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Self-Assembly of Biological Macromolecules

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Phil. Trans. R. Soc. Lond. B 1975 **272**, 123-136
doi: 10.1098/rstb.1975.0075

Phil. Trans. R. Soc. Lond. B. **272**, 123–136 (1975) [123]
Printed in Great Britain

Self-assembly of biological macromolecules

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The genetic apparatus of the cell is responsible for the accurate biosynthesis of the primary structure of macromolecules which then spontaneously fold up and, in certain circumstances, aggregate to yield the complex tertiary and quaternary structures of the biologically active molecules. Structures capable of self-assembly in this way range from simple monomers through oligomers to complex multimeric structures that may contain more than one type of polypeptide chain and components other than protein.

It is becoming clear that even with the simpler monomeric enzymes there is a kinetically determined pathway for the folding process and that a folded protein must now be regarded as the minimum free energy form of the kinetically accessible conformations. It is argued that the denatured subunits of oligomeric enzymes are likely to fold to something like their final structure before aggregating to give the native quaternary structure and the available evidence would suggest that this is so. The importance of nucleation events and stable intermediates in the self-assembly of more complex structures is clear.

Many self-assembling structures contain only identical subunits and symmetry arguments are very successful in accounting for the structures formed. Because proteins are themselves complex molecules and not inelastic geometric objects, the rules of strict symmetry can be bent and quasi-equivalent bonding between subunits permitted. This possibility is frequently employed in biological structures. Conversely, symmetry arguments can offer a reliable means of choosing between alternative models for a given structure.

It can be seen that proteins gain stability by growing larger and it is argued in evolutionary terms that aggregation of subunits is the preferred way to increase the size of proteins. The possession of quaternary structure by enzymes allows conferral of other biologically important properties, such as cooperativity between active sites, changes of specificity, substrate channelling and sequential reactions within a multienzyme complex. Comparison is made of the invariant subunit compositions of the simpler oligomeric enzymes with the variation evidently open to, say, the 2-oxoacid dehydrogenase complexes of *E. coli*. With viruses, on the other hand, the function of the quaternary structure is to package nucleic acid and, as an example, the assembly and breakdown of tobacco mosaic virus is discussed. Attention is drawn to the possible ways in which the principles of self-assembly can be extended to make structures more complicated than those that can be formed by simple aggregation of the component parts.

Introduction

In this contribution to the Discussion and in keeping with the spirit of the meeting I shall deal with principles and ideas as I see them and not burden the arguments unnecessarily with the details of the supporting experimental evidence. These can be obtained from the literature cited. I intend to tackle two principal questions

(*a*) Why do biological macromolecules have subunits?

(*b*) How do these structures form?

A specific three-dimensional structure has long been accepted as *sine qua non* for the biological

activity of macromolecules. It is now more than fifteen years since the first demonstration that an enzyme, the monomeric ribonuclease-A of ox pancreas, possessed the ability to refold spontaneously from the unfolded (denatured) state obtained in strong solutions of urea and 2-mercaptoethanol (Sela, White & Anfinsen 1957). When the reducing and denaturing agents were removed, the enzyme recovered its biological activity and its native structure, as shown, for example, by the exact regaining of its four disulphide bridges – 1 out of 105 possible arrangements. Contemporary experiments with the coat protein of tobacco mosaic virus (reviewed by Anderer 1963) demonstrated that the denatured polypeptide chain could, under appropriate conditions, refold and aggregate to form the well-known virus-like rods in the absence of viral RNA. A little earlier it had been found that the separated protein and RNA from tobacco mosaic virus could reassociate spontaneously *in vitro* to yield active, infectious virus (Fraenkel-Conrat & Williams 1955).

Numerous comparable experiments have since been carried out on systems as diverse as enzymes, flagella, microtubules and viruses (Wolstenholme & O'Connor 1966; Wolstenholme & O'Connor 1972; Markham *et al.* 1973). These may contain more than one type of polypeptide chain and components other than protein. As a result of such work we can erect a general concept of self-assembly: the genetic apparatus of the cell is responsible for the accurate biosynthesis of the primary structure of macromolecules (see, for example, Crick 1966) which then spontaneously fold up and, in certain circumstances, aggregate to yield the complex tertiary and quaternary structures we see in the biologically active molecules. Such an idea is very obviously at one with the essentially linear nature of the information encoded in the base sequence of DNA. Inevitably, not every biological structure can be accounted for in such simple terms but the concept provides an experimentally verified basis for the description of many important structures and offers an attractive starting point for analysis of the formation of higher orders of macromolecular organization.

The specificity of molecular interaction is a striking feature at all levels of the folding and assembly processes but I should like to emphasize at this point that biological structures as we know them are realized in an aqueous environment. The structure and properties of water as a solvent, although imperfectly understood, are of critical importance to a detailed description of any molecular interaction (see M. C. R. Symons, this Discussion). Our models for folding and assembly must take account of this fact.

