11

Quaternary Structure

INTRODUCTION

The subunit nature of many proteins was first demonstrated by the ultracentrifugation experiments of Svedberg. In studies of the molecular weights of a variety of proteins, he observed that in some cases the apparent molecular weight changed, depending on the nature or concentration of the solvent or other physical conditions that did *not* lead to polypeptide cleavage. This led to the suggestion that some proteins may be made up of aggregates of smaller proteins. Noticing that these smaller "subunits" tended to group into defined categories led to the hypothesis that all proteins were in fact assembled from a limited number of molecular weight class subunits. In the light of later knowledge this is obviously erroneous: The generalization that large proteins are built up from smaller entities does, however, remain valid. (It might be noted that in many instances there is evidence that the original suggestion of a limited number of subunits retains some appeal at the level of tertiary-structure domains rather than at the level of subunits. For example, the adenine binding domain which appears to be common to the dehydrogenases and other classes of proteins that bind adenine nucleotides may well have evolved from a common ancestral gene coding for a nucleotide binding site which subsequently became incorporated into the wide variety of proteins that make up this group).

In 1958, Bernal first adopted the designation of quaternary structure to describe a macromolecular system built up from noncovalently linked subunits. A scheme was proposed by which such subunit assemblies could be described consisting of the following designations.

Stoichiometry: A description of the types and number of each sort of subunit involved in the quaternary structure. Proteins can be classified as homo-oligomers, made up of a single type of polypeptide chain, or hetero-oligomers, made up of two or more types.

Architecture: A description of the geometric arrangements of the subunits and of the types of symmetry found in the quaternary structure.

Assembly: A description of the energetics of subunit interactions within a quaternary structure and of the nature of the interface contacts between the subunits.

Inter-subunit Communications: A detailing of the ways that conformational changes within one subunit of an oligomer affect the conformations of other subunits within the same oligomer.

Functional Aspects of Subunit Proteins: A natural corollary to the last point. What is the need for subunits in oligomeric proteins, and how do inter-subunit communications affect the biological functioning of the entire oligomer? An extension of the latter point is the question of multienzyme complexes, where individual "subunits" in an oligomer are involved in quite separate (though often related in some metabolic pathway) chemical processes.

STOICHIOMETRY

The determination of the types and number of subunits within an oligomer might seem to be quite straightforward. Intellectually, of course, it is; one establishes the number of chemically distinct polypeptide chains and how many of each are present. Practically, however, such an endeavor may be difficult. Initially, one must define the oligomer; in many instances this presents the most problems. Mammalian glutamate dehydrogenase is now regarded as a hexameric enzyme of molecular weight 336,000 Da. For many years the molecular weight could not be accurately determined because the hexamer unit undergoes a concentration-dependent polymerization, and there were questions as to whether the *active* form of the enzyme was a hexamer or some higher oligomer. The issue was finally resolved with the advent of reacting enzyme centrifugation, which showed that the minimum unit capable of catalyzing the oxidative deamination of glutamate was the hexamer. Subsequent studies demonstrated that the concentration-dependent polymerization of hexamers did not affect the enzymatic activity. Perhaps the simplest definition of quaternary structure is the size of the minimum unit capable of catalyzing the reaction supported by the protein. The demonstration that a number of proteins contain regulatory subunits which themselves have no catalytic activity but are an integral part of the native oligomer suggests that this definition should be modified to "the size of the minimum unit having the biological properties of the protein." Even with this definition, care must be taken that regulatory subunits are not lost during purification and thus go unrecognized.

A second problem arises from the possibilities of proteolytic degradation of some subunits during isolation, generating what might appear to be multiple-subunit

types in an oligomer which in reality consists of only a single type of polypeptide chain.

This is illustrated by the example of bovine galactosyltransferase, a protein that is conveniently purified by a variety of affinity chromatography procedures, giving a number of bands on SDS-PAGE with molecular weights ranging from 42,000 to 50,000. These bands copurify through all types of purification procedures and all are active, suggesting an oligomeric enzyme with dissimilar but related subunits. When care is taken to add protease inhibitors at the early steps in the purification, and to maintain their presence throughout, a single higher-molecular-weight band is isolated, indicating that rather than consisting of dissimilar subunits, this galactosyltransferase contains a single polypeptide chain. Subsequently, it was shown that the native enzyme is a monomer.

A variety of experimental techniques may be used in establishing the stoichiometry of an oligomer. In many instances these involve molecular weight determinations under native and denaturing conditions, with simple inference of the stoichiometry. In some cases the determination of the native molecular weight may present problems other than those just examined for glutamate dehydrogenase. Proteins such as yeast hexokinase or glyceraldehyde-3-phosphate dehydrogenase have oligomers that are dissociated by their substrates or regulatory ligands. More accurately, one should say that the oligomers exist in equilibrium with the subunits and, for example, glucose in the case of hexokinase, or ATP in the case of glyceraldehyde-3-phosphate dehydrogenase, displace the equilibrium toward the subunits. In other systems—for example, cytidine triphosphate synthetase (ATP or UTP) or homoserine dehydrogenase/aspartokinase (threonine, isoleucine, methionine, and divalent metal ions)—substrates or regulatory ligands displace the equilibrium toward the oligomeric form. In either instance the determination of the molecular weight under "native" (usually meaning nondenaturing) conditions can give results indicative of multiple species.

In bovine glutamate dehydrogenase an even more complex situation holds. The protein undergoes a concentration-dependent polymerization that can be affected towards either the hexamer form (the "monomer" in the concentration-dependent polymerization) or higher-molecular-weight forms. The polymerization can be displaced in either direction by various substrates or regulators. The adenine nucleotides ADP and ATP enhance polymerization, while the coenzyme NADPH or the inhibitor GTP increase dissociation. In a number of instances an oligomeric form of an enzyme can be made to dissociate into its constituent subunits by application of Le Châtelier's principle: Dilution leads to depolymerization. Examples of this type of behavior include enolase, glyceraldehyde-3-phosphate dehydrogenase, and tryptophanase, where in each case dilution yields increased amounts of monomers.

A number of chemical approaches have been successfully used to investigate subunit composition. These can be divided into two groups: (1) chemical modifications to *increase* dissociation of subunits, and (2) chemical cross-linking reactions to covalently "lock" an oligomer into its maximal molecular weight.

