinases other than hexokinase, phosphoglycerate kinase (42,43) and adenylate kinase (44), while the proposal appears to be incorrect for phosphofructokinase (45). Evidence from small-angle X-ray scattering experiments also suggests ligand-induced domain motion in arabinose binding protein (38).

We now concentrate on citrate synthase, the condensing enzyme which catalyses the reaction between acetyl-coenzyme A and oxaloacetate to form citrate. The molecule is a dimer of two identical subunits with 437 amino acid residues each (39). It is a large globular molecule that is formed almost entirely of α-helices (36). Figure 13 is a schematic representation of the helix arrangement in one subunit. The two subunits pack tightly via eight helices in an antiparallel arrangement. Each subunit folds into two domains, a large one mediating the dimer aggregation and a small domain of about 110 residues comprising helices N, O, P, Q, and R.

Citrate synthase has been crystallographically analysed in two forms which differ by the relative arrangement of large and small domains as shown in Fig. 14 in skeletal and space filling representations. The structural change can be described to a good approximation as rotation of the small domain by 18° around an axis close to residue 274, which represents the hinge (Fig. (15). However small deviations from a rigid rotation of the two domains are also involved in the structural change between open and closed forms. Figure 16 summarizes the CA deviations of large and small domains (P4:open form, C2: closed form), including a third crystal form analysed recently (G. Wiegand and S.J. Remington, unpublished). The latter is also a closed form . As the new crystal form has a dimer in the asymmetric unit, the two subunits can also be compared; the differences between them reflect the relatively small influence of lattice packing on the structure of the two domains (C4/1,C4/2). It is clear that the small domain has a much less rigid structure than the large domain; it appears to respond to the domain arrangement and to functional states of the enzyme by changes in tertiary structure.

What is the functional significance of these molecular forms? The open form (P4) crystallizes from phosphate or citrate; these crystals crack in the presence of oxaloacetate. The closed form (C2) crystallizes when the products citrate and CoA-SH or an analog of citroyl-CoA is present. The third form (C4) crystallizes with oxaloacetate. Neither the P4 nor the C2 crystals are enzymatically active, while the C4 crystals have not yet been tested. Citrate in C2 is bound in a cleft between the large and small domains and is completely enveloped in a highly polar pocket by the domain closure. CoA-SH is bound to the small domain, and the cysteamine part comes very close to the bound citrate. Only in the closed form is the CoA binding site completely formed, as contributions from the large domain of the other subunit are required. Domain closure thus provides a better binding site for the cofactor.

The binary complex with oxaloacetate (C4), demonstrates that oxaloacetate binds to the enzyme exactly as citrate does in the C2 crystals. Oxaloacetate binding apparently suffices to induce domain closure. It is interesting that the oxaloacetate binding site involves residues that form the hinge between the two domains; these interactions quite likely contribute to the process by which oxaloacetate triggers the conformational change. In addition, the binding site for citrate and oxaloacetate is surrounded and shaped by four histidine residues and three charged arginine residues. Formation of this binding site in the closed conformation is probably energetically

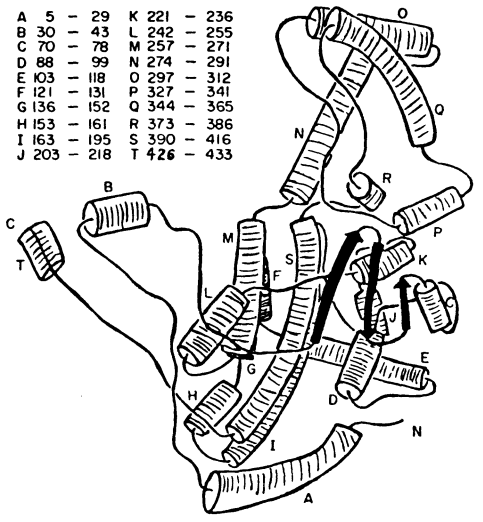


Fig. 13. Schematic diagram of the monomer of citrate synthase viewed from a point on the diad relating the two monomers. The insert indicates which residues form the helices to A to T (36).

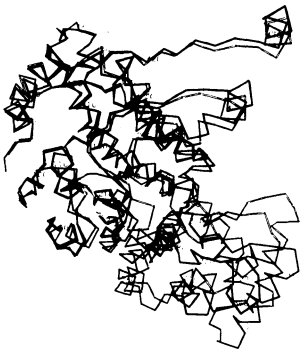


Fig. 15. The large domains of the open and closed forms of citrate synthetase are superimposed. The relative rotation of the small domain is obvious (36).

