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Review

Symmetry in F_1-type ATPases

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Contents

I. Introduction

Early in the 1960s it became evident that knob-like protrusions of the inner mitochondrial membrane were catalytically involved in the formation of ATP from ADP and P_i [1,2]. These knobs – referred to as coupling factor 1 or F_1 – were later shown to be part of a multisubunit enzyme complex which is now usually called F_0F_1 H^+-translocating ATP synthase. A passive flow of protons through the membrane spanning F_0 subcomplex of the synthase is coupled to the synthesis of ATP at the substrate binding sites on F_1, thus converting the electrochemical potential difference of a transmembrane proton gradient into the standard metabolic energy currency. In this article, we shall focus on structural and functional symmetry relationships in the F_1 subcomplex. For other facettes of current F_0F_1 research, the reader is referred to a number of excellent reviews that have appeared in recent years [3–13].

Using conventional negative stain techniques, initial electron microscopic analyses of mitochondrial F_1 were able to visualize individual enzyme molecules as particles of apparently globular shape [2,14,15]. Details of the quaternary structure were not analysed, however. Later, it became known that F_1 contains five different types of subunit, which were called α, β, γ, δ and ε in order of decreasing molecular mass [16]. The issue of subunit stoichiometry of F_1 is a classical example of controversial experimental results and, as a consequence thereof, of persistently contradictory structural models. For a long time it was unclear whether the large subunits α and β were present in two or three copies each. As amino-acid side-chains of the β subunits contribute to the catalytic sites, the solution to this problem was of central importance for an understanding of the enzymatic mechanism as well.

$\alpha_3\beta_3\gamma\delta\varepsilon$ and $(\alpha\beta\gamma\delta\varepsilon)_2$ were the two major models for the subunit stoichiometry in F_1, and experimental evidence in favour of either of them was about equally

Abbreviation: P_i, inorganic orthophosphate.

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strong until the early 1980s (Refs. 16–20 in support of the former model, Refs. 21–26 in support of the latter). An $\alpha_2\beta_2\gamma_{1\text{-}2}\delta_{1\text{-}2}\varepsilon_2$ stoichiometry was championed in particular by investigators of CF_1, the respective enzyme that is isolated from thylakoid membranes of chloroplasts [25,26]. Reports on the apparent molecular mass of CF_1 seemed to support this view: a protein of 325 kDa [27] would simply be too small to accommodate more than two α and two β subunits. This value has proved false, however: it is now clear that the correct molecular mass of CF_1 is in the range of 400 kDa [28] to 420 kDa [29], and the value of 325 kDa may have reflected a fragmentation product. Moreover, as will be discussed below, it has been established in the meantime that for virtually all known ATPases of the F_1 type (including CF_1) the stoichiometry of the large subunits is $\alpha_3\beta_3$.

The ultimate determination of the three-dimensional structure of F_1 will rely on the knowledge of the primary structure of its subunits. Sequence data, obtained by both protein and DNA sequencing, are now available for subunits of F_1 from a variety of sources (compiled in Ref. 30). The established amino-acid sequences confirm the expected high inter-species homologies of the α and β subunits as well as a lower degree of homology of these subunits inter se. In *Escherichia coli,* the genes encoding the subunits of the ATP synthase are arranged in a single polycistronic operon [31]. All genes exist as single copies in the operon, even those coding for polypeptides such as α and β that are present in multiple copies in the oligomeric enzyme. Thus, all three copies of both α and β can be expected to be a priori identical. It is a major concern of current work on F_1 to determine the extent to which this equivalence is preserved in the quaternary assembly of the enzyme complex. Clearly, the answer to this question will be of great significance also with regard to the catalytic mechanism which is known to involve multiple substrate binding sites on the large subunits of F_1.

II. X-ray crystallography

In terms of resolving power, the potential of X-ray diffraction analysis of single crystals is still unmatched by any other contemporary technique for the determination of three-dimensional protein structures. However, with an oligomeric enzyme of close to 400 kDa, the technical obstacles are considerable. In particular, the crystallisation of such a complex protein poses severe problems. As a consequence, detailed X-ray studies have to date been undertaken only with single crystals of the rat liver mitochondrial enzyme [32]. Microcrystals of some other F_1 type ATPases [33,34] were not suitable for X-ray analysis.