Various proteins—for example, glyceraldehyde-3-phosphate dehydrogenase and pyruvate carboxylase—dissociate into subunits when their sulfhydryl groups are

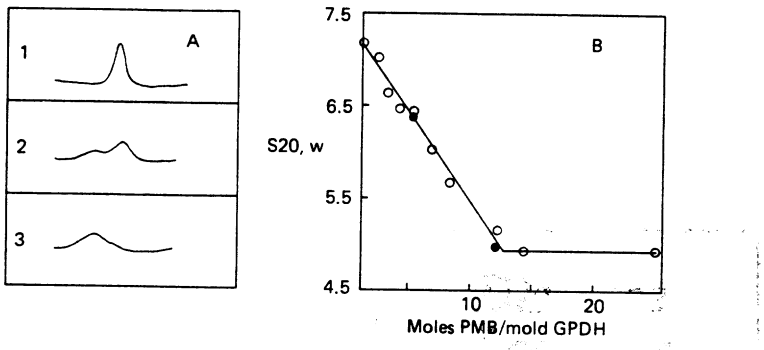

Figure 11-1 (A) Sedimentation velocity patterns of glyceraldehyde-3-phosphate dehydrogenase in the native form (panel 1), with partial modification (panel 2) and with complete modification (panel 3) by pHMB; (B) dependence of $S_{20,w}$ on moles of pHMB per mole of protein. (Adapted with permission from: G. D. Smith and H. K. Schachman, *Biochemistry, 10*, 4576–4588. Copyright 1971 American Chemical Society, Washington, D. C.)

altered by chemical modification. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, which sediments as a tetramer (Fig. 11-1A, panel 1), is dissociated by modification with *p*-hydroxymercuribenzoate (*p*-HMB). At intermediate levels of modification a mixture of monomer and tetramer is seen (panel 2), while at higher levels the tetramer is completely dissociated to the monomer (panel 3). As shown in Fig. 11-1B, there is a linear dependence of the change in the sedimentation coefficient on the modification reagent concentration. In addition, the presence of coenzyme does not affect this induced dissociation of the tetramer.

Although the actual basis for such effects has not been established, they presumably involve conformational changes induced in the proteins by the modification, which leads to decreased interactions between subunits and hence increased dissociation into the constituent subunits. More easily explained is the increased dissociation produced by acylation of reactive lysine residues in a variety of subunit-containing proteins by dicarboxylic anhydrides such as succinic anhydride, maleic anhydride, or tetrahydrophthalic anhydride, all of which result in the introduction of negative charges in place of the formal positive charges of the lysine side chains. This presumably leads to increased electrostatic repulsion between the subunits, which, depending on the stability of subunit interactions, may lead to increased dissociation. Simple determination of molecular weight before and after such modifications may, however, be misleading since it is possible that despite modification of each polypeptide chain in the oligomer by such reagents, dissociation is only prompted to some intermediate stage rather than to the level of individual subunits. As will be discussed later, such information can be invaluable in studying the architecture of an oligomer.

Chemical cross-linking studies, using the types of reagents described in Chap. 7, can be most useful in establishing subunit composition. In principle one can get cross-links occurring (1) between polypeptide chains within an oligomer, (2) within a single

polypeptide chain in the oligomer, and (3) between polypeptide chains in different oligomers. Clearly, this latter situation is not desirable and can be controlled against by making use of Le Châtelier's principle to overcome oligomer polymerization. In addition to the already mentioned effects of cross-linking, monofunctional reaction may be obtained which, although it may alter enzymatic parameters, does not effect the overall molecular weight distribution. In essence, such studies make use of the molecular weight of the highest cross-linked aggregate to indicate that of the oligomer. In conjunction with the polypeptide-chain molecular weight the stoichiometry is determined; with homopolymers unequivocal results can be obtained. With heteropolymers, however, it is possible that confusion can arise if the dissimilar subunits have molecular weights not well separated from one another. In such cases chemical cross-linking with cleavable cross-linking reagents, followed by diagonal mapping using SDS-PAGE with cleavage of the cross-links between the dimensions, may be of use.

Perhaps the most useful approach for establishing subunit composition is *hybridization.* The essence is to mix variants of the same protein under conditions where interchange of subunits can occur and then to determine the number of hybrid forms obtained. In a protein containing S subunits it can readily be shown that one obtains $S + 1$ hybrid forms of the oligomer. Although the situation is slightly more complex when the oligomer contains dissimilar subunits, the number of hybrid forms (N) is given by

$$N = \frac{[m + (S - 1)]!}{S!\,(m - 1)} \tag{11-1}$$

where S is the total number of subunits in the oligomer and m is the number of subunit *types* in the oligomer.

A number of important experimental criteria are involved in this approach. The hybrids formed must be experimentally distinguishable. Usually, charge differences are used and the hybrids separated by electrophoresis (either native or isoelectric focusing) or ion-exchange chromatography. With lactate dehydrogenase, two homogeneous isoenzymic forms are available which differ by charge. When the isoenzymes are hybridized (Fig. 11-2), five bands are obtained, indicating that each isoenzyme is

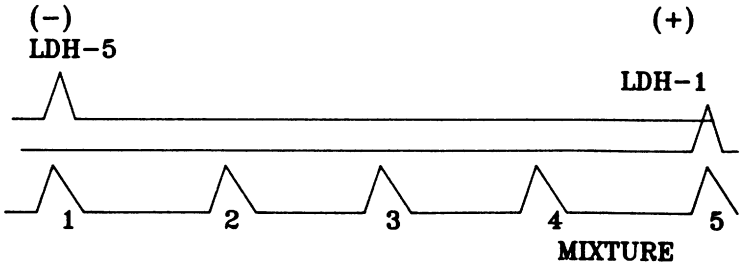

Figure 11-2 Gel scan of LDH-1 and LDH-5 isoenzymes and the hybrids obtained from an equal mixture of LDH-1 and LDH-5 after dissociation and recombination.

tetrameric. Such hybridization may also use genetic or species differences of proteins having different charges.

