Molecular and Cellular Biochemistry 70, 3–10 (1986).
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Current Thinking

Are proteins made of modules?

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Abstract

Analysis of a set of well characterized enzymes shows that the size of a protein subunit is directly related to the number of unique ligand binding functions described for the particular enzyme. The average size increment is about 5000 Da per ligand binding function. This value corresponds very well to: (a) the amount of polypeptide chain required to form a stable folded structure, and (b) the size of polypeptide coded by the average exon. This reinforces the hypothesis that exon-coded modules are basic architectural units for proteins. Key predictive elements of this hypothesis are: 1) generally each module has a unique function, such as the ability to bind a specific ligand; 2) the size of an enzyme subunit should be determined by the number of modules required to accomplish the enzyme's biological role.

Introduction

Subunit sizes for eukaryotic proteins vary from about 10000 Da to 480000 Da; the mean is at 31700 Da, and 90% are smaller than 60000 Da (1). Prokaryotic proteins have a similar distribution, though specific enzymes are usually smaller than their eukaryotic counterpart, and the mean subunit size is about 24000 Da (1). The terms domain and module will both be used to refer to units of globular structure that are smaller than a protein subunit. The commonly used term domain will always be used when citing specific papers from the literature; the average size of a domain is about 20000 Da (2). Modules are smaller units of tertiary structure, and it will be explained further on that they have an average size of 5000 Da.

Is this range of protein subunit sizes merely a distribution resulting from a stochastic non-ideal process for generating all the known, different, catalytic functions, or is the size of an enzyme related to its biological functions? The preeminent property of proteins is that their amino acid sequences specify stable forms that bind one or more ligands with high specificity. Ligands that can be bound include macromolecules as well as the obvious small metabolites that constitute the substrates, prosthetic groups, or allosteric effectors of enzymes. Most enzymes exist in their native form as polymers of the same subunits, or in complexes with other proteins (3, 4). Therefore, proteins should have specific binding sites for recognizing themselves (homopolymers), and other proteins (heteropolymers). Proteins also bind specifically to other cellular components such as lipid bilayers, complex carbohydrates, and nucleic acids. These properties lead to the suggestion that the size of a protein may be related to the number of its distinct ligand binding functions.

To test the hypothesis that modules have specific functions and that proteins are modular assemblies a set of well characterized enzymes has been analyzed. The following assumptions were used: 1) in the assembly of protein polymers at least one specific binding site is required to form dimers, a second to form trimers or tetramers, a third to form octamers, etc.; 2) in estimating the number of allosteric regulatory sites, effectors of similar struc-

Table 1.

Enzyme	Subunit Mr Da ($\cdot 10^{-3}$)	Polymer sites[a]	Substrate sites	Effector sites ($\times 2$)	Total ligand binding sites (Σ)	Mr/Σ	References
Brain nucleoside diphosphate kinase	18	2+1	1[b]	0	4	4500	53
Liver adenylate kinase	23	2+1	2	0	5	4600	53, 54
Fibroblast thymidine kinase	28	1+1	2	2	6	4667	55–58
Ehrlich ascites uridine kinase	31	4	2	2	8	3875	59
Liver pyruvate kinase	52	2	2	4	8	6500	60, 61
Liver phosphofructo-kinase	83	4+3	2	6	15	5533	19–21, 43, 44, 62
						Av. = 4946	

[a] Sites for formation for homopolymers + heteropolymers.
[b] Donor and acceptor bind to the same site, sequentially.

ture and function are assumed to bind at the same site; effectors that differ significantly in structure, or in function, are assumed to bind at different sites; 3) as will be explained later, two ligand binding functions are counted for each allosteric regulatory site. The first assumption will tend to underestimate the total number of polymerization sites, since even dimers may have 3 distinct interface regions (5). The second assumption will tend to overestimate the number of effector sites, since there are known examples where effectors having opposite results bind to the same allosteric site: e.g., ATP (positive effector) and CTP (negative effector) bind to the same site on *E. coli* aspartate carbamoyltransferase (6). More definitive data were used when available from crystallographic studies.

Results and discussion

Analysis of enzymes

In Table 1 are listed 6 kinases, enzymes that have a similar function of transferring the terminal phosphate from a donor (usually ATP) to some acceptor. It is immediately apparent that a particular type of catalytic function (i.e. phosphotransfer) does not itself specify a fixed subunit size since these range from 18000 to 83000 (these enzymes were selected to illustrate this size range). The enzymes are all from rat or mouse tissues, so that the size diversity is not likely to be due to the source. However, there is a direct correspondence between the subunit size of each enzyme and the total number of its ligand binding functions, with an average of one ligand binding function per 5000 Da.