Crystals of the rat liver enzyme were grown from an ATP- and P_i-containing buffer by dialysis against ammonium sulfate for several weeks [32]. They were described as rhombohedral (space group R32), and the molecular mass of the asymmetric unit was found to be 190 kDa. This result, together with the molecular mass of rat liver F_1 (384 kDa), was interpreted to indicate that the asymmetric unit contained just one half of the enzyme, and it was suggested that a 2-fold molecular symmetry axis coincided with a two-fold crystallographic symmetry axis of space group R32. (In contrast, it was later reported that crystals of F_1 from bovine heart mitochondria were orthorhombic, space group $P2_12_12$ for the predominant crystal form, and contained one molecule of F_1 per asymmetric unit [35].) The work on the rat liver enzyme was extended by X-ray diffraction analysis of the crystals and led to the conclusion that the molecule was formed by two crystallographically equivalent halves with each half being composed of three masses of approximately equal size [36,37]. The three masses were found to be neither equivalent nor symmetrically arranged; rather, they combine with the three masses of the other half to form a distorted hexagonal assembly which is thought to represent the complete F_1 [36].

As has been emphasized by the authors, these results are directly compatible with a dimeric stoichiometry of the kind $(\alpha\beta\gamma\delta\varepsilon)_2$. On the other hand, the presence of three α and three β subunits can be accommodated only after the implications of the crystallographic data have been relaxed [36,37]. Consequently, the authors postulate that neither the three α nor the three β subunits are structurally equivalent: they are thought to occupy positions that cannot be related by three-fold radial symmetry [37]. The authors have coined the term *chemical asymmetry* [38] to underline their view that the three α/β pairs reside in permanently different environments, and they have arrived at the conclusion that the chemical asymmetry may be the basis for a functional asymmetry of F_1 in the form of permanently different reactivities of the nucleotide binding sites [38]. However, the notion of a permanent structural asymmetry of the large subunits is in need of further substantiation as the resolution limit of the currently available X-ray data (0.9 nm) does not permit identification and localisation of individual subunits in the enzyme complex. Higher-resolution X-ray studies may become possible with crystals of the bovine heart mitochondrial enzyme which can now be grown to a sufficient size and homogeneity (Lutter, R. and Walker, J.E., personal communication).

III. Electron microscopy

The difficulties and uncertainties one commonly encounters when one tries to crystallise F_1 have prompted attempts to elucidate the three-dimensional structure of the enzyme by a number of other techniques, including

measurements of hydrodynamic properties, cross-link experiments, and electron microscopy. Most notably, a variety of electron microscopic techniques has contributed significantly to the elucidation of the quaternary structure of F_1 in recent years and will therefore be given prior consideration in the following.

F_1 preparations have been characterised by electron microscopy of negatively stained specimen [39–41]. In transmission micrographs the F_1 molecule appeared as a hexagonal particle, sometimes distorted, with or without an additional mass in the centre. Of course, such data are not sufficient to construct a three-dimensional model as a particle projection is two-dimensional and information about the relative projection angle is lost. This information can however be retained, at least in part, by tilting the specimen in the electron microscope under conditions of minimal beam exposure. Such tilting experiments have been performed with F_1 from bovine heart mitochondria [42] and, more recently, with F_1 from *Clostridium thermoaceticum* [43].

A three-dimensional model was established on the basis of tilting experiments with mitochondrial F_1 [42]. According to this model, the enzyme is composed of seven protein masses. Six of them are located peripherally and are of rather similar globular shape while the seventh mass, which will be discussed in detail below, is found in the central space that is surrounded by the peripheral masses. The six peripheral masses are arranged in two layers, each containing three identical masses at equivalent positions. The three masses in both layers are thus located at the vertices of equilateral triangles and are related by threefold radial symmetry. Both layers are stacked on top of each other and twisted by 60°. This type of three-dimensional mass distribution has been dubbed the *cyclohexane model* because of the similarity with the chair conformation of that molecule. In addition, it has been suggested (though not proved in Ref. 42) that F_1 contains three α subunits, confined to one of the layers, and three β subunits, confined to the other. In a two-dimensional projection, this arrangement would result in a symmetric pseudo-hexagonal pattern of alternating α and β subunits.