Yeast and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase do hybridize, giving five hybrid bands constituent with a tetrameric structure. Hemoglobin variants have been hybridized and the hybrids analyzed. In this instance, however, the results suggest that the tetramer dissociates only into dimers taking part in the hybridization. Furthermore, they indicate that the dimers were $\alpha\beta$ dimers rather than $\alpha\alpha + \beta\beta$. Where genetic, species, or isoenzymic forms of the protein are not available, it is possible to use this approach by generating molecular isomers through chemical modification. The modification procedure should be selected to change the charge of the polypeptide chains in one form, allowing easy separation of the hybrids. This method has been used with glutamate dehydrogenase. Tetrahydrophthalic anhydride modification leads to incorporation of negative charge on the subunits of the enzyme. When such a modified enzyme and unmodified native enzyme are mixed in the presence of low concentrations of guanidine hydrochloride removed by dilution, hybrids containing a native trimer and a negatively charged trimer are obtained. This indicates that the hexamer dissociates into two trimers. The hybrid forms in this example were separated by ion-exchange chromatography on DEAE-Sephacel.

The elution profile (Fig. 11-3) achieved with a salt gradient has three peaks, corresponding to the native hexamer, the hetero-hexamer (consisting of one native trimer and one acylated trimer) and the fully modified hexamer. This pattern is consistent with the predictions for a "dimer"-type system where one expects to obtain $S + 1$ hybrid forms. The trimer does not dissociate into individual polypeptide chains under these conditions. Implicit in the previous discussion is the fact that for this approach to be applied, the subunits in the oligomers must be reversibly dissociated and reassociated. This often requires some sort of reversible denaturation. The two

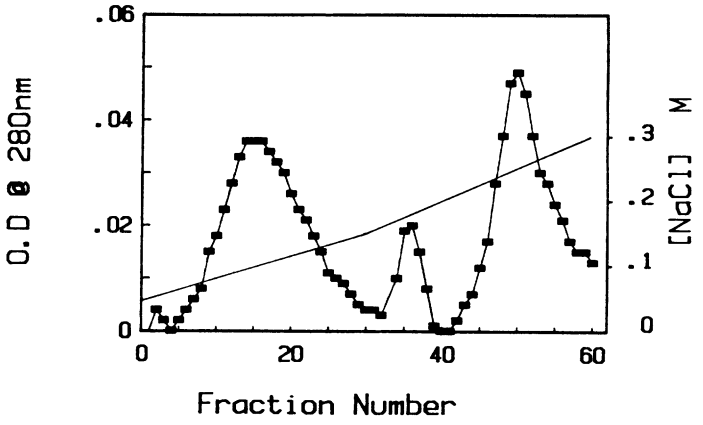

Figure 11-3 DEAE chromatography of GDH hybrids resulting from chemical modification.

parent forms of the protein are mixed, denatured, and then renatured prior to separation of the hybrids (the fact that it is possible to *reversibly* denature the hexamer of glutamate dehydrogenase only to the trimer stage and not to the monomer stage explains the previous observation that the hexamer behaves as a dimer in such experiments; this is an important limitation). Two other restrictions must be placed on this approach. First, it is essential that the association–dissociation equilibrium is *slow* relative to the time it takes to separate the hybrids, and second, it is important that complete equilibration among the variant forms occurs prior to separation.

Finally, before considering the geometric arrangement of subunits in oligomers, some comment must be made on the range of subunit compositions found in oligomeric proteins. Table 11-1 indicates some typical oligomeric enzymes, both homo- and hetero-oligomers, and their established subunit composition. Most proteins that contain subunits have two, four, or six, and almost all oligomeric proteins contain an *even* number. Excluded are multienzyme complexes, which must be dealt with separately. It would seem that there may be several exceptions to this generalization. Rat liver adenylate kinase appears to consist of three polypeptide chains of 23,000 Da

TABLE 11-1 Subunit composition of some oligomeric proteins

Protein	Mol. wt. (kDa)	Number of subunits	Subunit mol. wt. (kDa)
Homopolymers			
Azoferredoxin	55	2	27.5
Prealbumin	62	4	15.5
Malate DH	66.3	2	37.5
Glycerol-1-PO_4	78	2	40
Alcohol DH	80	2	41
D-Amino acid oxidase	100	2	50
Glycerol-6-PO_4DH	130	2	63
Phosphofructokinase	145	4	35
Glycerol kinase	217	4	55
Glutamate DH	336	6	56
Apoferritin	460	24	18.5
Thyroglobulin	669	2	335
Heteropolymers			
Lactose synthase	64	2	50, 14
Histidine decarboxylase	190	10	9 (5), 30 (5)
Aspartate *trans*-carbamylase	310	12	17 (6), 34 (6)
F_1 ATPase	380	9	55 (3), 50 (3), 31 (1), 19.5 (1), 15 (1)

each; 2-keto-3-deoxy-6-phosphogluconate kinase also appears to contain three polypeptide chains [24 kilodaltons (kDa) each], and histidine decarboxylase, while it does contain a total of 10 polypeptide chains, seems to contain five subunits of 9000 Da and five subunits of 30,000 Da, giving an oligomer of approximately 190,000 Da. The latter case is by no means the largest oligomeric protein known: Apoferritin has 24 subunits of 18,500 Da, each while erythrocruorin contains 162 subunits of 18,500 Da each.

The chemical identity of subunits within the oligomer may need to be established. The observation of two types having quite distinct properties leads to the conclusion that the oligomer contains dissimilar subunits. The converse situation—the observation of a single molecular weight of subunits—is not so easily interpreted. It is possible that two chemically dissimilar subunits have similar molecular weights. Separation by isoelectric focusing as well as by SDS-PAGE can resolve this problem. Identification of terminal amino acid residues may also distinguish identical subunits from dissimilar subunits. However, it is clearly possible that these approaches may still indicate a single type of subunit when in fact, dissimilar ones are present. Amino acid analysis and tryptic mapping represent the most declarative solution to this problem. As shown in Fig. 11-4, if the total number (N) of lysine + arginine for a protein sample is known, $N + 1$ peptides are obtained on a tryptic map, assuming

