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MINI-REVIEW

Chemical Modification of Active Sites in Relation to the Catalytic Mechanism of F_1

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Abstract

Recent studies of chemically modified F_1-ATPases have provided new information that requires a revision of our thinking on their catalytic mechanism. One of the β subunits in F_1-ATPase is distinguishable from the other two both structurally and functionally. The catalytic site and regulatory site of the same β subunit are probably sufficiently close to each other, and the interaction between the various catalytic and regulatory sites are probably sufficiently strong to raise the uni-site rate of ATP hydrolysis by several orders of magnitude to that of promoted (multi-site) ATP hydrolysis. Although all three β subunits in F_1 possess weak uni-site ATPase activity, only one of them (β') catalyzes promoted ATP hydrolysis. But all three β subunits catalyze ATP synthesis driven by the proton flux. Internal rotation of the $\alpha_3\beta_3$ or β_3 moiety relative to the remainder of the F_0F_1 complex did not occur during oxidative phosphorylation by reconstituted submitochondrial particles.

Key Words: F_1-ATPase; chemical modification of active sites; affinity reagent; submitochondrial particles; ATP hydrolysis; oxidative phosphorylation.

Introduction

Several comprehensive reviews of the chemical modification of F_1-ATPases have been published recently (Vignais and Lunardi, 1985; Satre *et al.*, 1986; Schäfer *et al.*, 1986; Allison *et al.*, 1986). The present mini-review will include only those chemical modification studies that have direct implications concerning the catalytic mechanism.

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Among the proposed catalytic mechanisms for F_1, the alternating-sites model with three equivalent catalytic sites is the most widely accepted (Gresser *et al.*, 1982; O'Neal and Boyer, 1984). According to this model, the binding of substrate at the catalytic site of one β subunit induces conformation change at the catalytic site of another β subunit and thereby facilitates the dissociation of the products. The catalytic sites alternate in function and act cooperatively in each catalytic cycle. Enzyme control via ligand-induced conformation change is a well-estabished phenomenon (Monod *et al.*, 1965; Koshland *et al.*, 1966). The assumption of substrate-induced conformation change in F_1 that regulates its activity is consistent with detailed kinetic studies (Grubmeyer *et al.*, 1982; Cross *et al.*, 1982) and fluorometric measurements (Wang, 1986). But experimental data are still needed for showing that the alternating sites indeed switch their functions in each catalytic cycle during the steady-state hydrolysis or synthesis of ATP.

On the other hand, athough the three β subunits of F_1 have identical amino acid sequence (Runswick and Walker, 1983; Kurawski *et al.*, 1982; Saraste *et al.*, 1981; Kanazawa *et al.*, 1982), interaction with other subunits could make them nonequivalent in the F_1 molecule (Williams *et al.*, 1984; Soong and Wang, 1984). Models of F_1 with nonequivalent β subunits are consistent with the available X-ray diffraction data (Amzel *et al.*, 1982), electron micrographs (Boekma *et al.*, 1986), the results of cold denaturation studies (Williams *et al.*, 1984), and experiments with various types of chemically modified F_1 (Fellous *et al.*, 1984; Lötscher and Capaldi, 1984; Matsuno-yagi and Hatefi, 1984; Snyder and Hammes, 1984; Soong and Wang, 1984; Williams *et al.*, 1984; Wang, 1984; Nalin *et al.*, 1985; Snyder and Hammes, 1985).

When 0.3 μM of ATP is added to 1 μM F_1 solution, the observed rate of product release is very slow but the catalytic exchange of oxygen atoms between water and P_i occurs readily (Choate *et al.*, 1979; Hutton and Boyer, 1979). Upon the binding of additional elements adenine nucleotides to F_1, this oxygen atom exchange is suppressed, but the overall rate of ATP hydrolysis can be increased by a factor of 10^6 (Grubmeyer *et al.*, 1982; Cross *et al.*, 1982). According to the alternating-sites model, the rate of product release at any active site can be greatly enhanced by ligand binding at the other sites which induces protein conformation change due to interaction between the subunits (binding change). According to one of the nonequivalent-sites models, the subunit β' is different from the other β'' subunits (Wang, 1985). Uni-site hydrolysis is also very slow at any of the three active sites, but promoted hydrolysis can only occur at the hydrolytic site of β' when ATP or ADP is bound to the regulatory site(s). Binding change is also assumed to take place via interaction between the catalytic and regulatory sites.