The folding of polypeptide chains

Monomeric proteins

The spontaneous folding of denatured polypeptide chains to reform native, biologically active structures was first taken to mean that native proteins must be at their lowest Gibbs free energy level commensurate with their aqueous environment. However, Levinthal (1968) pointed out that it would be impossible for a protein to sample all possible conformations of its polypeptide chain and yet arrive at the uniquely correct one in the comparatively brief time known to be required for the process. This led naturally to the concept of nucleation centres – regions, perhaps, of α-helix or β-pleated sheet – to direct the folding process, an idea that is receiving growing experimental support (Anfinsen 1973; Wetlaufer 1973) For example, intermediates have been detected and tentatively identified in n.m.r. studies of the unfolding of staphylococcal nuclease (Jardetzky *et al.* 1972) and ribonuclease-A (Benz & Roberts 1973) caused by a variety

of denaturing agents. Intermediates have also been recognized in the folding of ribonuclease (Tsong, Baldwin & Elson 1972), lysozyme (Wetlaufer & Ristow 1973), cytochrome *c* (Ikai, Fish & Tanford 1973) and pancreatic trypsin inhibitor (Creighton 1974); and this list is by no means exhaustive.

Since the accumulating evidence is in favour of the existence of folding pathways, kinetic constraints must be operating in the folding process and we conclude that the final biological structure is not that of lowest Gibbs free energy. It will, of course, be at a free energy minimum but this will be the free energy minimum of only those structures that can be visited as a consequence of the kinetic constraints.

Several of the proteins whose refolding has been most extensively studied are rich in disulphide bridges, e.g. lysozyme and pancreatic trypsin inhibitor. Although disulphide bridges, as covalent intramolecular cross-links, obviously contribute substantially to the stability of the molecule, there is no reason to believe that they change the product of the folding pathway in any particular fashion (Wetlaufer & Ristow 1973; Creighton 1974). Perhaps the most persuasive evidence for this is the observation that for a pair of homologous proteins with closely related three-dimensional structures as determined by X-ray crystallography, a disulphide bridge found in one protein but not the other can be fitted to the model of the latter without strain (Sigler, Blow, Matthews & Henderson 1968; McLachlan & Shotton 1971; Poljak *et al.* 1973).

Oligomeric proteins

When we turn to oligomeric structures, we see at once the specificity of recognition between protein subunits. Thus, *in vivo* we do not find adventitious mixtures of subunits in enzymes: aldolase, a tetramer in mammalian muscle, does not occasionally appear with one subunit derived from glyceraldehyde 3-phosphate dehydrogenase, another tetramer in the same tissue. Since the same specificity is revealed in reversible denaturation and refolding of mixtures of oligomeric enzymes *in vitro* (Cook & Koshland 1969), there is no need to invoke any special mechanism for the process *in vivo*. Indeed, the complexity of non-covalent interactions across the interfaces of subunits in oligomeric enzymes (Perutz 1970; Adams *et al.* 1972) makes the occurrence of such hybrid enzymes difficult to envisage: their absence is no more than to be expected.

On the other hand, hybrid formation is frequently observed *in vivo* and *in vitro* between closely related forms of an oligomeric enzyme, for example, between the isoenzymic forms of aldolase (Penhoet & Rutter 1971) or of lactate dehydrogenase (Markert 1963), found in different tissues of the same organism. Such hybridization clearly rests on the existence of a high degree of similarity of three-dimensional structure between the subunits of the parental forms, particularly at the subunit interface where recognition must occur. The generation *in vitro* of hybrids between preparations of the 'same' oligomeric enzyme from different species, e.g. glyceraldehyde 3-phosphate dehydrogenase from sources as widely separated phylogenetically as rabbit muscle and yeast (Spotorno & Hollaway 1970; Osborne & Hollaway 1974), can therefore be taken as evidence for structural homology between the enzymes.

On general grounds it is unlikely that interactions between protein subunits in oligomeric enzymes will cause wholesale structural changes in the polypeptide chains. There is impeccable evidence that small but critical changes in subunit contacts are sufficient to account for the subunit rearrangements that are at basis of the cooperativity in the oxygenation of the haemoglobin tetramer (Perutz 1970). It is clear that the α- and β_2-subunits of the tryptophan

