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The Number of Functional Catalytic Sites on F_1-ATPases and the Effects of Quaternary Structural Asymmetry on Their Properties

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Abstract

Recent structural and kinetic studies of F_1 and F_0F_1 are reviewed with regard to their implications for the binding change mechanism for ATP synthesis by oxidative phosphorylation and photophosphorylation. It is concluded that at least two and probably all three of the catalytic sites on F_1 are functionally equivalent despite permanent structural asymmetry in the soluble enzyme. A rotary mechanism in which all three catalytic subunits experience all possible interactions with the single-copy subunits during turnover is thought not to apply to soluble F_1 but remains an attractive model for the membrane bound enzyme.

Key Words: Oxidative phosphorylation; photophosphorylation, binding change mechanism.

Introduction

The membrane-embedded ATP synthase, F_0F_1, found in mitochondria, chloroplasts, and bacteria couples proton translocation to ATP synthesis during oxidative phosphorylation and photophosphorylation. Catalytic sites for ATP synthesis are located on the F_1 component. F_1 can be readily solubilized, but when unplugged from the transmembranous proton current, it catalyzes the hydrolysis of ATP.

The binding change mechanism (Boyer, 1979 and 1985; Senior and Wise, 1983; Cross *et al.*, 1984) has found widespread use as a working model for the mechanism of ATP synthases. The main features of the proposal are:

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(1) that ATP synthesis occurs spontaneously at catalytic sites on F_1 with the major energy-requiring step being a conformational change necessary to convert the catalytic site from a form with very high affinity for ATP to a form with low affinity (Boyer *et al.*, 1973), (2) that substrate binding, as well as product release, is associated with the energization step (Rosing *et al.*, 1977), and (3) that substrate binding and product release occur simultaneously on separate but interacting catalytic subunits (Kayalar *et al.*, 1977). In this review, recent structural and kinetic evidence relating to various aspects of the binding change mechanism will be discussed. These include the number of functional catalytic sites, the order in which they participate in catalysis, and the effects of quaternary structural asymmetry on catalysis by F_1 and F_0F_1.

The Number of Functional Catalytic Sites

Chemical modification studies have provided evidence that catalysis is associated with the β subunit of F_1. The subunit structure of F_1 from mitochondria (MF_1), chloroplasts (CF_1), and bacteria (BF_1) appears to be $\alpha_3\beta_3\gamma\delta\varepsilon$. The three copies of β are encoded by a single gene, so they are structurally identical. However, as will be discussed in a later section, the β subunits may be conformationally heterogeneous due to the presence of single copies of subunits lacking threefold symmetry (Walker *et al.*, 1985). Hence, various models have been proposed for F_1 that invoke one, two, or three functional catalytic sites.

Evidence for a Single Functional Site

In recent years, Wang and Hammes and their coworkers have been the leading proponents of a one-site model. Wang's proposal that F_1 has a single functional catalytic subunit (β') and two latent catalytic subunits (β'') is based mainly on studies of Nbf-Cl[2] modification of β-Tyr-311 on MF_1 (Andrews *et al.*, 1984; Sutton and Ferguson, 1985). The stoichiometry for inhibition of ATPase activity is 1 mol of reagent per mole of MF_1, and strong negative cooperativity in the modification reaction makes Nbf-Cl behave as a 1/3-of-the-sites reagent (Ferguson *et al.*, 1975). These results have been interpreted

[2]Abbreviations: Nbf-Cl, 7-chloro-4-nitrobenzofurazan; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)-adenosine 5′-triphosphate; FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; DCCD, *N,N′*-dicyclohexylcarbodiimide; FSBI, 5′-*p*-fluorosulfonylbenzoylinosine; PLP-ADP, pyridoxal-5′-triphospho-5′-adenosine; NAB-GTP, 3′,2′-O-(2-nitro-4-azidobenzoyl)guanosine-5′-triphosphate; BzATP, 3′-O-(4-benzoyl)benzoyl ATP; lucifer yellow, 4-amino-*N*-[3-(vinylsulfonyl)phenyl]naphthalimide-3,6-disulfonate.

by Wang as being due to the modification of the single active β' subunit, while the two latent β'' subunits remain resistant to modification. Based on this interpretation, Wang predicted that treatment of Nbf-modified MF_1 with 3 M LiCl to cause partial dissociation and reassociation of β from the asymmetric core would result in reactivation of the enzyme since Nbf-β might reassociate in the latent β'' position while unmodified β might associate in the active β' position. The predicted reactivation was observed (Wang, 1985). Furthermore, the reactivated enzyme was shown to be sensitive to further modification and inactivation by Nbf-Cl (Wang *et al.*, 1986).