More recent tilting experiments with F_1 from *Cl. thermoaceticum* [43] and with CF_1 from spinach chloroplasts (Tiedge, H. and Lünsdorf, H., unpublished results) have shown that the overall structural organisation of F_1 from different eucaryotic organelles and from a phylogenetically ancient bacterium are closely related. The similarities are indeed eye-leaping as is illustrated by a gallery of F_1-type ATPases obtained from seven different sources (Fig. 1). The latest addition to this gallery, the F_1-like ATPase of the archaebacterium *Sulfolobus acidocaldarius* [44], demonstrates once again the universally conserved three-dimensional structure of this enzyme. As a note of caution, however, it should be added that negative stain techniques such as these find their limitations in the possibility that particles may become artifactually flattened before they can be observed in the electron microscope. Such a flattening may distort the overall dimensions and axial ratios of a particle projection. Indeed, a recent electron microscopic analysis of lyophilized and shadowed CF_1 ATPase (Kohring, G.-W., Schäfer, G. and Mayer, F., unpublished results) suggested axial dimensions (12 nm × 9 nm) that were slightly different from the earlier negative stain data. Eventually, the limitations of conventional negative stains may be overcome by using cryo-electron microscopy to observe unstained single molecules embedded in a layer of vitreous water. On the other hand, however, results that are generally compatible with the cyclohexane model have also been contributed by a number of techniques other than electron microscopy, including cross-link experiments [45,46], measurements of hydrodynamic properties [47], small-angle neutron scattering [48], and small-angle X-ray scattering [49].

The cyclohexane model is symmetric in the sense that a three-fold radial symmetry (pointgroup C_3) is characteristic of the distribution of both the α and the β subunits. This notion has been questioned by a second electron microscopic study of the bovine heart enzyme [50]. In one of several types of projection, the enzyme particles appeared as symmetric hexagons although the central mass was missing. It was nonetheless claimed that an averaged projection resulting from a computer assisted superposition of ten such particles was slightly asymmetric. There are two general objections to be raised against this and similar techniques of single particle averaging. (1) Prior to averaging, single particle

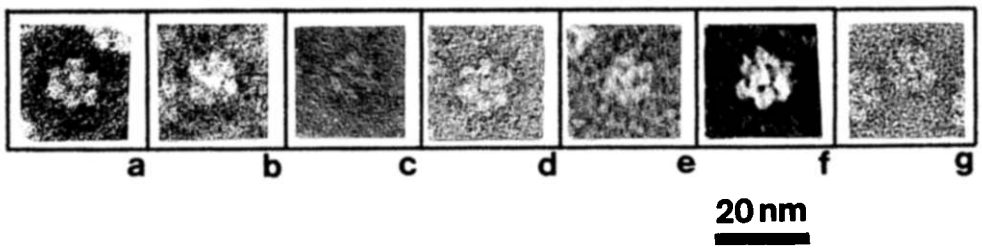

Fig. 1. Electron micrographs of F_1-type ATPases from seven different organisms. (a) CF_1 from spinach chloroplasts (Tiedge et al., Ref. 54); (b) F_1 from bovine heart mitochondria (Tiedge et al., Ref. 42); (c) F_1 from *Escherichia coli* (H. Lünsdorf et al., Ref. 51); (d) F_1 from *Rhodospirillum rubrum* (Strid, A., Lünsdorf, H. and Schäfer, G., unpublished); (e) F_1 from *Wolinella succinogenes* (Bokranz et al., Ref. 105); (f) F_1 from *Clostridium thermoaceticum* (Mayer et al., Ref. 43); (g) F_1-like ATPase from *Sulfolobus acidocaldarius* (Lübben et al., Ref. 44).

projections have to be selected from a field and to be classified according to arbitrarily chosen reference images. Thus, computer assisted averaging of single particles is by no means more objective than visual inspection of the field itself. (2) As stated above, no information is available about the relative projection angle of single particles in transmission micrographs. Consequently, as long as this information cannot be obtained in some other way, superposition of a mixed population of such particles will inevitably and falsely lump together different types of projection and may in this way generate an apparent asymmetry in the averaged projection even if the original particles were per se perfectly symmetric. It is thus concluded that the asymmetry (if there is any) in the averaged image in Ref. 50 is most likely due to an asymmetry of projection rather than to a genuine asymmetry of the projected particles.