It is generally agreed that each bovine heart F_1 molecule, with subunit structure of $\alpha_3\beta_3\gamma\delta\varepsilon$, has altogether six binding sites for adenine nucleotides

with K_d values in or below the millimolar range. Three of these are believed to be either inactive (Wise and Senior, 1985) or regulatory (Schuster *et al.*, 1975; Bullough *et al.*, 1987; Xue *et al.*, 1987), and located either in β subunits (Harris, 1978; Cross and Nalin, 1982; Boulay *et al.*, 1985) or between α and β subunits (Williams and Coleman, 1982; Khananshvili and Gromet-Elhanan, 1985; Bullough and Allison, 1986).

In uni-site hydrolysis measurements, less than equivalent amount of ATP is added to F_1 samples containing 2.5–3 mol of endogenous ATP and/or ADP per F_1. The observed rates are expected to be dependent on the distribution of the added ATP among the 3–3.5 vacant sites, and special procedures have been developed to put substrate molecules to only one type of sites (Grubmeyer *et al.*, 1982; Kironde and Cross, 1986, 1987). For the fast steady-state hydrolysis of ATP, the measurement can be made more easily reproducible by preincubation of F_1 with a large excess of ATP in the absence of Mg^{2+} and subsequently starting the hydrolysis reaction by adding Mg^{2+} (Wu and Wang, 1987). A full understanding of the detailed catalytic mechanism of F_1 will require the determination of the locations and binding free energies of all the catalytic, regulatory, and inactive binding sites in F_1 for adenine nucleotides. But a number of general questions concerning the catalytic mechanism of F_1 can already be answered by chemical modification studies.

Modification with NBD-Cl

The reagent NBD-Cl (7-chloro-4-nitro-2,1,3-benzoxadiazole or 4-chloro-7-nitrobenzofurazan) has been widely used as a potent inhibitor of F_1-ATPases. When F_1-ATPase was labeled with NBD-Cl in the dark at pH 7, the NBD-label was almost exclusively attached to a specific tyrosine residue until about one label is covalently attached per F_1 molecule (Ferguson *et al.*, 1975a). The NBD-labeled F_1-ATPases have negligible ATPase activity, although the labeled sites still bind ATP or ADP (Cantley and Hammes, 1975; Cross and Nalin, 1982). Cleavage of the labeled protein, followed by isolation and sequencing of the labeled polypeptide and comparison with the known amino acid sequence of the β subunit, showed that the NBD-label is initially attached to Tyr-β311 (Runswick and Walker, 1983; Andrews *et al.*, 1984a; Sutton and Ferguson, 1985). But how does the NBD-label inhibit catalytic hydrolysis? Is Tyr-β311 at the hydrolytic site such that the NBD-label interferes with the catalytic mechanism or is Tyr-β311 quite far from the hydrolytic site but its labeling triggers a long-range conformation change that inactivates the enzyme?

In order to answer this question, the compound P^1-(5′-adenosyl)-P^2-N-(2-mercaptoethyl)diphosphoramidate (AMEDA), with a structure similar to