synthetase of *E. coli* (see below) adopt new conformations when they form the $\alpha_2\beta_2$ structure of the native enzyme (Faeder & Hammes 1971). Similarly, a study of hybrid formation between native tetrameric rabbit muscle aldolase and a structurally distorted tetramer obtained by chemical modification of the protein amino groups has shown that the structure of a distorted, chemically modified subunit can be improved by interaction with its native neighbours in a hybrid form (Gibbons & Perham 1974). But these are small changes in the folding of the individual polypeptide chain, dramatic though the results may be in terms of the biological activity of the oligomeric protein. It seems likely, therefore, that the denatured subunits of oligomeric enzymes will fold to something like their final structure before aggregating to give the native quaternary structure. The available evidence (Teipel 1972; Shifrin & Parrott 1974) would suggest that this is so. Indeed, there is a preliminary report that aldolase subunits that have been allowed to refold from a denatured state but prevented by chemical modification from aggregating to the native tetrameric form, exhibit enzymic activity (Chan, Kaiser, Salvo & Lawford 1974). This would be very strong evidence that a structure closely resembling the individual subunit in the native oligomer is an intermediate in the assembly process.

More complex structures

With yet more complex structures containing more than one type of polypeptide chain or components other than protein, stable intermediates in the assembly are widely recognized. Many such structures contain distinct, identifiable functions associated with clusters of protein subunits although these functions may be very closely integrated in the final particle. A simple example would be the two catalytic trimers and the three regulatory dimers in the aspartate transcarbamylase of *E. coli*, which can be separated and recombined *in vitro* (Schachman 1972). Similar considerations apply to more complicated structures, such as the 2-oxoacid dehydrogenases of *E. coli* which are considered below in more detail. It would be surprising if the assembly process did not take account of these preferred interactions although it must be emphasized that the pathway of disaggregation established *in vitro* and the pathway of assembly *in vivo* need not be the same. The principle of microscopic reversibility would need to be satisfied only if the two sets of conditions were identical.

On the other hand, preferred interactions in the final structure are not always evident, for example in the helical array of identical protein subunits that comprises the protein coat of tobacco mosaic virus. Yet stable intermediate aggregates play a prominent role in the self-assembly of the virus (Butler & Klug 1971) and good structural (and biological) reasons can be advanced for their existence. This point is also taken up in more detail below.

The types of quaternary structure permitted

Many structures we know to be capable of self-assembly have large numbers of protein subunits but the number of different types of subunit in any given structure is generally small. Many structures, indeed a majority if one takes into account the large number of oligomeric enzymes, contain only identical subunits.

Most simply, we can make large organized structures by packing identical subunits in identical environments so that the bonding pattern is the same for all subunits. The final structure is then necessarily a symmetrical one. This was first clearly recognized by Crick & Watson (1956) in their consideration of the possible structures of simple viruses. It has since been

developed and extended as a concept, most notably and elegantly by Klug and Caspar (Klug 1969; Caspar & Klug 1962).

For the purposes of this present discussion, let me briefly state some of the conclusions:

(1) Since biological structures are different from their mirror images, the only permitted spatial symmetry operations are rotations and translations. This implies that all such structures must in principle be represented by the enantiomorphic line, point, plane and space groups.

(2) Biological structures with line group symmetry are found in the helices. A helical arrangement of identical subunits is infinitely extendable if nothing acts to terminate it. It is therefore not likely to be favoured in biological systems, which deal in general with finite structures. I shall return to this point later in connexion with tobacco mosaic virus.

(3) For a particle of finite extent, the only symmetry possible is that of the point groups: the number of subunits is then a certain and definite integer. There are three types of point group

(*a*) Cyclic symmetry. The subunits are arranged head to tail in a circular array and the number of subunits is unrestricted (and can therefore be even or odd).

(*b*) Dihedral symmetry. A twofold axis can be combined at right-angles with any n-fold axis and the number of subunits, $2n$, must be even.

(*c*) Cubic symmetry. The three types are

(i) tetrahedral (23), which requires 12 subunits,

(ii) octahedral (432), which requires 24 subunits,

(iii) icosahedral (532), which requires 60 subunits.

(4) The plane and space groups describe structures that are theoretically of infinite extent in two and three dimensions and we therefore need not concern ourselves further with them here.

(5) Protein subunits are themselves complex molecules and not rigid geometric entities. A structure of lower free energy can therefore sometimes be arranged by systematically deforming the bonds between subunits in a number of slightly different ways so that a larger number of stable bonds can arise. The subunits are then no longer related by strict equivalence but are in quasi-equivalent environments. This concept of quasi-equivalence has been spectacularly successful in explaining the presence of more than the nominal maximum of 60 identical subunits in the protein shells of some icosahedral viruses (Caspar & Klug 1962).

Most oligomeric enzymes are dimers or tetramers of dihedral symmetry and the bigger ones all show dihedral or cubic symmetry (Valentine 1969; Green 1972). Making the reasonable assumption that multimeric structures have evolved from monomeric proteins, we may account for this by the fact that intersubunit bonds are generally hydrophobic in nature and probably arose by mutations to form a hydrophobic patch on the surface of the monomer. Hydrophobic interaction would then lead to symmetrical dimers which, by a similar process, could dimerize once more to yield tetramers. The primary importance of the symmetrical (isologous) bond is therefore clear (Monod, Wyman & Changeux 1965; Valentine 1969; Green 1972). Limited structures with point group symmetry can only arise if at least one of the intersubunit bonds is symmetrical (Green 1972).