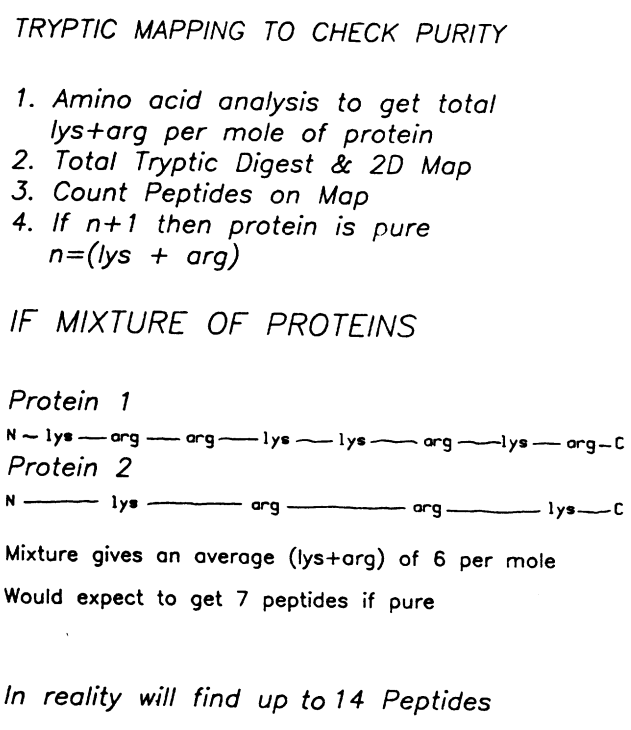

Figure 11-4 Scheme of tryptic mapping of a mixture of proteins.

that *all* the molecules in the sample are identical. If two or more different types of polypeptide chain are present, greater than $N + 1$ peptides on the map are obtained.

ARCHITECTURE

An oligomer consisting of a definite number of subunits could have any one of a finite number of spatial arrangements. With some oligomeric proteins it is possible that the molecular architecture can be observed directly using x-ray crystallography or electron microscopy. In other cases such approaches may not be possible and alternative techniques must be used to infer an architecture.

Clearly, the number of possible arrangements a molecule can have increases with the number of subunits. However, they can be limited by two assumptions which appear to hold in many cases (it is important to note, however, that there are exceptions to these assumptions and that they can have significant implications for some considerations of the functional aspects of oligomeric proteins; these are discussed as appropriate).

Assumption 1: *All subunits are in equivalent, or pseudo-equivalent environments.* Strictly speaking, this applies only to homo-oligomers and not to oligomers containing dissimilar subunits. In practice, hetero-oligomers can frequently be considered as consisting of an oligomer of $\alpha\beta$ building blocks, where α represents one type of subunit and β represents a different type. Each $\alpha\beta$ block would be in an equivalent or pseudo-equivalent environment to all other $\alpha\beta$ blocks in the hetero-oligomer.

Assumption 2: *The bonding potentials of the subunits must be saturated.* As a direct consequence of the first assumption, one can disregard linear arrays of subunits or variants of the idea of linear arrays. The second assumption limits the categories of subunit architecture that one must consider to closed sets for homo-oligomers or hetero-oligomers. In the absence of this second assumption, aggregates containing multiple oligomers could be obtained. Such is the case in sickle-cell hemoglobin, where the substitution of a valine for a glutamate at the B_6 position creates an additional bonding potential on the β subunits in the tetramer that is not satisfied by the normal subunit–subunit interactions; the result is an uncontrolled polymerization of the tetramer and resultant precipitation of the variant hemoglobin. Another example, found normally, is the case of glutamate dehydrogenase, a hexamer, but one that undergoes a concentration-dependent polymerization:

$$n(\text{GDH hexamer}) \longleftrightarrow (\text{GDH hexamer})_n \qquad (11\text{-}2)$$

Although this is very well documented and is affected by a variety of ligands and substrates for the enzyme, it appears to have no enzymatic function. Because the polymerization results in long linear arrays of hexamers it seems that the hexamer has some unsaturated bonding potential at its apexes, which results in its ability to undergo this polymerization.

The result of both assumptions is that the architecture of the oligomers is restricted to those that have the subunits arranged in a regular fashion about a central point: *Only point symmetry is possible.* Point groups contain rotation axes and inversion axes; however, for polypeptide chains, which contain *L*-amino acids, inversions are not allowed (these would lead to enantiomorphic differences) and only *rotation axes* passing through the point need to be considered. There are three types of point symmetry that we consider: cyclic symmetry, dihedral symmetry, and cubic symmetry.

Cyclic Symmetry

In a system showing cyclic symmetry (which is the simplest type) there is an n-fold rotational axis, where n = the number of subunits in the oligomer. As illustrated by Fig. 11-5, an n-fold rotational axis indicates that the molecule must be rotated about a point by $360/n°$ to effect a transposition into an equivalent environment for the subunits.

For a *dimer* (where $n = 2$) there is a single two-fold rotation axis and the molecule is said to show C_2 symmetry. A rotation by $360/2 = 180°$ transposes the molecule into itself again. With a trimer (where $n = 3$) there is a single three-fold axis (the

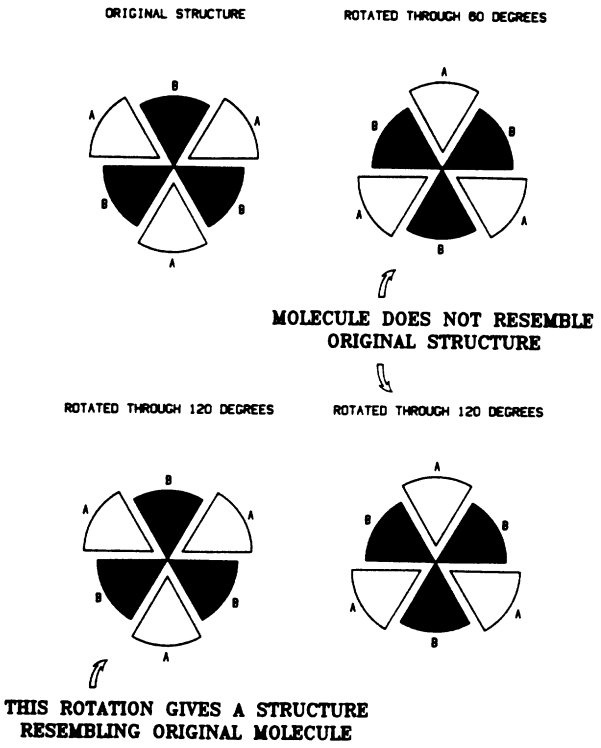

Figure 11-5 Point-symmetry operations.