More of a problem was the remaining discrepancy between the cyclohexane model and the model derived from the X-ray data. While the latter denies the possibility that the α and β subunits may be grouped as symmetric trimers, the former suggests that exactly this is the case. The issue was resolved by use of subunit-specific monoclonal antibodies in immuno-electron microscopy. The rationale of this approach was a direct visualisation of specific subunits by tagging them with monoclonal IgG molecules. Identical results were obtained with monoclonal antibodies directed against the α subunits of F_1 from *E. coli* [51,52] and those of CF_1 from thylakoid membranes [53,54]: (1) A maximum of three monoclonal anti-α antibodies was found to bind to the enzyme, a result which definitely ruled out any dimeric stoichiometry of the kind $(\alpha\beta\gamma\delta\varepsilon)_2$. (2) The antibodies were seen to bind symmetrically to the ATPase, yielding angles of 120° between them. These results clearly corroborate the symmetric cyclohexane model as the symmetry inherent in the immune complexes reflects a symmetric distribution of the major subunits in the enzyme. In summary, we believe that the model that is schematically illustrated in Fig. 2 adequately describes the juxtaposition of the large subunits in F_1. It should be noted once again that the juxtaposition of α and β subunits as revealed by immuno-electron microscopy is the same for a procaryotic and an organelle type eucaryotic enzyme.

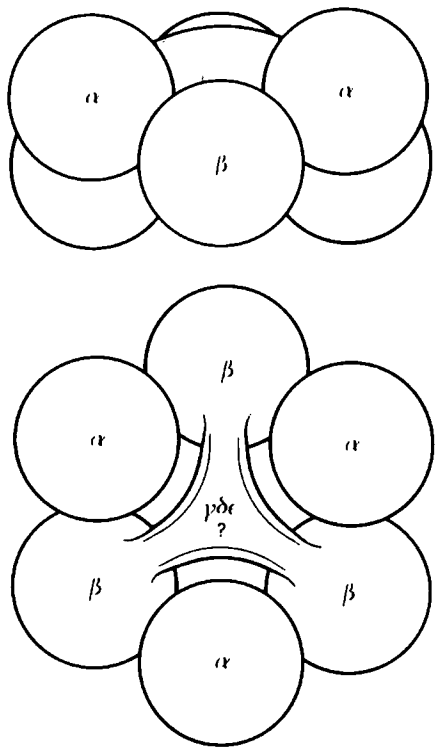

Fig. 2. Schematic representation of the subunit juxtaposition in F_1-type ATPases. The molecule is shown in a side-view projection (above) and in a front-view projection (below). The peripheral subunits α and β are drawn as spheres to emphasize their globular shape. As is indicated by the question mark in the centre of the molecule, composition and shape of the central mass are still conjectural. For details, see text and Refs. 42 and 54.

IV. The central mass

The question mark in the diagram of Fig. 2 not only denotes the centre of the molecule, but also indicates the current centre of uncertainty and speculation about the structure of this enzyme. Unclear is both the composition and the shape of the central mass. Even its existence, however, was disputed for a long time, as X-ray studies [36] as well as electron microscopic analyses [17,50,55] reported a central cavity devoid of protein. In contrast, a central mass was detected in a number of more recent electron microscopic studies (see Fig. 1 and references quoted in the legend), and with the possibility in mind that the central mass might have been averaged out in the X-ray study because it did not conform to crystal symmetry [38], it seems fair to say that its existence is now a well-established fact.

The peripheral masses being identified as α and β subunits, it appears most natural to think of the central mass as being composed of one copy of each of the minor subunits γ, δ, and ε. Although corroborated by isotope labelling experiments of the *E. coli* enzyme [56], evidence in support of this notion is incomplete and far from conclusive. A monoclonal antibody has been produced that is specific to the γ subunit of CF_1. In immuno-electron microscopic studies similar to those performed with the α and β subunits, it was found that no more than one monoclonal anti-γ IgG molecule would bind per molecule CF_1 (Lassen, A., Lünsdorf, H. and Tiedge, H., unpublished results). Also, in view of the size of the central mass (which is comparable to one of the peripheral masses) and of the upper limit of the molecular mass of the holoenzyme, it may seem safe to assume that no more than a single copy of γ contributes

to the central mass. Of course, this argument does not hold for δ and ε, and while it has indeed been reported that there might be more than one copy of the δ subunit in CF_1 [57], the exact stoichiometry of the minor subunits has yet to be established.