ATP but with the γ-phosphate group of ATP replaced by the mercaptoethylamino group, has been synthesized and tested (Wu *et al.*, 1987). It is well known that the NBD-label on Tyr-β311 can be rapidly removed by sulfhydryl compounds such as dithiothreitol or N-acetylcysteine. If Tyr-β311 is at the hydrolytic site, we would expect AMEDA to be bound very close to the [^{14}C]NBD-label so that its sulfhydryl group could react *in situ* with the latter to form AMEDA-[^{14}C]NBD and regenerate the active F_1-ATPase. The results from a series of experiments show that AMEDA indeed reactivated O-NBD-F_1, and that the reactivation was effectively prevented by either ADP or ATP. A kinetic analysis of the data showed that AMEDA first binds to O-NBD-F_1 with $K_d = 15\,\mu$M, then reacts *in situ* with the label to produce AMEDA-[^{14}C]NBD and the reactivated enzyme. The product AMEDA-[^{14}C]NBD was isolated, identified, and shown to bind even more tightly to F_1 with $K_d = 2\,\mu$M. These observations and the structural similarity between AMEDA and ATP may be regarded as compelling evidence for the presence of the NBD-labeled Tyr-β311 near the phosphate groups of ATP bound at the hydrolytic site of F_1-ATPase, because it would be inconceivable for an AMEDA molecule bound at the hydrolytic site to react with a far away NBD-label to form the product AMEDA-NBD and then to be bound again to the site with even higher affinity. We may conclude that labeling this Tyr-β311 residue at the site interferes with the hydrolytic mechanism, even if this Tyr-β311 does not participate directly in the catalysis. Since the NBD-label on this Tyr-β311 can be transferred spontaneously to Lys-β162 at pH $>$ 9 (Ferguson *et al.*, 1975; Andrews *et al.*, 1984b), we may conclude that Lys-β162 is also at or near the hydrolytic site. The conserved residues Tyr-β311 and Lys-β162 of bovine mitochondrial F_1 (MF_1) correspond to Tyr-β297 and Lys-β155 of *E. coli* F_1 (ECF_1). Directed mutagenesis experiments on the *E. coli* enzyme show that the amino acid substitutions Lys-β155 $\rightarrow$ Gln and Lys-β155 $\rightarrow$ Glu reduced the membrane F_1-ATPase activities of the mutant enzymes to 11 and 16%, respectively, of that of the wild-type enzyme, and reduced the ATP-driven pH gradient formation to 22 and 43% of that of the wild-type enzyme (Parsonage *et al.*, 1987). The amino acid substitution Tyr-β297 $\rightarrow$ Phe reduced the membrane F_1-ATPase activity of the mutant enzyme to 76% and the ATP-driven pH-gradient formation of the mutant to 72%, respectively, of that of the wild-type enzyme. The substitution Tyr-β297 $\rightarrow$ Phe also reduced the rate of inactivation of the ATPase with NBD-Cl by a factor of 7. These results suggest that Lys-β155 may be essential, but Tyr-β297 not essential, to the catalytic mechanism.

The observation that a single NBD-label attached to one Tyr-β311 is sufficient to inhibit completely the ATPase activity on MF_1 has been interpreted in two ways. According to the alternating-stress model, the rapid hydrolysis of an ATP molecule bound to an active site requires a protein

conformation change at the site coupled to ligand change at two equivalent sites which alternate with the first. Labeling the Tyr-β311 at any one of the three equivalent sites would block one of the necessary consecutive steps in the catalytic cycle and thereby stop the steady-state hydrolysis of ATP. According to the model with only one efficient hydrolytic site and one or more regulatory sites, the highly efficient promoted hydrolysis of ATP bound to the hydrolytic β' site also requires a protein conformation which is stabilized by the adenine nucleotides bound at the regulatory site(s). But, unlike in the alternating-sites model, the nucleotides bound at the hydrolytic and regulatory sites in this model are not required to turn over at the same rate during the steady-state hydrolysis of ATP. Direct labeling of F_1 by NBD-Cl is assumed to take place predominantly at the β' site because of the enhanced reactivity of its Tyr-β311 toward this reagent. Therefore, according to the nonequivalent-sites model the F_1 would also be inhibited completely by NBD-Cl at a label-to-F_1 molar ratio of $n = 1$.

In order to test these alternative models for F_1, a procedure was developed for inducing the rearrangement of subunits in F_1. It was found that when unlabeled F_1 was treated with 3 M LiCl for 2–4 min at 0° C and subsequently separated from LiCl in the presence of 5 mM ATP at 25° C by centrifugal gel-filtration, 64–89% of the ATPase activity of the F_1 could be restored (Wang, 1985; Wang *et al.*, 1987). Since prolonged treatment of F_1 solution by 0.85 M LiCl at 20° C was known to dissociate the subunits (Verschoor *et al.*, 1977), it was speculated that a brief treatment of F_1 by 3 M LiCl at 0° C could cause the subunits to rearrange and change their partners of close interaction. Since the ATPase activity of unlabeled F_1 is also decreased up to 36% by the LiCl treatment, the observed changes in ATPase activity of the labeled enzyme is best expressed in terms of the relative activity r defined by

$$r = \frac{\text{specific activity of labeled } F_1}{\text{specific activity of control unlabeled } F_1}$$

The control unlabeled F_1 could be a sample of unlabeled F_1 which had been subjected to the same treatment. An alternative control would be an aliquot of the labeled F_1 with all of its label removed by dithiothreitol (DTT) at the last step before assay.