These considerations of symmetry and closed structures are not at variance with our recognition of intermediates in the self-assembly of organized structures. Structures with dihedral and cubic symmetry formally possess three classes of intersubunit bond although only two classes are essential for the closed structure to cohere. Stable intermediates involving only one class of bond can therefore form and these in turn can aggregate by virtue of the second bond class to yield the final structure with a fixed number of subunits.

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The consequences of quaternary structure

Biological structures are the product of a long period of evolution and knowledge of the selection pressures on a given molecule through time is unavailable, although attempts to bring this subject within reach of experiment are now meeting with some success (Betz, Brown, Smyth & Clarke 1974; Rigby, Burleigh & Hartley 1974). In considering the consequences of quaternary structure for biological macromolecules, one must therefore beware the dangers of excessive reliance on teleological argument, the confusion of necessity with chance. However, with that reservation in mind, I should now like to pursue this theme some way since, at its worst I believe it offers a rationalization of much diverse information and, at its best, it brings new insights into biological organization (Monod 1969).

The principal consequences of the quaternary structure of enzymes are as follows:

(1) Each subunit retains its active site and the active sites remain independent.
(2) The active sites exhibit cooperativity.
(3) The specificity of the enzyme becomes changed.
(4) The reactions catalysed by individual subunits can form part of a coherent sequence, i.e. a multienzyme complex is generated.

Obviously the first and second consequences in this list are mutually exclusive but in many nzymes a mixture of properties will be observed.

Independent active sites

It is now clear that there exists a large class of oligomeric enzymes in which each subunit catalyses the enzymic reaction independently of its neighbours in the oligomer (Gibbons & Perham 1974; Holbrook & Gutfreund 1973; Knowles, Leadlay & Maister 1972). Why then do the subunits aggregate? Why do they not remain monomeric, as some enzymes do? The answer may lie in the fact that the stability of proteins in aqueous solution depends largely on the multiplicity of weak hydrophobic and other secondary interactions in the 'interior' of the protein. As a globular protein increases in size its volume grows more rapidly than its surface area, with a consequent increase in stabilizing interactions. There are two ways for a protein to increase in size; the polypeptide chain can grow longer or subunits can aggregate. Nature appears to have favoured the latter course and again one can advance good reasons for this. First, longer polypeptide chains demand longer genes to code for them: in due course this becomes too high a price to pay because of the limitations it will impose on the structure and activity of DNA or the chromosome (or both). Secondly, the longer a polypeptide chain the more likelihood there will be that a mistake in its biosynthesis will lead to an inability to fold correctly with a corresponding loss of enzymic activity. Formation of quaternary structure can conceivably provide an 'editing' step whereby incorrectly folded subunits are rejected. And, lastly, protein biosynthesis is an endergonic process. To make a protein bigger by lengthening the polypeptide chain can therefore be seen as a wasteful alternative to growing bigger by aggregation where that is possible.

It is also worth remarking that if a given amount of catalytic activity has to be packed into a cell, the osmotic consequences of introducing these polypeptide chains can be reduced by causing the chains to aggregate appropriately.

The argument developed above is supported by the fact that the monomeric enzymes tend to be extracellular and generally enjoy the extra stability conferred by disulphide bridges

whereas the oligomeric enzymes are intracellular and lack disulphide bridges (Monod 1969). Moreover, separated enzymically active subunits of the normally tetrameric rabbit muscle aldolase are more easily denatured than is the intact tetramer (Chan *et al.* 1974).

Cooperative effects

An adequate description of this phenomenon is impossible to encompass in a few words. Cooperativity has been widely observed between the active sites of oligomeric enzymes that consist of identical and non-identical polypeptide chains and two principal theories have been advanced to account for it (Monod *et al.* 1965; Koshland, Nemethy & Filmer 1966). Both rely on interactions between subunits and associated conformational changes in the oligomeric enzyme (for a review see Koshland (1970)). A molecular basis for cooperativity is available in some detail for haemoglobin (Perutz 1970), a tetrameric ($\alpha_2\beta_2$) structure. In the present context, it is worth recalling that the full quaternary structure of haemoglobin is necessary for cooperativity in oxygenation, the $\alpha\beta$ dimer being non-cooperative. Given our present knowledge of protein structure, it is difficult to envisage such interactions evolving in a single polypeptide chain with multiple catalytic sites.