Unfortunately, the nomenclature of the minor subunits δ and ε has turned out to be inconsistent. While mitochondrial δ and bacterial ε are homologous proteins, there is no known counterpart of mitochondrial ε in bacterial F_1. Bacterial δ corresponds to the mitochondrial oligomycin sensitivity conferring protein (OSCP) which is, however, not copurified along with mitochondrial F_1 and is therefore usually not considered a subunit of that enzyme. Bacterial δ and ε also seem to be homologous to the respective subunits of CF_1. In any case, however, are these homologies weak as compared to the inter-species homologies of the α and β subunits [30], and they seem to be restricted to rather short stretches of the polypeptide chains.

Clues as to the location of γ, δ, and ε within the enzyme have come from electron microscopic studies. In an analysis with subunit deficient CF_1 [58], it has been demonstrated that a three-subunit enzyme (deficient of δ and ε) lacks the central mass, while a four-subunit enzyme (deficient of δ only) does not. Apparently, ε is needed to preserve the integrity of the central mass and to keep it in its proper position. This view is in line with the finding that ε can be cross-linked to a β subunit [59]. Electron microscopy with ferritin labelled mitochondrial F_1 has also suggested an interaction of γ and/or ε with one of the β subunits [60]. It remains to be established, though, whether this asymmetric reactivity reflects the individual character of a single β subunit or rather a transient functional state that can be assumed by any of the three β subunits. It has been suggested recently that the ε subunit may bind transiently to the carboxy-terminal domain of a β subunit and that, upon dissociation of ε from β, product release from a catalytic site on that β subunit may be accelerated [61].

With respect to both subunit composition and polypeptide sequences, the inter species diversity of the central mass appears to be much greater than that of the peripheral masses. It came as no surprise, then, that reports on the shape of the central mass were of a similar diversity. In the study of the mitochondrial enzyme [42] that led to the cyclohexane model for the peripheral subunits, the central mass was given a star-like shape with three protrusions radiating to the peripheral masses. While it was made clear that the number of these arms and the points of attachment to the peripheral masses were conjectural (see, for example, Fig. 1b, in which the central mass is of a rather bananoidal shape) it was noted that the central mass was slightly elevated, thus adding polarity to the enzyme complex (see also Fig. 2). Similarly, when CF_1 was analysed in the electron microscope, the central mass looked globular in a number of projections (see Fig. 1a) although Y-shaped forms are likewise prominent in the original micrograph [54].

In a later report about the mitochondrial enzyme [62], the central mass was described as having only two arms pointing to two neighbouring peripheral masses. However, as these results were based on single particle averaging with no information about the relative projection angles available, the aforementioned considerations apply here as well. More recently, the same group has used their averaging technique to reinvestigate the molecular shape of CF_1 [63]. The authors discussed the influence of particle tilting and uneven stain distribution on the appearance and symmetry characteristics of the averaged images. They described the central mass as a structure of variable shape, sometimes with two or three protrusions, sometimes broken down into two submasses. The arrangement of the peripheral masses was again compatible with the cyclohexane model.

Other investigators have applied computer assisted single molecule averaging techniques to analyse wild-type and subunit-deficient F_1-ATPases from *E. coli* and spinach chloroplasts [58]. Although the central mass appeared globular in their averaged reconstructions, these authors were careful not to claim that this had any relevance to the physical shape of that mass. Much like CF_1 or mitochondrial F_1, the central mass of the *E. coli* enzyme seems to be of variable shape; as with CF_1, both holo-F_1 [51] and a δ-deficient subcomplex [64] have been analysed using electron microscopic techniques. Finally, the central mass of F_1 from *Cl. thermoaceticum* has again a number of protruding spokes [43], and the authors suggested that each of the six peripheral subunits may be contacted by one of them.

What may be the reason for the admittedly bewildering variability of the central mass, even within the same species? There are at least three plausible possibilities: (1) The observed variability might result from experimental artifacts, due to a selective loss of subunits or some other damage that may occur during isolation and processing of the enzyme. Also, as has been stressed above, uncritical application of averaging techniques is clearly a potential source of artifactual results. (2) The central mass may be a Janus head with its two different faces looking up and down, respectively, along the enzyme's axis of pseudo-hexagonal symmetry. (3) As in a number of cases the enzyme was prepared for the electron microscope in a state of active ATP hydrolysis, the variability of the central mass may reflect conformational fluctuations occurring in the course of the catalytic cycle. However attractive possibilities (2) and (3) may appear to the biochemist, a solution of this problem has certainly to await new advances and refinements in electron microscopy and X-ray crystallography.