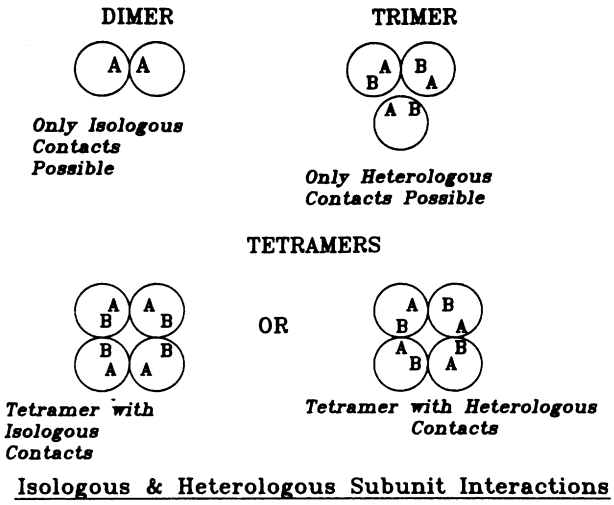

Figure 11-6 Isologous and heterologous subunit interactions for (A) a dimer, (B) a trimer, and (C) a tetramer.

molecule has C_3 symmetry) and a rotation about a point of $360/3 = 120°$ results in an equivalent molecule. C_2 and C_3 symmetries are the only geometries possible for dimers and trimers, respectively, that result in identical environments for the subunits.

In considering cyclic symmetry for dimers or trimers (Fig. 11-6) an important point is apparent. In a dimer the contact sites between the subunits are identical in both subunits—the subunits are said to have *isologous bonding.* In the trimer, however, such a situation is clearly not possible—the subunit contact regions between any two subunits are not identical and the intersubunit bonding is *heterologous*: Any oligomer containing an odd number of subunits must have heterologous bonding. In a system with an even number of subunits (>2), either isologous or heterologous bonding can exist (to maintain the condition of identity in subunit environments, however, a single oligomer cannot have isologous *and* heterologous contacts). Consider the case of a tetramer—in cyclic symmetry there are two ways of orienting the subunits that fulfill both assumptions: one has two types of isologous bonding and the other heterologous bonding.

Dihedral Symmetry

If a molecule has n twofold axes at right angles to a single n-fold axis, it is said to exhibit dihedral symmetry. In such cases the number of subunits must be equal to $2n$. As a result, only oligomers with an even number of subunits can have dihedral symmetry.

The simplest and most common form of dihedral symmetry is *tetrahedral,* where the oligomer contains four subunits and is said to have D_2 symmetry. In tetrahedral

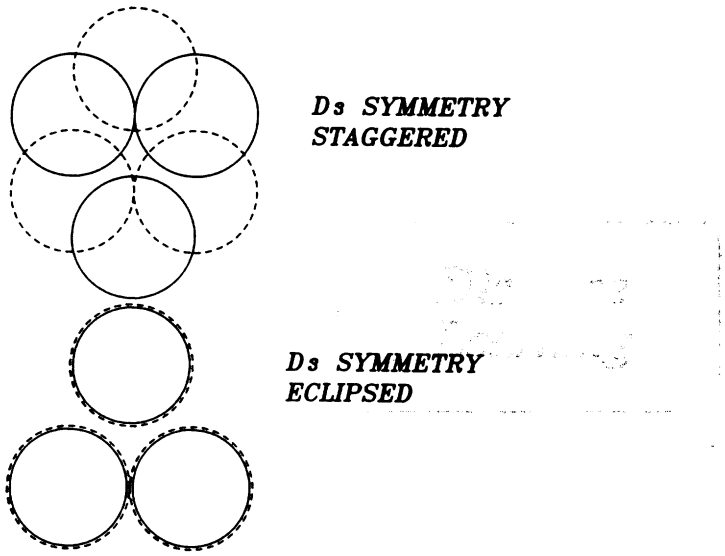

Figure 11-7 D-3 symmetries of a hexamer.

symmetry the subunits have isologous bonding. For a simple molecule such as methane (CH_4) one gets cubic symmetry with four threefold axes and three twofold axes. However, with proteins containing only L-amino acids (which as a result are inherently asymmetric), the threefold axes cannot exist, and as a result one gets three twofold axes.

With six subunits (i.e., $n = 3$ for dihedral symmetry) there are two possible noncyclical architectures, both of which are D_3 symmetry. These are represented in Fig. 11-7 and are the *trigonal prism*, which has an eclipsed orientation of the two apparent trimers that make up the hexamer, and an *octahedron*, which has a staggered orientation of the two apparent trimers. In addition one can also get cyclic symmetry in the form of a hexagon, which has C_6 symmetry.

Cubic Symmetry

In addition to quaternary structures with cyclic or dihedral symmetry, oligomers may have cubic symmetry. However, for an oligomer to exhibit this, it must possess $12n$ subunits; as a result, not many have been described showing cubic symmetry. It is an important symmetry, however, in macromolecular assemblies such as the spherical viruses.

Before considering some experimental approaches to establishing the symmetry of an oligomer there are several comments to be made about subunit arrangements and the types of bonding between subunits.

Earlier it was indicated that very few, if any, oligomeric proteins had odd numbers of subunits, yet we have considered symmetries for trimers. This was done be-

cause in a number of instances both homo- and hetero-oligomers overall have an even number of subunits but have substructures containing elements with odd numbers of subunits. Aspartate transcarbamylase serves as an example of a hetero-oligomer. It contains a total of 12 subunits, but the architecture of the oligomer indicates that the six catalytic subunits are arranged in two trimers separated from one another by the regulatory subunits. Glutamate dehydrogenase, a hexamer of identical polypeptide chains, appears to have D3 symmetry but consists of two trimers—a dimer of trimers. These considerations are important when examining subunit–subunit interactions since the architecture of the oligomer indicates something about the nature of the contacts between subunits.

If one considers the bonding between subunits in an oligomer, there is no thermodynamic reason to a priori prefer isologous or heterologous bonding. The driving force for the association of subunits into an oligomer *must* come from increased stability due to the formation of inter-subunit bonds. As a result, one might expect the spatial architectures giving the largest numbers of inter-subunit contacts to be the most stable. This would result in a tetrahedral architecture being the most stable form for a tetramer and an octahedral architecture being optimal for a hexamer. Although these are common architectures (thus supporting the idea), there are exceptions. This results from the fact that the energy of interaction in the oligomer contains an intensity factor as well as a quantity factor. Finally, it must be noted that in cyclic symmetry the inter-subunit contacts are *all identical*, whereas in dihedral or cubic symmetry one can get several different bonding regions within a subunit.