Although these results are by themselves well accommodated by the model proposed by Wang, alternative interpretations consistent with catalysis at multiple sites are possible. For instance, the LiCl treatment may increase the structural flexibility of the β-subunit allowing Nbf-Tyr-311 to assume a more favorable orientation. Such a reorientation might allow resumption of catalysis by removing the Nbf moiety from the catalytic site. In addition, if the presence of this nucleotide analog at one catalytic site originally rendered the other two sites resistant to modification through ligand-induced negative cooperativity, then removing the Nbf moiety from the site would be expected to cause the other sites to regain their susceptibility to the reagent. It should be noted that although β-Tyr-311 appears to be at or very near the catalytic site of MF_1 (Wu *et al.*, 1987), a site-directed mutagenesis study of the equivalent residue on the *E. coli* enzyme has shown that it is not essential for activity (Parsonage *et al.*, 1987).

Hammes' laboratory has characterized three nucleotide binding sites on CF_1 (Hammes, 1983). Based on the slow rate of exchange of nucleotide bound at two of the sites, Hammes concluded that only the single fast-exchanging site was a normal catalytic site. Evidence obtained in other laboratories suggesting strong positive catalytic cooperativity between multiple sites was interpreted as being due to a promotive effect resulting from reversible binding of nucleotide at noncatalytic sites. Recently, however, Leckband and Hammes (1987) have reported that the slow rate of dissociation of an endogenous ADP from one of the sites occurs at the same rate as an activation process. They further demonstrated that during steady-state catalysis, exchange of nucleotide at this site is as fast as the turnover rate. On the basis of these results, they concluded that there is in fact more than one catalytic site capable of rapid turnover, but they suggest that the sites might not be equivalent. The presence of multiple catalytic sites on CF_1 is in agreement with earlier results obtained in Boyer's laboratory (Kohlbrenner and Boyer, 1983; Feldman and Boyer, 1985; Xue *et al.*, 1987), but functional heterogeneity among the catalytic sites is inconsistent with substrate-modulated intermediate $P_i \rightleftharpoons H_2O$ exchange data which show that the sites behave as a homogeneous population (Kohlbrenner and Boyer, 1983).

Evidence for Two Functional Sites

Extensive studies in Boyer's laboratory of the oxygen exchange reactions catalyzed by soluble and membrane-bound F_1 have provided evidence that substrate binding at one catalytic site promotes product release from an adjacent interacting site during both ATP synthesis and hydrolysis (Boyer, 1985). Under conditions for ATP hydrolsis by MF_1, the importance of cooperative interactions between multiple sites has been quantitated. ATP binding accelerates the rate of ADP and P_i dissociation by approximately one-million fold (Cross *et al.*, 1982). Using the ATP analog TNP-ATP, Grubmeyer and Penefsky (1981) directly demonstrated the presence of at least two interacting sites capable of hydrolyzing substrate.

Some investigators in the field believe there are no more than two functional catalytic subunits. From X-ray crystallographic data, Amzel and Pedersen and coworkers conclude that the single-copy subunits of F_1 interact specifically with one $\alpha\beta$ pair rendering that β nonfunctional (Amzel *et al.*, 1982). On the basis of their finding that FSBA acts as an all-of-the-sites reagent, Allison's laboratory proposed that the catalytic subunits of F_1 function independently rather than cooperatively (Esch and Allison, 1979; Bullough and Allison 1986a). In addition, since they found DCCD to be a 2/3-of-the-sites reagent with F_1 from a variety of sources, they concluded that only two of the three copies of the catalytic subunit were functional (Yoshida *et al.*, 1982). These conclusions, however, were based on the premise that DCCD and FSBA reacted with residues located at the catalytic site. This has proven not to be the case. FSBA modifies a tyrosyl residue that has been shown to be present at noncatalytic nucleotide binding sites (Cross *et al.*, 1987). Taking advantage of the very high specificity of noncatalytic sites for adenine, Allison's laboratory introduced FSBI as a catalytic-site-specific affinity probe (Bullough and Allison, 1986b). The incorporation of only 1 mol of this reagent per mole of enzyme was found to be sufficient for complete inhibition. This led Allison's group to conclude that catalytic sites are indeed interactive; however, they continue to favor the presence of only two normal sites (Bullough *et al.*, 1987).