The central mass is most likely involved in the transmission of energy from the F_0 part to the catalytic sites on the peripheral subunits on F_1. Moreover, also a regulation of energy transmission and catalytic function has been fairly well documented for the minor subunits γ, δ, and ε of CF_1 from thylakoid membranes [65–67], of F_1 from *E. coli* [68], and of TF_1 from PS3 [69], respectively. Clearly, different demands for regulation in different organisms may at least in part explain the inter-species diversity of the central mass. Irrespective of its exact composition and shape, however, the central mass is remarkable in that it adds an element of asymmetry to the structure of F_1. At the level of polypeptide chains, the central mass must be assumed to lack radial symmetry. As a consequence, a priori equivalent peripheral masses may find themselves in non-equivalent environments as they contact different regions of the central mass. Hence, symmetry relationships between the major subunits α and β may be affected. It will be of relevance to our understanding of the functional mechanism of the enzyme to establish whether the asymmetry of the central mass actually results in a functional non-equivalence of the major subunits and, if so, whether this non-equivalence remains unchanged throughout the life time of an F_1 molecule, thus conferring individuality to the α and β subunits, or rather fluctuates or rotates in a state of active catalysis, thus retaining symmetry on an averaged time scale.

V. Functional implications

The conspicuous rosette-like design seems to be common to all F_1-type ATPases that have been described so far. Apparently, stringent mechanistic requirements made this design necessary and indispensable through evolution. Functional models have been put forward to illuminate these requirements in the context of the structural data. They may be classified as 'asymmetric' and 'symmetric' concepts depending on whether the three α/β pairs are assumed to display permanently different reactivities or not.

Asymmetric concepts postulate asymmetric locations and permanently different environments for the three – a priori identical – α/β pairs. This kind of structural non-equivalence means, according to this model, that the nucleotide binding sites on these subunits are of invariably different reactivities. Evidence in support of such a functional asymmetry has come from a number of different experimental approaches, including a variety of labelling and ligand binding studies.

NBD-Cl modification of F_1 ATPase results in the labelling of one β subunit and simultaneously in a complete inactivation of the enzyme [70,71]. Partial dissociation of the modified enzyme with 3 M LiCl followed by reassociation of the fragments restored the enzymatic activity to a significant extent [72,9]. The result of this 'scrambling' experiment was interpreted to indicate that only one particular β subunit, due to its special location or interaction with the central mass, is catalytically competent. This β subunit is, according to this model, the one that is labelled (and inactivated) by NBD-Cl. Upon 'scrambling', a non-labelled β subunit may end up occupying the 'special position', thus restoring enzymatic activity. In this model, the two β subunits in the non-competent positions are catalytically silent and may have a regulatory role. Alternative interpretations of the experimental data have been offered [8].

Similarly, asymmetric models were used to explain results of photoaffinity labelling studies [73,74]. In harmony with an earlier nucleotide binding site model [75], labelling data obtained with 8-azido adenine nucleotides [74] seemed to support the view that in addition to two catalytic sites which do not interact with each other the enzyme contained a set of non-catalytic nucleotide binding sites which were suggested to have a regulatory function. Also compatible with this model were more recent results from kinetic studies with CF_1 [76]. It was concluded that two sites participate in catalysis while a third, non-catalytic site may play some yet unknown regulatory role. This and other asymmetric models seem to share the notion that at least one of the three binding sites on the β subunits has a noncatalytic, possibly regulatory function [77]. However, the precise nature of this function has yet to be established.