According to the nonequivalent-sites model, the LiCl treatment could cause the labeled subunit in the hydrolytic β' position to switch place with an unlabeled subunit in the β'' position as illustrated in Fig. 1, and hence could increase the value of r. But according to the alternating-sites model, r should not be affected by the LiCl treatment, since all β subunits are assumed to be equivalent. Such experiments were repeated many times with highly reproducible results. An example is given in Table I, which shows that the LiCl

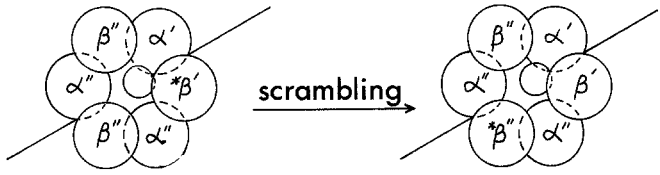

Fig. 1. Possible rearrangement of the subunits due to LiCl treatment. The undesignated sphere represents the smaller subunits which make β' uniquely efficient for the promoted catalytic hydrolysis of ATP. The straight line represents the approximate 2-fold symmetry axis of F_1. The asterisk (*) represents radioactive NBD-label. The smaller subunits could have been dissociated from their original $\alpha^*\beta$ pair in the presence of LiCl, but reassociated with a new $\alpha\beta$ pair when the salt was removed in the presence of ATP. Such a scrambling process could be visualized as a wobbling rotation of the $\alpha_3\beta_3$ moiety relative to the smaller anchoring subunits. This type of internal rotation did not take place during oxidative phosphorylation by submitochondrial particles.

treatment can raise the value of r from 0.02 to 0.37 without removing the covalent label. Subsequent studies show that the NBD-label was still attached to Tyr-β311 after scrambling by LiCl (Wang *et al.*, 1986). These results contradict the alternating-sites model, but are consistent with one efficient hydrolytic site assisted by one or more regulatory sites.

The NBD-label on Tyr-β'311 can be removed in a well-controlled way with N-acetyl-L-cysteine. For example, when (O-β'-NBD)$_{1.0}F_1$ was incubated with 20 μM of N-acetyl-L-cysteine at pH 7.0, 25° C, in the dark, the value of r increases as the number n of label per F_1 decreases. The plot of r versus n as illustrated in Fig. 2 is linear with a slope equal to -1, which shows that the removal of each NBD-label completely reactivates a fully inhibited F_1 molecule. Treatment of the O-β'-NBD-F_1 with LiCl increased its r value from less than 0.1 to about 0.6 without changing n, presumably because some of the labeled subunit in β' position has been rearranged to the weakly hydrolytic β'' position, and some unlabeled subunit in β'' position had been rearranged to the fully hydrolytic β' position. Treatment of this scrambled mixture of O-β'-NBD-F_1 and O-β''-NBD-F_1 with 20 μM N-acetyl-L-cysteine removed

Table I. Effect of LiCl Treatment on the ATPase Activity of (Tyr-β'311-[^{14}C]NBD)$_n F_1$ (Wang *et al.*, 1987)

Sample	n	ATPase activity (μmol ATP min^{-1} mg^{-1})	r
Untreated	0	72.3	(1)
O-β'-NBD-F_1	0.97 ± 0.01	1.52	0.021
Scrambled F_1	0	64.3	(1)
Scrambled O-NBD-F_1	0.95 ± 0.01	23.8	0.37