The advantages of cooperativity for certain enzymes, in particular those concerned with the regulation of metabolic pathways, have been well documented (Newsholme & Start 1973). Most conspicuously, the response of such an enzyme to interactions with small molecules that govern its activity is greatly sharpened and the regulatory effect thereby enhanced. A notable example of the complexity a regulatory enzyme can attain is the aspartate transcarbamylase of *E. coli* where the catalytic and regulatory functions reside in distinct polypeptide chains, associated as trimers and dimers respectively within the enzyme (Schachman 1972).

Modification of enzymic specificity

Association of polypeptide chains can cause changes of enzyme specificity. Perhaps the most impressive example is that of the widespread enzyme UDP-galactose: *N*-acetylglucosamine galactosyl transferase, which catalyses the reaction

$$\text{UDP-galactose} + \textit{N}\text{-acetylglucosamine} \rightarrow \textit{N}\text{-acetyllactosamine} + \text{UDP}.$$

When this enzyme associates with the milk protein, α-lactalbumin, the synthesis of lactose can be catalysed as follows:

$$\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP}.$$

The synthesis of lactose uniquely in the mammary gland is a consequence of the presence there of the regulatory protein, α-lactalbumin. The structure of α-lactalbumin is remarkably similar to that of hen egg-white lysozyme and there can be little doubt that the two proteins have evolved from a common ancestor (Hill *et al.* 1972).

Less dramatic but none the less important changes in specificity can occur when the component enzymes of a multienzyme complex come together. An example of this is given in the following section.

Multienzyme complexes

The soluble multienzyme complexes of cells represent a novel answer to some of the problems of organization and control in intermediary metabolism (Reed & Cox 1970). In general, the complexes catalyse two or more reactions in a metabolic pathway and one can envisage several advantages conferred by aggregation of the component enzymes:

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(i) *Enhancement of catalytic activity*

Association of enzymes makes it physically easier for the product of one enzyme to become the substrate of the next. It may also be that the most effective conformations of the individual enzymes can only be arrived at by interactions in the complex, although whether this is because evolutionary change has been unable to achieve them for the separate subunits is obscure.

(ii) *Substrate channelling*

The advantage of proximity reaches a new level if the product of one enzyme passes direct to its successor without having to diffuse off and then on again. This mechanism implies that the substrate could be channelled through a series of reactions to avoid competition with other enzymes for the intermediate products. As a system of selecting the metabolic fate of the substrate, this has obvious attraction.

(iii) *The 'hot potato' hypothesis*

This somewhat fanciful title describes another possible reason for substrate channelling, namely, that an intermediate product in a reaction sequence might prove to be unstable in aqueous solution and therefore not survive the passage between active sites if diffusion were required.

Not surprisingly, most multienzyme complexes show a combination of these properties. As examples, I shall take tryptophan synthetase and the 2-oxoacid dehydrogenase multienzyme complex of *E. coli*, respectively one of the simplest and one of the most elaborate enzymes of this type. A full account of several other multienzyme complexes may be found elsewhere (Reed & Cox 1970).

Tryptophan synthetase (reviewed by Yanofsky & Crawford 1972) is a tetramer ($\alpha_2\beta_2$), the α- and β-chains being coded for by the two most distal genes of the multigene *trp* operon. The enzyme can be resolved into active α- and β_2-subunits and the reactions catalysed by the various forms of the enzyme are as follows:

(1) indole-glycerolphosphate $\rightarrow$ indole + glyceraldehyde 3-phosphate $\quad \alpha : \alpha_2\beta_2$

(2) indole + serine $\rightarrow$ tryptophan $\quad \beta_2 : \alpha_2\beta_2$

(3) indole-glycerolphosphate + serine $\rightarrow$ tryptophan + glyceraldehyde 3-phosphate $\quad \alpha_2\beta_2$.

The intact $\alpha_2\beta_2$ complex is about 100 times as active as the free α-subunit in reaction (1) and 10–50 times as active as the β_2-subunit in reaction (2). These enhancements can be attributed to the formation and stabilization of new subunit conformations by interaction in the complex (Faeder & Hammes 1971) and mutant α- and β_2-subunits that are themselves devoid of catalytic activity can fully activate the complementary subunit in this way. Similarly, side reactions of the free β_2-subunit, such as its ability to deaminate serine, are suppressed in the complex. Association proceeds through an $\alpha\beta_2$ intermediate and this has half the activity of the $\alpha_2\beta_2$ complex (Creighton 1970). And, finally, the tryptophan synthetase of *Neurospora crassa* has recently been demonstrated to channel indole very efficiently between the two active sites of the enzyme (Matchett 1974).

There are two 2-oxoacid dehydrogenase multienzyme complexes of *E. coli*, one specific for pyruvate and the other for 2-oxoglutarate (Reed 1974). Each is a multimeric structure about the size of a ribosome (30 nm across) that catalyses the reaction shown schematically in figure 1 and comprises three different types of polypeptide chain identified with E1, E2 and E3 respectively.