Symmetric concepts assume that in spite of the asymmetry of the central mass, the catalytic sites operate nonetheless with equal potency during turnover. The *binding change hypothesis,* first formulated in 1977 [78] on the basis of earlier ideas [79], has evolved into a symmetric model par excellence. It was proposed that (1) product release, not product formation was the energy-requiring step [80], and (2) binding of ligands to one site might promote product release at a neighbouring site of the same class [79]. Although these suggestions had to face a fair amount of initial scepticism, supporting evidence has accumulated ever since. Thus, it has indeed been demonstrated that ATP formation at the active sites occurs with negligible changes in free energy [81]. It has also been shown, both with ATP and TNP-ATP as substrates [82,83] and with isolated F_1-ATPase and membrane bound F_1 [84] as enzymes, that the overall rate of catalysis is drastically increased when more than one binding site is occupied by the substrate. Also, when the release of ligands from the enzyme was monitored directly, it was found that binding of a ligand to one site did indeed accelerate the release of ligand from a neighbouring site of the same type [85]. Moreover, fluorescence energy transfer studies with CF_1 have shown that nucleotide binding sites on the β subunits indeed switch properties during ATP hydroly-

sis [86]. Thus, the general picture emerges that anticooperative ligand binding is expressed as positive cooperativity in catalysis.

While the earlier *alternating site model* relied on two catalytic centres [87], it was suggested in 1982 that the enzyme may work with a repertoire of three catalytically competent nucleotide binding sites and that each of these sites may in turn be recruited for the different stages of the catalytic process [88–90]. Thus, it became now apparent that the threefold radial symmetry of the α/β pairs might indeed be of significance for the catalytic mechanism. Meanwhile, the existence of three anticooperatively interacting substrate binding sites has been confirmed by a variety of ligand binding studies [85,91,92]. Accordingly, the symmetric model assumes that three active sites are equipotent in that they contribute equally to the catalytic process, proceeding successively through the three catalytic stages of substrate binding, product formation, and product release. Although the dissociation of products is the energy requiring step in catalysis, input of protonic energy will not only render one site competent for product release but simultaneously switch the two adjacent ones to a state of active substrate binding and product formation, respectively. There are no regulatory sites in this model as coordination and modulation of the catalytic sequence is achieved, possibly under the auspices of the central mass, by binding of ligands to the catalytic sites themselves.

Does the *three-site binding change mechanism* operate on a random or a sequential, i.e., cyclical basis? To answer this question, a number of experimenters have tried to establish whether cooperative multisite catalysis required the continual participation of all three sites in such a way that inactivation of only one site would inactivate catalysis at the other two sites as well, or whether, in contrast, inactivation of one site would leave the two remaining sites unaffected in their catalytic cooperativity. Two strategies have been used to create an enzyme with one-third of the catalytic centres modified. (1) A variety of nucleotide analogues was reported to inactivate catalysis completely after incorporation of 1 mol analogue per mol enzyme [92–97]. (2) Hybrid *E. coli* F_1, reconstituted from mutant and wild-type subunits and containing an average of one-third mutant α [98] or one-third mutant β [99], catalysed ATP hydrolysis at rates that were significantly lower than the rate for multisite catalysis at two sites. Therefore, it may be justified to conclude that the enzyme cannot tolerate an incapacitation of one out of three catalytic sites because this would disrupt the strictly sequential turnover cycle.

We suggest calling the catalytic sites *equipotent* to indicate that any of these sites can perform any functional step of the catalytic cycle and is compelled to do so in a manner strictly coordinated and synchronised with the other sites. Although this concept may provide the most coherent explanation for the vast majority of experimental data, it should, at least for the present, not be taken as anything more than a working hypothesis to direct future experiments. Furthergoing speculations are nonetheless tempting. It has occurred to a number of investigators, by apparent analogy with the bacterial flagellar motor and some man-made machines, that a physical rotation of different subunits of the F_0F_1 complex with respect to each other might provide the basis for a genuine rotational catalysis [88,100–102]. Supporting evidence is not entirely convincing, however, and the crucial experiment, reversible inhibition of rotation using cleavable bifunctional agents to crosslink one of the peripheral subunits with the central mass, has produced contradictory results [103,104]. It could be imagined, however, that a finely tuned job rotation of the catalytic sites may be superior to conventional catalysis in achieving optimal reaction rates. Although entirely conjectural, this view may offer a plausible answer to the question why the enzymatic machinery of F_1 has to be of such an extraordinary complexity.

Note added in proof (Received 14 September 1989)

Two papers on the molecular structure of *Escherichia coli* F_1-ATPase were published after this manuscript went to press [1,2]. Another recent publication [3] is elucidating the evolutionary relation between F_1-ATPases, archaebacterial ATPases and eucaryotic vacuolar ATPases.

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