Evidence for Three Functional Sites

Based on our finding that three out of a total of six adenine nucleotide sites on MF_1 exchange rapidly with medium nucleotide (Cross and Nalin, 1982), we proposed a model for the binding change mechanism that included three functional catalytic sites (Cross, 1981). In support of this model, evidence was presented that MF_1 is capable of catalysis in three different modes, namely, where one, two, or three sites function simultaneously (Cross *et al.*, 1982). Uni-site catalysis was measured under conditions

where substrate was added in substoichiometric amounts to the enzyme. The affinity of ATP at the first site to bind substrate is exceptionally strong ($K_d = 10^{-12}$ M), but the rate of catalysis is very slow ($V_{max} = 10^{-3}\,s^{-1}$) and limited by the rate of product release (Grubmeyer *et al.*, 1982). As ATP concentrations are increased, substrate binding at a second site promotes product release from the first site to the extent that catalysis can be measured by a coupled enzyme assay. Kinetic plots of such measurements are biphasic, yielding two K_m's in the micromolar range (Cross *et al.*, 1982). These were interpreted as being due to turnover at two sites (bi-site catalysis) or three sites (tri-site catalysis).

Other laboratories, however, have reported triphasic kinetics for ATP concentrations in the micromolar range. Hatefi's laboratory reports three K_m's (Wong *et al.*, 1984) while Boyer and coworkers report two K_m's plus a $K_{0.5}$(ATP), which characterizes a transition between high and low activity for the intermediate $P_i \rightleftharpoons H_2O$ oxygen exchange reaction (Gresser *et al.*, 1982).

The discrepancy between these results and ours would appear to have two possible explanations. The first would be that the tight binding site for ATP characterized under conditions for uni-site catalysis does not exist during steady-state turnover or that it is a property of a unique catalytic site which does not contribute in any significant way to multisite catalysis. Evidence both for (Bullough *et al.*, 1987) and against (Cross *et al.*, 1984; Wu and Boyer, 1986) this explanation has been published. Alternatively, kinetic measurements made using micromolar concentrations of ATP might reflect only bi-site and tri-site catalysis. One of the three K_m's measured by Wong *et al.* (1984) could reflect a transition between low- and high-activity forms of F_1 obtained with the filling of a single vacant noncatalytic nucleotide site that is present on native MF_1 (Kironde and Cross, 1986). The $K_{0.5}$ measured by Gresser *et al.* (1982) for the oxygen exchange reaction might reflect a kinetic partitioning between resynthesis of ATP at the catalytic site, which is required for exchange, and substrate-promoted product release, which terminates the exchange.

So How Many Sites Are There?

In light of the evidence favoring cooperative interactions between a minimum of two functional catalytic sites, one-site models would appear difficult to defend. Although the intriguing results obtained with Nbf-modified MF_1 by Wang's laboratory are not predicted by multisite models, they can perhaps be accommodated as discussed above. With regard to the question of whether there are two or three functional catalytic sites, the answer may be either depending on the conditions. As will be discussed

in connection with the rotary mechanism, all three catalytic subunits of membrane-bound F_1 may pass through identical states during turnover. An artifact of stripping F_1 from the membrane may be to induce permanent structural asymmetry which persists even during catalysis. This in turn may or may not render one of the catalytic sites nonfunctional or nonequivalent. Although a number of laboratories, including our own, favor the view that all three sites are functional even on soluble F_1, it seems clear to us that this has not yet been firmly established and that additional experiments will be necessary.

The Order of Turnover of Sites during Multisite Catalysis

If there are only two functional interacting catalytic sites per F_1, then the question of the order of participation of the sites is a trivial one since they will alternate. However, if there are three functional interacting sites, then the order of their participation may be sequential or random.