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The complexes can be resolved into their constituent enzymes and reassembled *in vitro*. The enzymes E1 and E2, a decarboxylase and a transacylase, are specific for their respective complexes and are not interchangeable, whereas E3, lipoamide dehydrogenase, from either complex can be used to assemble active complexes (Reed & Oliver 1968). There is now strong chemical evidence that the two lipoamide dehydrogenases are in fact identical (Brown, Harrison & Perham, unpublished work) and the product of a single structural gene (Guest & Creaghan 1973). Since E3 fulfills the same service function in both complexes (figure 1), this makes good sense for the economy of the cell and, in the present context, it is worth recalling that this principle of parsimony finds further expression in the existence of a common subunit in a number of pituitary hormones of widely differing specificity (Pierce *et al.* 1971).

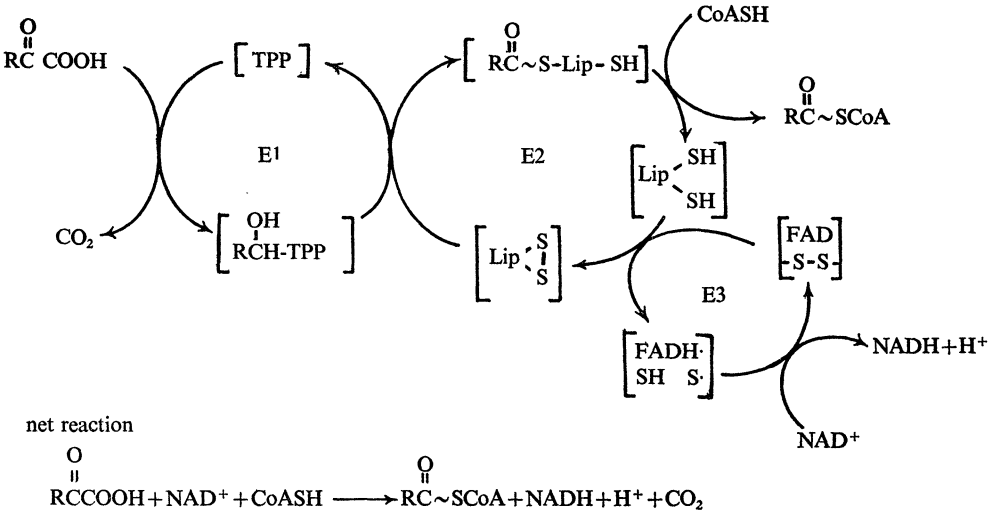

FIGURE 1. The reaction mechanism of the 2-oxoacid (pyruvate and 2-oxoglutarate) dehydrogenase multienzyme complexes of *E. coli*. E1 (a decarboxylase), E2 (a lipoyl transacylase) and E3 (lipoamide dehydrogenase) represent the three enzymic activities that make up the complex. The other abbreviations are: TPP, thiamine pyrophosphate; Lip, lipoic acid.

The transacylases evidently form a structural core for both enzymes since E1 and E3 do not associate with one another but do combine with E2 to yield intact complexes that closely resemble the native enzymes (Reed & Oliver 1968). The symmetry of E2 from the 2-oxoglutarate dehydrogenase complex has been established as octahedral (432) which, as we have seen, implies 24 subunits (DeRosier, Oliver & Reed 1971) although it has been pointed out that the same symmetry can be achieved with 12 polypeptide chains if each chain behaves as though it were composed of two independent packing units (Perham & Thomas 1971). The transacetylase of the pyruvate dehydrogenase complex is almost certainly of similar construction although it has been suggested, on the basis of estimates of molecular masses of the complex and of the subunits, that there are 16 polypeptide chains in the transacetylase (Vogel, Hoehn & Henning 1972). If further work reveals the symmetry of the transacetylase to be truly octahedral – its cube-like appearance in the electron microscope is not conclusive evidence – a structure based on 16 constituent polypeptide chains will necessarily be excluded. It should be noted here that the transacetylases of the pyruvate dehydrogenase complexes from mammalian

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sources are probably of icosahedral symmetry, which implies 60 subunits (Reed 1974). However, the lipoamide dehydrogenases from pig heart and *E. coli* do show amino acid sequence homology, indicating evolution from a common ancestor (Brown & Perham 1974) and lipoamide dehydrogenase from pig heart can substitute with reasonable efficiency for the bacterial enzyme in the assembly of active *E. coli* complex *in vitro* (Harrison & Perham, unpublished work). Despite the putative symmetry differences, the binding sites for E3 in the octahedral and icosahedral transacetylases must therefore have much in common.