Table 2 shows a similar analysis for 15 proteins that were selected to reflect widely different sources (bacteria, fungi, birds, mammals) and a variety of different catalytic reactions. Again, an almost identical result is obtained with the size of enzyme subunits increasing by an average 5200 Da per ligand binding function. The range of average module sizes in these Tables (3300 Da–7500 Da) is consistent with the requirement for stable, independent units of protein structure. From studies on protein folding, Wetlaufer has estimated that at least 20–40 amino acid residues (2300–4600 Da) are required to form a stable folded structure (7). It has generally been found from X-ray crystal structures that domains in proteins are formed by continuous portions of the polypeptide chain, and that in enzymes with more than one substrate, the different binding sites usually occur on different domains. I therefore suggest that for each enzyme in Tables 1 and 2 each ligand binding function corresponds to a specific protein module.

Table 2.

Enzyme	Subunit Mr Da ($\cdot 10^{-3}$)	Polymer sites[a]	Substrate sites[b]	Effector sites ($\times 2$)	Total ligand binding sites (Σ)	Mr/Σ	References
Yeast uridine nucleosidase	16.5	1	3	0	4	4125	63
Erythrocyte Zn-Cu superoxide dismutase	17	1	3	0	4	4250	64
Erythrocyte adenine phosphoribosyl-transferase	17.5	2	2	0	4	4375	65, 66
Erythrocyte hypoxanthine phosphoribosyltransferase	24	2	2	0	4	6000	67
Neurospora adenylosuccinate-AMP lyase	27.6	3	2	0	5	5250	68
E. coli aspartate carbamoyl transferase:							
regulatory subunit	16.5	1+2	0	2	5	3300	69, 70
catalytic subunit	33	3+2	2	0	7	4714	
Erythrocyte P-Rib-PP Synthetase	33	4	2	2	8	4125	71
B. stearothermophilus phosphofructokinase	33.9	2	2	2	6	5650	21
Heart malate dehydrogenase	35	1	3	2	6	5833	72
Yeast glyceraldehyde-3-P dehydrogenase	36	2	4	2	8	4500	73
Yeast hexokinase	50	2+1	2	4	9	5556	74
Erythrocyte glutathione reductase	52	3	6	0	9	5780	5
Avian liver amido phosphoribosyl-transferase	56	2	4	2	8	7000	75, 76
Muscle glycogen phosphorylase	97.4	3	3	6(+1)[c]	13	7492	17, 77
						Av. = 5196	

[a] Sites for formation of homopolymers + heteropolymers.
[b] Includes tightly bound metal cofactors, nicotinamide cofactors, etc.
[c] Phosphorylation site.

Defining domains and modules

As described in recent reviews (2, 9, 10) procedures used to identify protein domains include visual inspection of the structure derived by X-ray crystallography, identification of functional sites, and various algorithms that measure folding or surface area. When applied to the same protein structure, these different procedures do not always produce concordant results. Except for the smallest proteins, domains as detected by most current procedures vary in size from 10000 to 70000 and their average size is closer to 20000 (2, 9).

The term module has previously been used to designate units of structure smaller than domains (11, 12), and will be defined here as a minimal polypeptide chain that can assume a stable folded structure and bind some ligand with high specificity;

this would require at least 2000–5000 Da of polypeptide. Recent work has demonstrated units of protein structure this size by proteolytic cleavage of proteins (7); by using an algorithm for α carbon distances in hemoglobin and lysozyme (11, 12); and by measuring the heat capacity during temperature dependent denaturation of proteins (13). These last studies by Privalov and colleagues have shown that many domains in the 10000–20000 range contain at least 2 stable globular regions (13). These studies suggest that structural units the size of modules exist (7, 11, 12, 13). Modules as defined here represent a fundamental unit of protein architecture; domains represent an intermediate level of organization, containing 2 or more modules.

Does one ligand binding site equal one module?