EXPERIMENTAL APPROACHES

As discussed previously, direct establishment of molecular architecture by means of x-ray crystallography or electron microscopy gives an unequivocal answer, but it is often not possible or is laborious. A wide variety of other experimental approaches have been used in various systems to give information about molecular architecture.

One of the most used comes from assumption 2. In certain symmetries one finds different types of subunit–subunit interactions within the oligomer. In a tetramer with D_2 symmetry there is one type of dimer bond and two types of tetramer bonds. As a result of the intensity factor discussed, one might expect that a relatively stable dimer could be produced in such a situation. Thus an idea of the tetramer symmetry can be established if the dissociation stages of the oligomer can be identified. This approach has been used in a number of cases, such as hemoglobin, glyceraldehyde-3-phosphate dehydrogenase, aspartate transcarbamylase, and glutamate dehydrogenase. In the former two cases stable dimers have been identified, while in the latter trimer intermediates are found, indicating D_2 or D_3 symmetry respectively. In addition to assisting in the assignment of the molecular architecture, such studies have inherent implications for the strength of different contact regions between subunits in oligomers. This is discussed in more detail later in this chapter.

Chemical cross-linking studies may also be useful in distinguishing between different molecular architectures. In studies with glutamate dehydrogenase the subunits

can be covalently cross-linked with bifunctional reagents such as dimethylpimelimidate. If the cross-linked subunits within the oligomer can be experimentally quantitated by, for example, SDS-PAGE, it is possible from computer modeling studies to distinguish between C_6 and D_3 symmetries. Equations giving the probabilities of the distribution of the monomers, dimers, trimers, tetramers, pentamers, and hexamers that from random cross-linking of the subunits in D_3 or C_6 symmetries can be

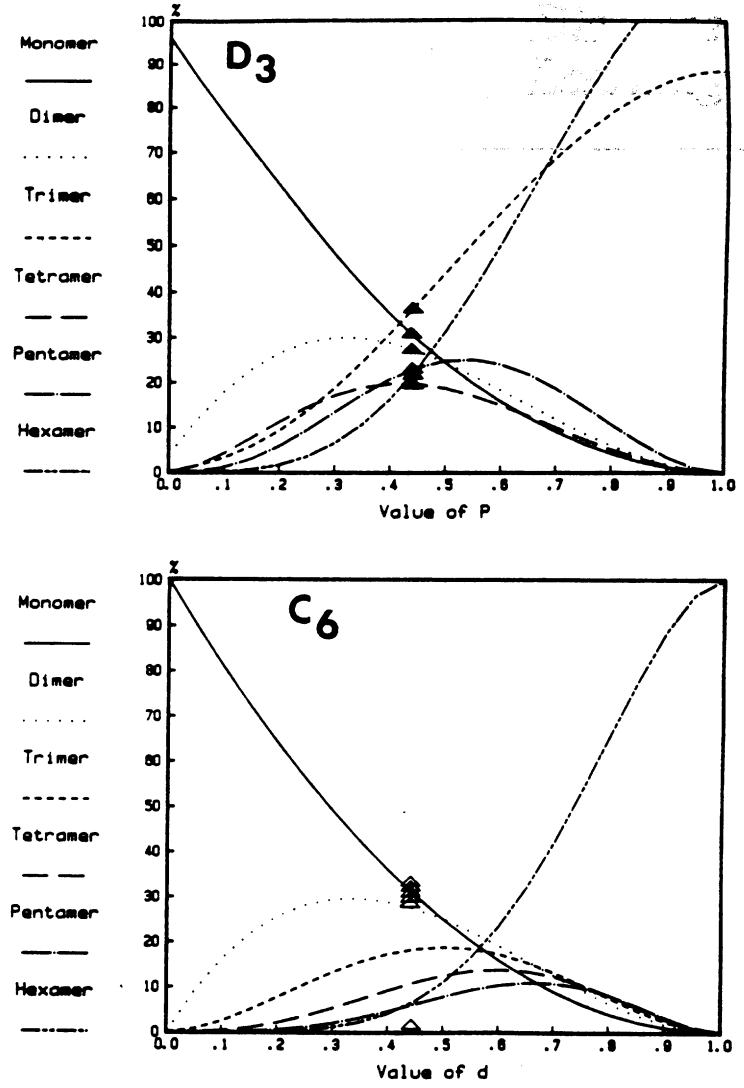

Figure 11-8 Comparison of experimentally observed distribution of cross-linked species of glutamate dehydrogenase with predicted patterns for C-6 or D-3 symmetries.

derived. With D_3 symmetry two parameters, p, the probability of intra-trimer modification, and r, the probability of inter-trimer modification, are required. With C_6 symmetry only the probability of modification between adjacent subunits, d, is required. Figure 11-8 shows computed profiles for each model together with experimental data that clearly indicate that the molecule has D_3 rather than C_6 symmetry. It should be pointed out that distinction between D_3 trigonal prism and D_3 octahedron is not possible by such an approach.

A variation has been used with avidin, a tetrameric molecule of molecular weight 68 kDa which binds biotin. A series of bis-biotin derivatives synthesized with differing chain lengths between the biotin rings were examined for their ability to aggregate avidin. It was established that with a $(CH_2)_n$ chain length of $n = 1$ between the biotin moieties, cross-linking between two tetramers occurs and the aggregated material can be observed by electron microscopy. The polymers are linear chains, with a width as expected for a single avidin tetramer, and have *no* branch points. If the subunits in avidin are in equivalent environments, only C_4 or D_2 symmetries need to be considered. With C_4 symmetry one expects the possibility of branch points and also that the width of the chain is larger than observed. The results therefore indicate that the avidin oligomer has D_2 symmetry.