The ratio of component polypeptide chains in the 2-oxoacid dehydrogenase complexes is also of particular interest. Although the number of protein subunits in E2 must remain fixed at 24 (or 12, see above) if it is to have octahedral symmetry, there is no agreement on the chain ratio of E1:E2:E3 in the pyruvate dehydrogenase complex. A ratio of 2:2:1 is favoured by Reed and his colleagues (Reed 1974) whereas a ratio of 1:1:1 has been proposed by Vogel *et al.* (1972) for a 'core' complex. Work in our laboratory (Bates, Brown, Harrison & Perham, unpublished work) is consistent with the observation that some preparations of the pyruvate dehydrogenase complex carry a molar excess of E1 compared with E2 (Vogel *et al.* 1972). In our hands, the ratio may rise as high perhaps as 2:1. Similarly, some preparations of the complex have less than a molar equivalent of E3. Since the overall complex reaction can be catalysed by complexes deficient (although not, of course, totally lacking) in both E1 and E3, it is evident that a structurally complete complex is not required for biological activity. The opportunity to vary the subunit composition of these multienzyme complexes within limits posed by symmetry marks them out as different from the simpler oligomeric enzymes where subunit composition is invariant. It raises interesting questions about the state of the complexes *in vivo* and the organization of the active sites of the component enzymes in the complex. On the other hand, some multienzyme complexes, e.g. fatty acid synthetases, contain only one copy of each component enzyme in the functional assembly and omission of any one would lead to loss of the overall activity.

Although the association of enzymes into complexes can be seen to offer a number of advantages for biological organization, it raises new problems of its own. In particular, there is the need to arrange for the biosynthesis of the different polypeptide chains in the appropriate amounts. This appears to have been achieved in two ways. First, in most microorganisms the genes for the various enzymes in a complex are closely linked in an operon and can therefore be placed under common control. More important, however, for the present Discussion is the observation that a putative event of gene fusion has occasionally led to the occurrence of a 'double-headed' enzyme, i.e. a polypeptide chain containing two different active sites. There is good evidence (Matchett 1974) for this phenomenon in the tryptophan synthetase of *Neurospora crassa*, a dimer instead of the more usual tetramer ($\alpha_2\beta_2$, see above), and for the aspartokinase-I: homoserine dehydrogenase-I of *E. coli* (Veron, Saari, Villar-Palasi & Cohen 1973). It has also been achieved experimentally in the histidine operon of *Salmonella typhimurium*: two frameshift mutations near the intercistronic region cause the original punctuation signals to be misread and the two polypeptide chains of enzymes 2 and 3 of the operon are then synthesized as one (Yourno, Kohno & Roth 1970). The polypeptide regions responsible for the different enzyme activities must, of course, retain the ability to fold independently but the folding domains found in immunoglobulins which probably arose by a comparable process of gene duplication and fusion (Amzel *et al.*, this Discussion) lend support to the proposal.

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Tobacco mosaic virus

I should now like to turn briefly to tobacco mosaic virus since it is both a helical structure and one of the most celebrated examples of a self-assembling system containing nucleic acid and protein (Caspar 1963). Moreover, the quaternary structure serves a new function, to package RNA.

The virus is rod-shaped and consists of a single strand of RNA winding in a helical groove between successive turns of the right-handed helical array of identical protein subunits (Finch 1972). In the absence of RNA, the coat protein aggregates at slightly acidic pH to form rods that closely resemble the virus but with one major difference: whereas the virus has a defined length of 300 nm, the protein rods have no fixed length and can grow well beyond 300 nm. As pointed out earlier, helical structures can in theory grow indefinitely although shearing forces in solution would be expected to prevent this happening. It has been suggested (Valentine 1969; Kellenberger 1969) that a helix might be terminated more specifically by growth occurring with increasing misalignment of inter-subunit bonds. Successive turns of the helix would be gradually less well bonded than the ones before and growth would eventually stop. Considerations such as these may have to be invoked for some helical structures such as flagella or microtubules. For tobacco mosaic virus, however, there is the additional component, RNA, incorporated into the final structure and it is the interaction between RNA and protein that serves to determine the length of the helix.

The mechanism of virus assembly has recently been analysed in some detail (Butler & Klug 1971; Klug 1972; Butler 1974). It has become clear that at neutral pH and moderate ionic strength – physiological conditions – the preferred aggregate of the protein is a two-layer disk with 17 subunits in each layer. The bonding pattern of subunits in the disk resembles that of subunits in the helix but the patterns cannot be identical. Virus assembly is nucleated by interaction between disks and the RNA at a specific sequence of bases at the 5′-end of the nucleic acid and can continue by sequential addition of further disks until the RNA is completely coated. At each addition, the two-layer aggregate of protein subunits must be rearranged into the characteristic helical form of the virus coat through interaction with the nucleic acid.