A mechanism to transmit binding of a regulatory ligand into conformational change in the protein could be constructed as follows. Two modules, each binding a different part of the ligand, are positioned in the protein such that they are separated by a distance that is somewhat larger, or somewhat smaller, than the size of the effector ligand. It would be possible for 2 ligands to produce opposing results: the larger ligand pushing the regulatory site open, the smaller ligand pulling it closed. These conformational movements are transmitted to the catalytic site (see Fig. 1) since recent reviews (14, 15)

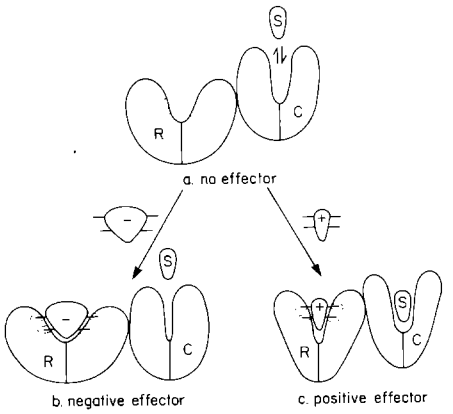

Fig. 1. A two module binding site will mechanically transmit conformational changes proportional to the size of the regulatory ligand. Both the regulatory site (R) and the catalytic site (C) contain at least 2 modules. a) In the absence of effectors the substrate (S) may bind poorly; b) A negative effector leads to closure of the catalytic cleft, and c) a positive effector opens the catalytic cleft to increase access by S.

have emphasized that the greater part of any conformational change is normally the movement of one domain relative to another.

The above is a reasonable description of how ATP (positive effector) and CTP (negative effector) are thought to regulate the *E. coli* aspartate carbamoyltransferase by binding at the same regulatory site (6, 16). This type of mechanism could also account for the activator (A) site of glycogen phosphorylase *b*, if this site were formed by distinct modules specific for 1) a purine base and 2) ribose-P. Binding of AMP at this A site is necessary to produce a conformational change that facilitates binding of substrates at the catalytic site (17). Binding of the larger ATP has negative results. Much smaller ligands do not promote a conformational change in phosphorylase *b* if they bind to only 1 module or subsite: this suggests that caffeine or glucose-6-P are negative effectors because they displace AMP, the obligatory positive effector.

Because of the above analysis, it was assumed in Tables 1 and 2 that each regulatory site is composed of 2 modules. There is also evidence that other ligands, such as the NAD(P)H cofactors, may require 2 modules. Rossman has shown that many proteins contain a domain-like region designated as a mononucleotide binding fold for binding single nucleotides such as AMP, or nicotinamide mononucleotides, and has concluded that duplication of such a structure would produce the commonly observed dinucleotide binding fold for NADH (18). The average size of the dinucleotide fold is about 12000 (2).

The binding of one substrate at the catalytic site might also require 2 modules, since it has been observed that binding of some substrates leads to closure of the binding cleft, suggesting a domain movement similar to the description for a regulatory site. Since this may not be a general feature for catalytic sites this consideration was not included in the analysis of enzymes in Tables 1 and 2. It needs to be emphasized that the analysis shown in Tables 1 and 2 can only serve as an approximation.

Is there a specific binding site for each ligand?

Complex regulatory proteins respond to a large variety of ligands: for mammalian phosphofructokinase, 23 distinct effectors have been reported (19). Based on similarities in structure, and kinetic

studies, Sols has concluded that these 23 effectors act at 7 distinct sites (19). Even this estimate may be too large; a separate review (20) supports the interpretation that there are probably 3 allosteric regulatory sites per subunit. On the principle of parsimony the lower number was used in Table 2. For the mammalian enzyme, no crystallographic data are available to make a definitive interpretation. For phosphofructokinase from *B. stearothermophilus*, a much smaller protein of Mr = 34000, it has been shown that a single regulatory site serves both positive and negative effectors (21).

Exons and modules

More compelling support for the proposed modular architecture could be obtained if this pattern could be discerned at the level of DNA. A recent analysis (22) of the sizes of exons and introns for about 80 genes yielded surprising results. Introns vary in size from 40 bp to 4900 bp, with the mean near 700 bp. Exons had a similar overall size range, but about 70% of all exons are between 52 and 223 bp, with a mean of 140 bp. Exons, therefore, predominantly code for polypeptide sequences of 2000 Da to 8500 Da, the average exon corresponding to ~5400 Da. The correspondence with the module sizes suggested in Tables 1 and 2 is quite strong.

After the discovery of intervening sequences in eukaryotic DNA, Gilbert (23) proposed that the expressed coding regions (exons) correspond to functions in the finished protein, and that recombination of exons would assort these functions independently as a mechanism for evolution, leading to different protein products. For various reasons (e.g. the need for splicing mechanisms) discussed by Doolittle (24), the early, simultaneous origin of exons and introns is consonant with Gilbert's exon recombination hypothesis.