		C2	P4	C4/1	C4/2	
	C2		0.72	0.56	0.54	
			2299	2336	2320	
	P4	1.35		0.77	0.75	Large Domain
		748		2341	2338	
Small Domain	C4/1	1.00	1.87		0.29	
		697	662		2360	
	C4/2	0.99	1.86	0.32		
		697	662	666		

Fig. 16. A comparison of four different subunits in three crystal structures of citrate synthase (C2: the closed form with citrate and CoA bound; P4: the open form in phosphate; C4/1, C4/2: the two halves of the binary complex with oxaloacetate). Each box shows the r.m.s. distance between equivalent atoms, after atoms with d 2 were rejected. The number of equivalent atoms is also given (36).

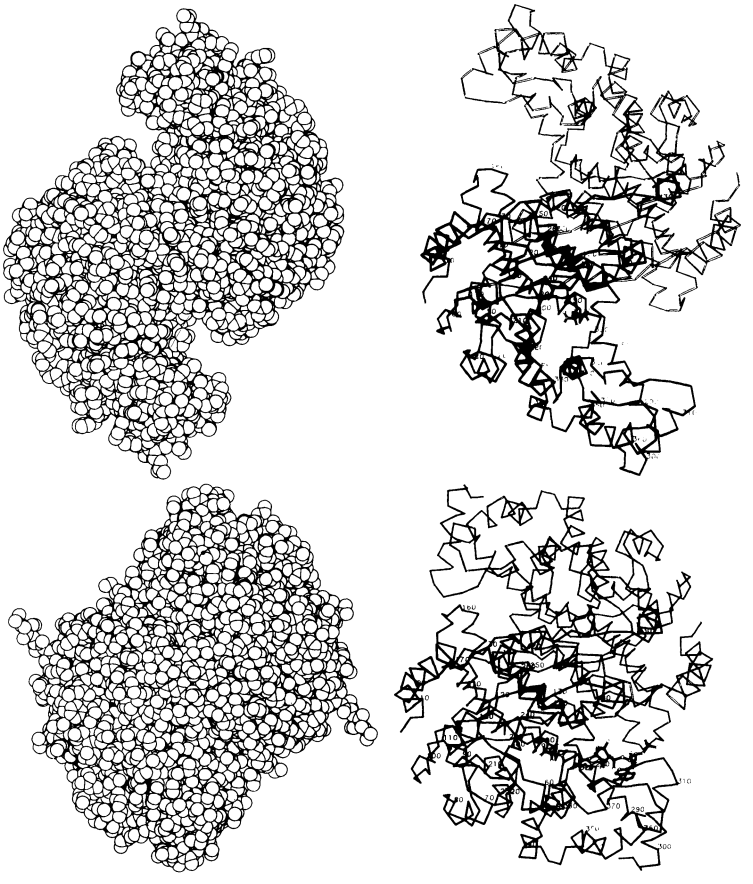


Fig. 14. Open and closed form of citrate synthase (36).

unfavourable in the absence of the counter-charges provided by the negatively charged substrates. Kinetic studies (40) show that citrate synthase has an ordered mechanism with oxaloacetate binding first and show very strong cooperativity between oxaloacetate and acetyl coenzyme A. This observation can be understood in terms of the structural features just discussed.

Catalytic action, condensation of oxaloacetate and acetyl-CoA to form citryl-CoA and hydrolysis of citryl-CoA to citrate and CoA, proceeds in the closed form. There may be subtle differences in structure between the form of the enzyme which acts as a ligase and that which acts as a hydrolase. The small differences in the tertiary structure of the small domain observed between the C2 and C4 crystal forms might be related to functional differences of this sort. The open form (P4) is probably the substrate binding and product release form. Domain motion must occur in each catalytic cycle, i.e., a few thousand times per second under optimal conditions. It is unclear however whether this is the rate limiting step in the enzymatic reaction.

As we have mentioned above, the occurrence of domain motion has been well established in a few examples. It is probably a widespread phenomenon, as it offers a unique way to create a binding site well shielded from the surroundings where catalysis can proceed without the interference from water.

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