ASSEMBLY OF SUBUNITS

The assembly of subunits into a quaternary structure can occur in a variety of ways: In homo-oligomers the process may be a gradual, stepwise transition, with one subunit being sequentially added to the growing "core" until the final oligomer is reached. Alternatively, stable, intermediate forms may be formed which then associate to give the final form. With hetero-oligomers this latter process seems inherently more probable. The formation of relatively stable sub-structures, either in the case of homo- or hetero-oligomers, can themselves be considered as structures that must be assembled from subunits, and as initially stated the process presumably consists of the stepwise addition of subunits to a preexistent core. In such schemes, at each aggregation stage the succeeding subunit may be attached to the core with the same intrinsic free-energy change as its predecessor, with a greater intrinsic free-energy change or with a lesser intrinsic free-energy change. These situations can be correlated with an assembly process showing *no* interactions between subunits in self-assembly, cooperative interactions, and antagonistic interactions, respectively. In these cases there is a smooth transition between initial and final states, an extremely sharp transition, or a particularly gradual transition. Figure 11-9 is a schematic energy diagram showing the intrinsic free-energy change for each subunit coming into the oligomer for a tetramer in each of these cases. The intrinsic free-energy change per subunit is of course always negative; otherwise, the oligomeric form would not be favored. The experimental determination of these free energies requires that the equilibrium constants between the various intermediate stages in the assembly process be determined. Any method that can quantitate the concentrations of, for example, monomer, dimer,

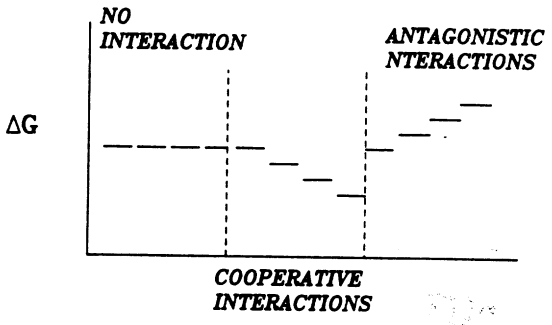

Figure 11-9 Schematic energy diagram for subunit assembly, showing the stepwise free-energy changes for the formation of a tetramer.

trimer, and tetramer, for a tetrameric protein, as a function of the protein concentration, could be used to determine these equilibrium constants.

Such associations are best followed using techniques such as sedimentation or gel filtration of the protein under native conditions, or by using an approach such as light scattering or fluorescence polarization measurements. The first three approaches, essentially based on molecular weight determinations, were discussed in Chap. 4. The final approach is outlined, for the example of the association of α-lactalbumin with galactosyl transferase to form the hetero-dimer lactose synthase, in Fig. 11-10.

If we consider in more detail the case of a tetrameric protein, two extremes become apparent.

1. There may exist, in equilibrium with monomer and tetramer, significant amounts of intermediate forms; for example, in the case of lactate dehydrogenase a significant concentration of dimers is observed.
2. Only monomers and tetramers may exist in significant concentrations. An example is *hemerythrin*, which although it has the final oligomeric size of an octamer, seems to be assembled from two tetramers that appear to exist in equilibrium only with monomers.

These two extremes represent cases of either noncooperative (or antagonistic) interactions between subunits and extremely cooperative interactions during assembly.

A *cooperativity parameter* α can be defined by

$$\alpha = \frac{K_{i+1}}{K_i} \tag{11-3}$$

where K_i is the equilibrium constant for the addition of monomeric A to a molecule containing A_{i-1} subunits.

$$A + A_{i-1} = A_i \qquad \text{with } K_i = \frac{[A_i]}{[A] \cdot [A_{i-1}]} \tag{11-4}$$

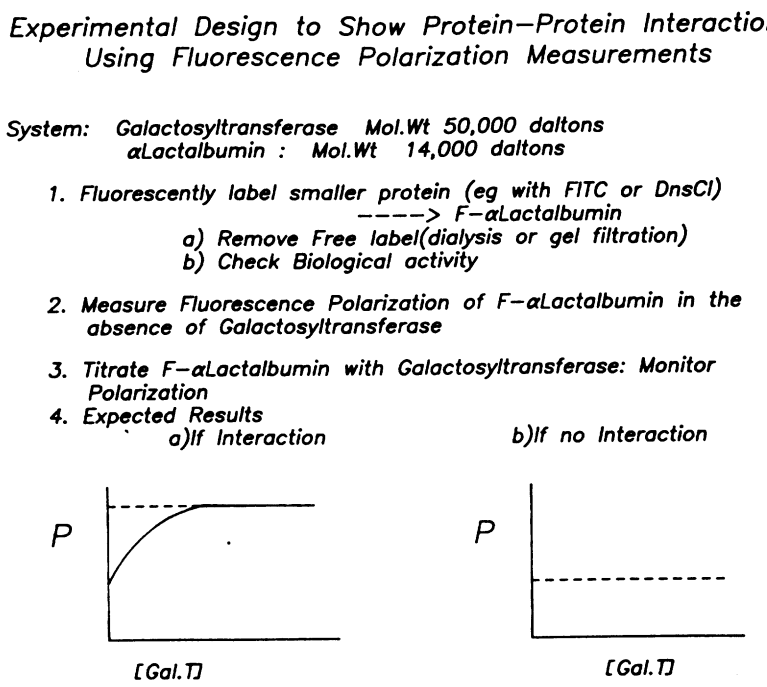

Figure 11-10 Use of fluorescence polarization measurements to study protein–protein interactions in the lactose synthase system.

Such an equation can be written for each step in the assembly process, and from the expression for α [Eq. (11-3)] we get $K_3 = \alpha K_2$, $K_4 = \alpha K_3 = \alpha^2 K_2, \ldots, K_i = \alpha K_{i-1} = \alpha^{i-2} K_2$. From Eq. (11-4) it is apparent that

$$A_i = K_2^{i-2} \alpha^{\tau} A^i \tag{11-5}$$

where $\tau = 1 + 2 + \cdots + (i-2)$; thus $\tau = [(i-1)(i-2)]/2$.