The great uniformity of size for exons, and the agreement between exon size and the size of modules suggest an underlying relationship that was optimized by evolution. If exons and introns were present very early, how were exons selected? They may have been selected for specifying modules endowed with a useful function, and since ligand binding is the principal function of proteins, it is reasonable that modules evolved and were selected for specific ligand binding.

Some of the ramifications of Gilbert's proposal were favorably extended by Blake (25–28), and studies of lysozyme (12, 29), hemoglobin (11), ovomucoid (30), IgG (31), and complement factor B (32), showed a correlation between exons and structural domains in the proteins. However, other studies claimed a lack of correspondence between exons and protein domains for hemoglobin (33), carboxypeptidase (34), ovalbumin and antitrypsin (35). In recent reviews Doolittle has described modular regions, corresponding to exons, distributed throughout many serum proteins (36, 37).

Benefits of protein evolution by assembly of modules

To evaluate the benefits inherent in the exon-module hypothesis, it is necessary to consider the consequences of this paradigm for the protein product. A rapid divergence to produce a great variety of proteins would appear inevitable, since a limited set of structural modules would readily permit the evolution of enzyme classes having a common function (e.g. phosphotransfer) combined with high specificity for many different acceptors. This does not mean that there must be only 1 type of ATP binding module; there will certainly be a small number of such modules. It does mean that for the several hundred different kinases, an ATP binding function did not need to be developed *ab initio* in each case.

Another benefit is that, as needed, a protein can be expanded to become more complex, as suggested by the comparison of phosphofructokinases from bacteria (Table 2) and mammals (Table 1). The 2 enzymes perform the same reaction. The bacterial enzyme (Mr 34000) contains one regulatory site; the mammalian enzyme (Mr 83000) probably contains 3 regulatory sites, and in addition will bind to 3 different types of regulatory proteins. Thus the mammalian enzyme, with its increase in size, has acquired 5 additional regulatory controls. In addition to ligand binding sites, modules for regulation by covalent modification may also exist. The latter is suggested by a comparison of glycogen phosphorylase from bacteria and mammalian muscle. The 2 enzymes have strong homology through 91% of the polypeptide chain (38). However, the muscle enzyme has an extra N-terminal extension which contains a site for phosphorylation by protein

kinase (38). This does not imply that all sites for chemical modification are products of specific modules.

The preceeding suggest that there are benefits derived from a strategy for permanently joining modules in a covalently linked assembly by clustering the corresponding exons into one gene. Evidence for this same strategy at a higher level is found in the subclass of enzymes known as multifunctional proteins; these are proteins that contain 2 or more different catalytic centers. The best example for illustration is fatty acid synthase: in bacteria 8 genes specify 8 different proteins which assemble into the active enzyme complex; in fungi 2 genes produce 2 proteins that are respectively homologous to 3 and 5 of the bacterial proteins; birds and mammals have one gene producing a 250000 Da protein equivalent to the 8 bacterial proteins (39). Although each of the bacterial genes codes for a stable protein structure that should contain 2 or more modules, using gene recombination plus fusion, birds and mammals have carried the process of module assembly to its conclusion for fatty acid synthase.

Another benefit of modular assemblies is that it suggests a rationale for the formation of catalytic or regulatory sites by portions of 2 neighboring subunits. As outlined in Fig. 2, if modules are appropriately arranged in sequence, then when the polypeptide chain forms into a stable overall tertiary structure, the catalytic site should be formed by the conjunction of substrate binding modules (Fig. 2A). If, however, the folded protein has these modules far apart, a functional catalytic site may still be formed if the subunits come together in an appropriate quaternary structure (Fig. 2B). Examples of enzymes where the catalytic site is formed by 2 subunits include *E. coli* aspartate carbamoyltransferase (40, 41), glutathione reductase (5), and citrate synthase (42). In essence, association of subunits to form homopolymers provides organisms an additional degree of freedom in the assortment of modules to yield functional catalytic enzymes.

Exceptions to the model?

A variety of proteins do not readily fit into Table 2, and are therefore important because they either define the limitations of the model, or provide insights for extending it.