A number of laboratories have attempted to discriminate between a sequential or random order for a three-site model. One approach has been to determine the minimum number of catalytic sites which must be derivatized in order to inhibit multisite catalysis. If the order is strictly sequential, then modification of a single site per F_1 should suffice to inhibit all three sites. However, if the order is random, then the modification of one site may allow the remaining two sites to alternate in rapid bi-site catalysis. In this case, the incorporation of a second mole of reagent per mole of F_1 would be required to inhibit the strong positive catalytic cooperativity obtained when substrate binding at one site promotes product release from a second site. Inhibition of multisite catalysis can be measured as the inhibition of activity measurable by a coupled-enzyme assay, since any remaining uni-site activity would be too slow to detect by such a method. A strict requirement for this type of experiment is that the modifying reagent must exhibit high specificity for the catalytic site and it must completely inactivate the site once incorporated. Several nucleotide analogs, used under the proper conditions, appear to satisfy these requirements. 2-Azido-ATP (van Dongen *et al.*, 1986; Cross *et al.*, 1987), FSBI (Bullough and Allison, 1986b), NAB-GTP (Kozlov *et al.*, 1985), PLP-ADP (Noumi *et al.*, 1987), and BzATP (Ackerman *et al.*, 1987) all require the incorporation of a single mole per mole of F_1 when the data are extrapolated to 100% inhibition.

An alternate approach, based on a similar rationale, has been to reconstitute hybrid F_1 containing mutant and wild-type subunits. Noumi *et al.* (1986) reported that a hybrid of *E. coli* BF_1 containing 1/3 mutant and 2/3

normal β-subunits showed the same very low activity as that seen with the homologous mutant enzyme. Another hybrid constructed using 1/3 mutant ad 2/3 normal α-subunit showed significantly depressed rates for multisite catalysis (Rao and Senior, 1987).

In each of these experiments, the presence of a single defective catalytic site prevented multisite catalysis by the two remaining catalytic sites. The following are possible interpretations of these data: (1) F_1 has three functional catalytic sites which turn over in a strictly ordered sequence; (2) F_1 has three functional catalytic sites which normally turn over in a random order; however, the presence of one modified or mutated catalytic unit per F_1 prevents a conformational change required for bi-site catalysis by the remaining two catalytic sites; (3) F_1 has only two functional catalytic sites, and the impairment of either blocks multisite catalysis.

In order for interpretation (3) to be correct, the incorporation of the affinity probe into the nonfunctional catalytic subunit would have to be excluded or the stoichiometry for inhibiting bi-site catalysis would be greater than one. Similarly, in forming the hybrid, the mutant subunit would have to bind to the asymmetric core exclusively in a "functional" position. One can reason against interpretation (2) on the basis that 2-azido-ATP very closely resembles the natural substrate. Hence, if the reaction order were random, modification of a single β-subunit by this substrate analog would not be expected to cause it to assume an abnormal or highly restricted conformational state that would prevent the other two sites from functioning. For these reasons, the first interpretation is favored, but again additional experiments will be needed to settle this question to everyone's satisfaction.

Structural Asymmetry and Rotational Catalysis

In order to accommodate the presence of three copies of each of the large subunits of MF_1 with the two-fold symmetry which they observed for their X-ray crystallographic structure at 9 Å resolution, Amzel *et al.* (1982) proposed that the single-copy subunits interact with one specific $\alpha\beta$ pair to give an $\alpha_2\beta_2X_2$ structure at this level of resolution. They further suggested that the asymmetry of F_1 might account for negative cooperativity in ligand binding and for various kinetic properties of the enzyme. Image processing of electron microscopic images of F_1 appears to confirm the asymmetric association of $\gamma\delta\varepsilon$ with $\alpha_3\beta_3$ (Boekema *et al.*, 1986).

Additional evidence for structural asymmetry comes from chemical modification studies. Tommasino and Capaldi (1985) found that the β subunit which can be cross-linked to the single ε-subunit is not labeled by DCCD

even when 2 mol are incorporated into β subunits per mole of BF_1. Stan-Lotter and Bragg (1986) found that this unique β-subunit also does not react with Nbf-Cl or thiol reagents. After labeling a single α-subunit on CF_1 with lucifer yellow, McCarty and coworkers determined distances between the probe and other loci on the enzyme by fluorescence transfer measurements (Nalin *et al.*, 1985). Their results showed that a unique α-subunit was labeled by lucifer yellow. We found that the three noncatalytic nucleotide sites on MF_1 behave asymmetrically in that a single site undergoes rapid exchange in the presence of EDTA. More significantly, we found that after approximately one thousand catalytic turnovers per MF_1, the same noncatalytic site retains the capacity for rapid EDTA-induced exchange. This indicates that the asymmetric determinants are permanent and that the three noncatalytic sites on soluble F_1 do not pass through identical states during catalysis (Kironde and Cross, 1987).