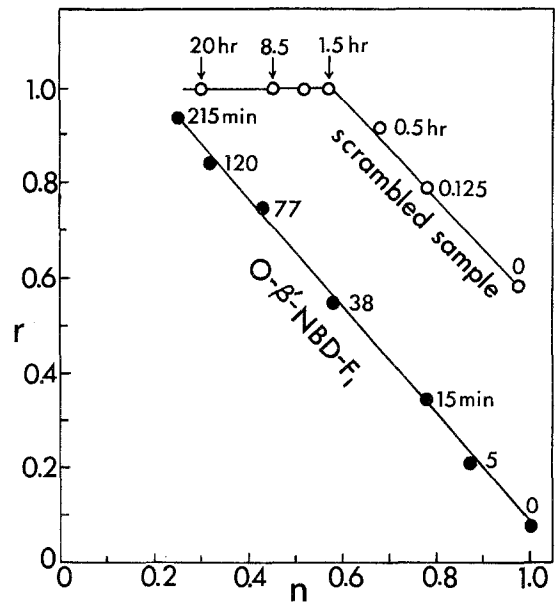

Fig. 2. Selective removal of O-NBD label from O-NBD-F_1 by N-acetyl-L-cysteine in the dark at 25° C. O-β'-NBD-F_1 (4.97 μM) was prepared by direct labeling with [^{14}C]NBD-Cl in the dark at pH 7.0. The scrambled enzyme sample (3.68 μM) was prepared by treating O-β-NBD-F_1 with 3 M LiCl for 3.5 min at 0° C, followed by gel-filtration at 25° C in the presence of 5 mM ATP. For each set of measurements, 20 μM N-acetyl-L-cysteine was added at time 0 and aliquots of the reaction mixture were taken at the indicated time intervals. Each aliquot was immediately gel-filtered in the presence of 5 mM ATP and assayed. n represents the molar ratio of NBD label to F_1, and r represents the ratio of the specific ATPase activity of O-NBD-F_1 before and after treatment with 2.5 μM DTT, respectively (Wang *et al.*, 1986).

the NBD-label at the same rate as before until all β' subunits were free of the label (upper curve in Fig. 2). Further removal of label from β'' subunits progressed at about 10-fold slower rate and no longer affected the value of r.

If the reaction mixture containing the scrambled sample was gel-filtered as soon as all NBD-labels on β' subunits had been removed, a labeled enzyme containing ~ 0.6 label per F_1 was obtained with $r = 1$ and presumably the formula $(O\text{-}\beta''\text{-NBD})_{0.6}F_1$. On the other hand, if the reaction mixture containing the unscrambled sample was gel-filtered when n had decreased to 0.5–0.6, a labeled enzyme was obtained with $r = 0.5$–0.6 and presumably the formula $(O\text{-}\beta'\text{-NBD})_{0.6}F_1$. In this way, two geometric isomers of covalently labeled F_1 were obtained with contrasting biochemical properties. At pH 9 in the dark, the NBD-label in O-β'-NBD-F_1 was found to transfer spontaneously from Tyr-β311 to Lys-β162 with a half-life of 1/2 hr, whereas the NBD-label in O-β''-NBD-F_1 was found not to transfer at all under the same conditions.

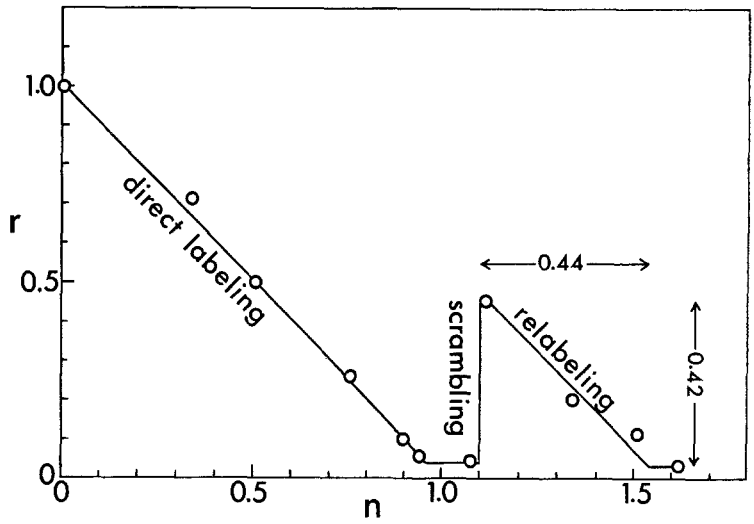

Fig. 3. Observed changes in n and r of the labeled F_1 sample due to direct labeling by NBD-Cl, scrambling of the β subunits by LiCl treatment, and subsequent relabeling (Wang *et al.*, 1986).

Both the horizontal portion of the biphasic plot in Fig. 2 and the observed extremely slow O → N transfer rate for O-β''-NBD-F_1 show that the latter was an isomer with contrastingly different property, not a mixture of labeled and unlabled forms of the enzyme. The two labeled enzymes must be geometric isomers, because cleavage of either of them with pepsin, separation of the [^{14}C]NBD-labeled peptides, and subsequent determination of its amino acid sequence showed that the label was covalently attached to Tyr-β311 in both isomers (Wang *et al.*, 1986). These data seem to rule out the proposed models for F_1 with either three or two equivalent sites for efficient ATP hydrolysis.