Several features of this assembly mechanism are of particular interest here. The two-layer disk is a limited structure despite the fact that it has no twofold axis perpendicular to the axes of the rings: it is limited by a pairing distortion between the rings that is relieved by interaction with the RNA (Finch & Klug 1974). The existence of the disk ensures that large helical protein aggregates cannot form under physiological conditions in the absence of the RNA and the initiation of assembly by interaction of RNA with disks makes possible the selection of the correct RNA by virtue of its base sequence at the 5′-end. And, finally, the addition of 34 protein subunits at a time (sufficient to coat 102 nucleotides) could enable short regions of unfavourable RNA-protein interaction to be overcome.

Variability in the interaction between RNA and protein along the virus rod has been inferred from the existence of intermediates in the process of virus degradation in detergent (Symington 1969) and at alkaline pH (Perham 1969; Perham & Wilson, unpublished work). These degradations proceed by sequential loss of protein subunits – presumably as monomers since there is no opportunity for disks to form – predominantly, if not exclusively, from one end of the virus. However, whereas degradation in detergent takes place initially from the 3′- to the

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5′-end of the RNA (May & Knight 1965), i.e. the reverse of assembly, degradation at alkaline pH proceeds principally from 5′- to 3′-end (Perham & Wilson, unpublished work). Thus, we have another example of the possibility of assembly and breakdown pursuing different pathways and it is worth remarking again that we do not offend against the principle of microscopic reversibility since the conditions for the two processes are not identical.

Concluding remarks

In this account of self-assembly I have restricted myself to systems where the structure we see *in vivo* can be shown to be capable of spontaneous assembly from its component parts *in vitro*. It is impressive and satisfying that we can usually account for the form of these structures by arguments from symmetry and, as a corollary, that symmetry arguments can provide us with a reliable means of choosing between alternative models for a given structure. The combination of techniques in protein chemistry, electron microscopy and X-ray diffraction is proving particularly powerful.

It is said of Metternich that when he was told of the eventual death of that arch political opportunist, Talleyrand, he commented, after due reflection, 'Now I wonder why he did that'. Teleological argument has its dangers but it is nevertheless encouraging that many of the properties of multimeric structures can now be interpreted in terms of the acquisition of quaternary structure and that this can be seen to be an economical solution to some of the problems of biological structure and organization.

Nucleation and intermediates have come to be recognized as important elements of many assembly and denaturation processes. This is true of the transient intermediates that determine the folding and unfolding pathways of simple proteins and of the stable intermediates in the assembly and breakdown of more complicated structures, such as multienzyme complexes and viruses. The intrusion of kinetics into protein folding means that the folded protein must now be regarded as the minimum Gibbs free energy form of the kinetically accessible conformations. Equally important is the realization that protein subunits are themselves complex molecules capable of quasi-equivalent bonding in aggregates, which can enable the rules of strict symmetry to be bent and more than the nominal number of subunits to be accommodated in the structure. Subtle changes in bond arrangements are the basis of the existence of the two-layer disk of coat protein subunits of tobacco mosaic virus, at first sight not a closed structure, and of its role in nucleation and incorporation into the growing helix during virus assembly. Similarly, in the polymerization of flagellin to form bacterial flagella, the same type of flagellin subunit can be utilized to form flagella of different wave-forms, the choice of wave-form being governed by that of the nucleating fragments of flagella that begin the filament (Oosawa, Kasai, Hatano & Asakura 1966), although under certain conditions different wave-forms can be observed in the same flagellum (Asakura & Iino 1972). The structure can therefore adopt a number of stable polymorphic forms that cannot differ greatly in free energy. Effects such as these add a new dimension to the considerations of symmetry and packing.

Finally, I have neglected structures that suffer modification after assembly. The simplest example would be the formation of the hormone, insulin: the hormone is synthesized and folds as a single chain precursor, proinsulin, from the interior of which a peptide is then cleaved by proteolysis to leave the well-known two-chain structure. In the absence of the connecting peptide, the denatured hormone reassembles poorly (Grant & Coombs 1970). Cleavage of

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proteins is observed in the assembly of some multi-component bacteriophages (Eiserling & Dickson 1972) and 'registration peptides' are discarded from the *N*-terminus of procollagen chains after they have nucleated the self-assembly of the collagen triple helix (Speakman 1971; Gallop, Blumenfeld & Seifter 1972). These observations lead on to the possible use of jigs and templates in the assembly of biological structures through the use of molecules that do not themselves appear in the completed structure. Interesting advances are being made in this direction (see, for example, Kellenberger 1972) and, when unravelled, it is likely that such systems will be seen as the evolutionary answer to the problem of making structures more complex than simple self-assembly will allow.

I am grateful to my colleagues (Mr D. L. Bates, Dr J. P. Brown, Dr R. A. Harrison and Mr T. M. A. Wilson) for permission to quote from our unpublished experiments and to the Science Research Council for financial support.

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