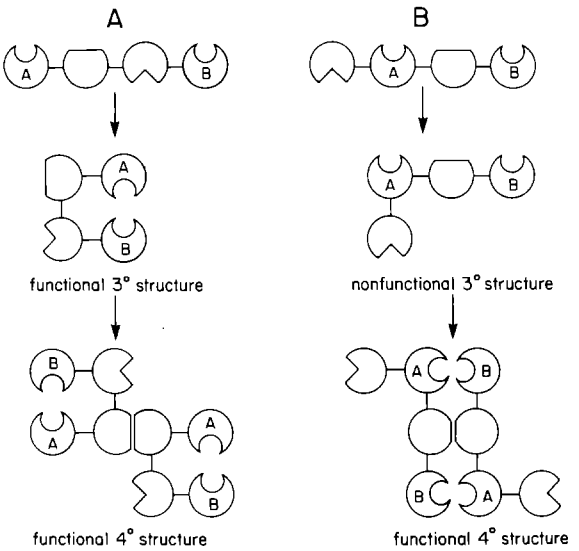

Fig. 2. Enzyme activity depends on the appropriate juxtaposition of ligand binding modules. The same four modules may be arranged as shown in schemes A and B. In A, folding into the tertiary structure brings ligand binding modules together for a functional structure; formation of a dimer may, or may not, lead to change in function. In B, the tertiary structure is not functional and only in the dimer are ligand binding sites brought into appropriate juxtaposition.

(i) proteins that are too large. There are a number of proteins with subunit sizes greater than 50000 Da that do not have enough known binding functions for their size. Perhaps these represent a subset of proteins that have escaped the size-function relationship suggested by Tables 1 and 2, and have accumulated extra mass. If this is the case, then proteolytic digestion of such proteins might produce a core fragment containing all the known activities.

It is also possible that large proteins are not adequately characterized, and that ligand binding functions remain to be discovered. Even for extensively characterized enzymes, new ligand binding sites are still being found: recent reports show that mammalian phosphofructokinase interacts with calmodulin (43), and with two other regulatory proteins (44); an NADP binding site has been reported for catalase (45) and for aldolase (46), though neither enzyme requires this cofactor for normal activity. Proteins may also be very large because of doubling by gene duplication plus gene fusion: yeast hexokinase and the mammalian hexokinase D have a subunit size of 49000–51000; the

other mammalian isozymes of hexokinase have subunits of 96000 to 98000 (47).

(ii) proteins that are too small. Several small proteins have ligand binding ratios approaching 1 per 1000 Da. Ferredoxin (Mr = 6000) binds 4 FeS (48). Metallothionein (Mr ≃ 6800) has 2 domains or modules that respectively bind 3 and 4 cadmium atoms (49). Insulin (Mr = 5600) forms hexamers that can bind 4 Zn^{2+} (50). This is not a general attribute of metal binding proteins, since calmodulin (Mr = 17000) contains 4 discrete calcium binding modules (51).

For ferredoxin and metallothionein one may suggest that each metal atom does not constitute an independent ligand. For example, the 4 Fe and 4 S form a coordinated cluster, and it is the latter that ferredoxin binds as one complex ligand. For insulin, however, the 4 metal atoms are dispersed about the hexamer.

(iii) proteins that have diverged. Myoglobin may be an example where divergence leads to loss of ligand binding functions. It has a structure very similar to the α and β subunits of hemoglobin, but has lost the ability to form polymers, or to bind 2,3-bisphosphoglycerate, the allosteric regulator of hemoglobin (52). The loss of such ligand binding properties are appropriate for the physiological role of myoglobin which does not require cooperativity for storing oxygen. A similar relation may be seen in isozymes where binding functions may change; for example, of the 4 hexokinases only isozyme A associates readily with membranes, while isozyme D is not significantly regulated by glucose-6-P (47).

Conclusion

Identified by some specific function, at least 3000 different proteins have been found. For about 100 of these crystallographic studies have been done. Even for this subset our ability to relate structure and function is far from complete, since new functions are still being discovered for well known proteins (e.g. catalase, aldolase, phosphofructokinase). Nevertheless, the rapidly expanding libraries of protein sequences will facilitate the identification of patterns in structure-function relations of the type proposed in this module hypothesis. The hypothesis proposes a unit for quantifying and relating size to function; an average module of ~5000 Da has been suggested. Where the protein subunit size is known, this relationship can be used to estimate the number of ligand binding functions for an enzyme. While a precise arrangement of modules may be critical for the overall tertiary structure and function in some proteins, different combinations might be possible, at least in larger proteins. This in turn suggests the possibility for protein engineering at the level of DNA, by the rearrangement, deletion, or insertion of exons to yield novel combinations of modular assemblies.

Acknowledgements

I thank the National Science Foundation (DMB 8310902) and American Cancer Society (BC-451) for research support. For their critical evaluation of this manuscript I am grateful to my colleagues: Charles W. Carter Jr., Howard Fried, Aziz Sancar, and Gwendolyn Sancar.

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Received 12 November 1985.