In attempting to defend the alternating-sites model, we could assume that although the NBD-label was still covalently attached to Tyr-β311, the treatment with LiCl had changed it to a noninterfering conformation so that the ATPase was partially reactivated. But if this is true, the Tyr-β311 residue which has already been labeled with a noninterfering NBD-group cannot be labeled again. On the other hand, if the LiCl treatment had indeed caused a labeled subunit in the β' position to switch place with an unlabeled residue in the β'' position, the new unlabeled subunit in the β' position should again be susceptible to labeling by NBD chloride. Figure 3 shows that the scrambled enzyme can again be labeled with $dr/dn = 1$. This indicates that, after the scrambling process, some unlabeled β subunits have indeed rotated to the hydrolytic β' position and thus can now be rapidly labeled by NBD chlorine at its Tyr-β311.

Modification with FSBA and FSBI

The affinity reagents 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) and 5′-*p*-fluorosulfonylbenzoylinosine (FSBI) have been used fruitfully in studying the catalytic mechanism of F_1-ATPase (Allison *et al.*, 1986). NBD chloride is not an affinity reagent. The rapid and specific labeling of F_1 by NBD chloride is due to the unusually high reactivity of Tyr-β'311 in F_1 toward this reagent. The rate of labeling is proportional to NBD chloride concentration, and the labeling reaction is not prevented by ATP or ADP (Ting and Wang, 1980). By contrast, F_1 is protected from FSBA by ATP or ADP, and the active sites labeled by this affinity reagent can no longer bind a full complement of ATP or ADP (Esch and Allison, 1978, 1979). FSBA was found to label specifically either Tyr-β368 or His-β427 but not both (Bullough and Allison, 1986a). FSBI was found to label specifically Tyr-β345 (Bullough and Allison, 1986b).

It was found that while the inactivation of F_1 by FSBI is accompanied by the labeling of a single Tyr-β345 residue, the inactivation of the ATPase by FSBA requires the labeling of all three copies of the β subunit in F_1. Even more surprising is the observation that the number of labeled copies of the β subunit required for total inhibition by FSBA depends on the substrate, as summarized in Table II. Bullough and Allison concluded that the Tyr-β345 labeled by FSBI is at the hydrolytic site whereas the Tyr-β368 and His-β427 labeled by FSBA are at the regulatory site. The data in Table II for efficient steady-state hydrolysis of ATP and ITP could be explained in terms of the alternating-sites model if we also assume that the SBA-label at a regulatory site has an attenuating effect on the catalysis at hydrolytic sites and that only ATP but not ITP can be bound to the unlabeled regulatory site (Bullough and Allison, 1986b).

Table II. Inactivation of F_1 by FSBA and FSBI (Bullough and Allison, 1986)

	FSBA	FSBI
Residues modified	His-β427 or Tyr-β368 but not both	Tyr-β345
Pseudo-first order kinetics	Biphasic	Linear
Number of labels required for total inhibition of steady-state ATPase[a]	~3	~1
Number of labels required for total inhibition of steady-state ITPase	~1	~1

[a]Nearest integer of the extrapolated values of 2.5 to 2.7 and 0.83, respectively.

Explanation of the data in Table II in terms of the single efficient hydrolytic-site model is simpler. Since FSBI labels Tyr-β'345 at the single efficient hydrolytic site, the enzyme should be completely inactivated when there is one SBI-label per F_1 no matter whether ATP or ITP is used as the substrate. On the other hand, since FSBA labels regulatory sites, its effect may depend on the structure of the substrate. With ATP as the substrate, the inhibitory perturbing effect of the SBA-label could be partially compensated by the stimulatory effect of ATP bound to the unlabeled regulatory site in the same F_1, and cause an attenuation rather than total inhibition of ATPase activity when there is one SBA-label per F_1. But with ITP as the substrate, since ITP does not bind or bind properly to the unlabeled regulatory site to offset the inhibitory effect of the SBA-label, total inhibition of ITPase activity would be possible when there is only one SBA-label per F_1.

Perhaps the most exciting observation from these chemical modification experiments is that the catalytic and regulatory sites are quite close to each other in the same β subunit. Hitherto only the interactions between the subunits of F_1 have been discussed in the published literature. Now we also have to treat more intimate interactions between the catalytic and regulatory sites responsible for ligand-induced changes in secondary and tertiary structure. If the polypeptide from Tyr-β345 to Tyr-β368 were an uninterrupted α-helix, the distance between the two terminal residues would be 34.5 Å. But since there are three proline residues in between where the α-helix should turn, the actual distance between the two terminal residues should be much shorter. Considering that an extended ATP molecule has a span of 16 Å (covalent) or 19 Å (van der Waals), the interaction between the catalytic and regulatory sites could be sufficiently strong to raise the uni-site hydrolysis rate by several orders of magnitude to that of efficient steady-state (multi-site) rate of catalytic hydrolysis.