The experimental single association constant for a monomer–i mer equilibrium is given by

$$K_i = \frac{A_i}{A^i} \tag{11-6}$$

From Eqs. (11-5) and (11-6) it is found that

$$K_i = K_2^{i-1} \alpha^{\tau} \tag{11-7}$$

If we consider now a hypothetical system, an octamer made up of identical subunits of 10 kDa each, and arbitrarily assign a value for K_2 of 1×10^5, we can compute the concentration dependence of the molecular weight of the system with values for α of 3.0 (indicating cooperativity between subunit self-assembly), 1.0 (each

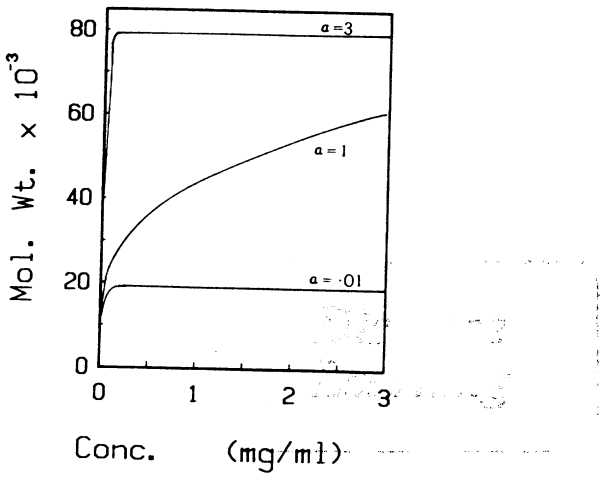

Figure 11-11 Weight-average molecular weight for an associating system, as a function of concentration, with different values of α.

subunit binds with the same affinity as the previous subunit), and 0.1 (antagonistic interactions during self-assembly). Figure 11-11 illustrates the results of such calculations.

With cooperative interactions the concentration dependence shows a rapid rise to the oligomer molecular weight of 80,000, and as expected, with antagonistic interactions the octamer is never formed—even formation of a dimer is unfavorable. Examination of Fig. 11-11 shows that a relatively small increase in the cooperativity parameter $\alpha = 1$ to $\alpha = 3$ results in a large effect on the concentration dependence of the molecular weight. The free energy associated with such a change is given by

$$\begin{aligned} \Delta(\Delta G^\circ) &= \frac{RT \ln K_{i+1}}{K_i} \\ &= RT \ln \alpha \\ &= RT \ln 3 \\ &= 0.65 \text{ kcal} \quad \text{at room temperature} \end{aligned} \tag{11-8}$$

This quantity, 0.65 kcal, is actually surprisingly small and represents, for example, a small increase in the area of contact between subunits in the oligomer compared to the dimer. Later, we consider where this energy change could come from. However, first we can represent the molecular weight versus concentration profile of Fig. 11-11 in terms not of the value of α, but in terms of the free-energy change associated with α. Such a plot is shown in Fig. 11-12.

As discussed previously and as calculated using Eq. (11-8), the energy changes associated with subunit–subunit interactions during self-assembly required to give

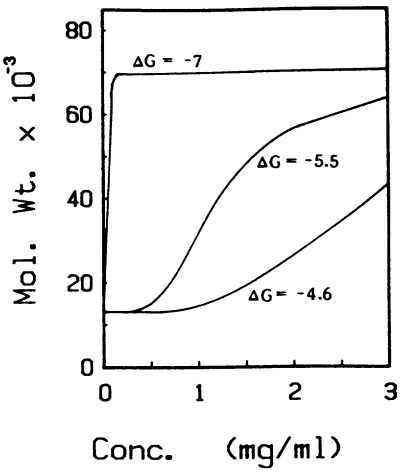

Figure 11-12 Dependence on concentration of weight-average molecular weight for an associating system with different energies of interaction between the subunits.

cooperative interactions are quite small. It is useful, in the current context, to consider some of the possible conformational changes that could be associated with subunit interactions during self-assembly.

Small conformational changes involving, for example, the change in environment of a carboxyl group or the change in flexibility of an amide group can easily account for free-energy changes in the range 1 to 2 kcal. The transfer of a carboxyl group from an aqueous to a nonpolar environment can be approximated, for the purposes of considering the free-energy change, by considering the pK of glutamate-35 in lysozyme. This residue is in a highly nonpolar environment, and as a result the pK of the side-chain carboxyl is shifted to approximately 6.0, compared to a side-chain pK of 4.5 in aqueous solution. This change corresponds to a free-energy change of approximately 2 kcal. The restriction of an amide group from a freely mobile, flexible conformation to an immobile amide group is basically an entropy-requiring process for which a $\Delta S°$ of -5 eu has been estimated. At room temperature the size of this entropy change corresponds to a ΔG of about 1.5 kcal.

Similarly, one might consider the effects of substituting, for example, an asparagine residue with an aspartate residue at a subunit interface. Such a process is accompanied by a substantial free-energy change as a result of the ionization of the carboxyl group. The ΔG can be estimated approximately by considering the free energy of ionization of a carboxyl, which is about 6 kcal.

As can be seen by considering these examples, quite large amounts of free energy (relative to the amount needed to produce highly cooperative subunit self-assembly) is available via a variety of seemingly inconsequential conformational changes. Large conformational changes upon subunit–subunit association are not needed to account for highly cooperative (or antagonistic) interactions.

Other Factors Affecting Subunit Interactions

In some cases the assembly of subunits into an oligomer requires some cofactor that allows assembly to take place. A typical example is provided by aspartate transcarbamoylase. The oligomer contains 6 mol of zinc, which is *extremely* tightly bound; it is not removed from the oligomer by dialysis against the chelating agent 1,10-phenanthroline and is not exchangeable with ^{65}Zn when the polypeptide subunits of aspartate transcarbamoylase are dissociated. Yet the zinc is *not* required for catalytic activity since the catalytic subunits can be prepared zinc-free, yet still have full activity. When zinc is removed from the isolated regulatory subunits (aspartate transcarbamoylase is a hetero-oligomer) however, they cannot interact with the catalytic subunits to reconstitute the regulatory properties of the holoenzyme. Zinc has no effect on the catalytic trimers but does promote the dimerization of the regulatory subunits. It is apparent that zinc is involved in maintaining quaternary structure, which in this particular case is essential not for catalytic purposes, but for the regulatory properties of the holoenzyme. Zinc apparently enhances the interface interactions between the catalytic and the regulatory subunits and thus allows the association of the two catalytic trimers with the three regulatory dimers in the holoenzyme.

In Chapters 9 to 11 we examined various aspects of the three-dimensional structure of proteins. Although secondary, tertiary, and quaternary structures have been dealt with separately, it is important to realize that each additional level of structure has some impact on the previous one. Thus in considering quaternary structure it must be emphasized that the association of two or more subunits into a quaternary structure *is expected* to have an influence on the tertiary structure of each subunit. It is via this coupling of tertiary and quaternary structure that conformational changes can be transmitted between subunits in an oligomer.