An important question relative to the binding change mechanism is how can multiple catalytic subunits pass through equivalent states during catalysis when it appears that they have unique interactions with other subunits? Several interesting answers to this question have been offered. The first is that the catalytic subunits are not equivalent. According to this view, only one or two of the catalytic subunits are functional at any one time. By analogy, this might be referred to as the "untuned engine model." The engine has three cylinders, but only one or two of them are capable of firing. Evidence against a one-site model has already been discussed, and the two-site model really does not solve the symmetry problem. This is because the two functional subunits would see the nonfunctional subunit differently. If the asymmetric determinants had sufficient effect to render one catalytic subunit nonfunctional, they might be expected to cause the two functional subunits to show nonhomogeneous kinetic properties. Attempts to demonstrate this by measuring the effects of ATP concentration on substrate modulation of the intermediate $P_i \rightleftharpoons HOH$ oxygen exchange have failed. The distribution of oxygen incorporated into the product shows a smooth transition, demonstrating that regardless of whether there are two or three functional catalytic sites, they behave in a homogeneous manner (Boyer, 1985).

For these reasons, we have offered a second possible explanation (Kironde and Cross, 1987). That is that the permanent structural asymmetry of soluble F_1 has no detectable effect on the three catalytic subunits during steady-state turnover. By analogy, this might be referred to as the "loaded wheel-of-fortune model." The wheel is divided into three numbers and, while spinning, no difference in turnover of the numbers can be detected. The effects of the asymmetric distribution of weight can only be seen as the wheel comes to rest, always on the same number. In this model, the numbers on the

spinning wheel represent catalytic sites passing through the various stages of catalysis. The sites turn over in a homogeneous manner despite asymmetry in the structure of the enzyme.

A third explanation is that during catalysis, β-subunits and perhaps other subunits which are present in multiples of three copies rotate relative to the rest of the subunits, so that after one complete enzyme cycle, all β will have experienced all possible interactions with the other subunits (Gresser *et al.*, 1982; Melese and Boyer, 1985; Cox *et al.*, 1984, 1986). During ATP synthesis, this rotary motion would couple the completion of a transmembrane proton channel to the conformational changes required for substrate binding and product release. A further feature of this model is that it would require participation of the three catalytic sites in a strictly ordered sequence.

Attempting to test the rotary model, Musier and Hammes (1987) have cross-linked the γ-subunit of soluble CF_1 to α- and β-subunits. They find that the level of inhibition of the enzyme is less than would be expected if rotation were required for catalysis (for an alternative view see Kandpal and Boyer, 1987). In addition, EcE_1 with α- and δ-subunits cross-linked shows full activity (Tozer and Dunn, 1986; Bragg and Hou, 1986). Finally, the rotary model does not explain the permanent asymmetry among noncatalytic nucleotide sites on MF_1. These sites are located at least in part on the β-subunit (Cross *et al.*, 1987). For these reasons we prefer the loaded wheel-of-fortune model for soluble F_1. However, a rotary mechanism remains an attractive possibility for the membrane-bound enzyme. A biological precedent for using an electrochemical gradient to drive rotation and conformational change is provided by the bacterial flagellar motor. The permanent asymmetry seen with soluble F_1 may be an artifact of separating it from F_0 subunits required for rotary motion. Hence attempts to test the rotary mechanism by cross-linking F_1 subunits should include measurements of ATP synthesis by reconstituted membranes.

Future Prospects

Over the next few years, intensive efforts in many laboratories are likely to provide answers to most of the questions raised in this review as well as to clarify the role of noncatalytic nucleotide sites, to define the path of protons through F_0, and to reveal the three-dimensional structure of F_1. However, the exact details of how proton movement through a membrane channel is coupled to the binding changes required for ATP synthesis is likely to remain a stimulating challenge to investigators in the field for some time to come.

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