Uni-site catalytic hydrolysis of ATP by chemically modified F_1 samples has also been examined by Allison and cowokers. Their data indicate a heterogeneity among the weakly hydrolytic uni-sites (Bullough *et al.*, 1987), a conclusion which is entirely consistent with the nonequivalence of β subunits in F_1 discussed in the last section.

Modification with Derivatives of ATP and ADP

The photoaffinity reagents 2-azido-ATP and 2-azido-ADP were chosen by Vignais and coworkers to modify F_1 (Boulay *et al.*, 1985; Dalbon *et al.*, 1985; Garin *et al.*, 1986) because of their "anti" conformation (Czarnecki, 1984) in preference to 8-azido-ATP and 8-azido-ADP with their "syn" conformation (Sarma *et al.*, 1974). Using [α-^{32}P]ADP as the specific photolabeling

reagent, they showed that radioactivity was localized on the four amino acid residues: Leu-β-342, Ile-β344, Tyr-β345, and Pro-β346.

Using specially prepared samples of bovine heart MF_1-ATPase with controlled distribution of adenine nucleotides bound at the catalytic and noncatalytic sites (Kironde and Cross, 1987), Cross, Boyer, and coworkers showed from their labeling experiments with 2-azido-[^{32}P]ATP that Tyr-β345 is at catalytic sites and Tyr-β368 is at noncatalytic sites (Cross *et al.*, 1987). It was estimated that 1/3 of the catalytic sites or 2/3 of the noncatalytic (presumably regulatory) sites must be modified to give nearly complete inhibition of the ATPase. 2-Azido-[^{32}P]ATP was also used to label CF_1-ATPase from spinach chloroplasts (Xue *et al.*, 1987). The results are in agreement with the conclusions from the MF_1 experiments. These conclusions are also in agreement with the conclusions from the labeling experiments with FSBA and FSBI discussed in the last section.

Possible tertiary structures of the polypeptide from Tyr-β345 to Tyr-β368 in F_1 have been proposed by Garin *et al.* (1986) and by Cross *et al.* (1987). In both proposals, the distance between Tyr-β345 and Tyr-β368 is less than the span of an ATP molecule in the "anti" conformation.

Tritiated adenosine triphosphopyridoxal (AP_3-PL) and adenosine tetraphosphopyridoxal (AP_4-PL) have been used by Futai and coworkers to label F_1-ATPase from *E. coli* with interesting results. The binding of AP_3-PL to a reactive Lys residue had a stoichiometry of 1 mol AP_3-PL/mol F_1 at the saturating level (Noumi *et al.*, 1987). About 2/3 of the label was bound to the α subunit and 1/3 bound to the β subunit. F_1 labeled with 1 mol AP_3-PL/mol exhibited essentially no uni-site or multi-site ATPase activity. Their results suggest that AP_3-PL or AP_4-PL was bound to a hydrolytic site and hydrolysis of ATP did not take place at the two remaining catalytic sites.

Reconstituted Submitochondrial Particles with Chemically Modified F_1

One way to find out whether the catalytic hydrolysis and synthesis of ATP take place in opposite directions along exactly the same reaction path is to compare the efficiency for ATP hydrolysis with that for ATP synthesis by submitochondrial particles containing chemically modified F_1. In order to avoid unnecessary complications, the usual procedure is to label the soluble F_1 first and then use it to reconstitute the ASU to form various forms of chemically modified submitochondrial particles (Steinmeier and Wang, 1979; Kohlbrenner and Boyer, 1982; Matsuno-Yagi and Hatefi, 1984).

Since oxidative phosphorylation can be measured only in energized membranes whereas ATP hydrolysis is often measured in de-energized membranes, we should first examine the effect of energization on the ATPase

Table III. ATP Hydrolysis and Reverse Electron Transport By Chemically Modified SMP at 30°C

Measurement	F_1-ASU	O-β'-NBD-F_1-ASU	r
ATPase (+FCCP, −oligomycin) (μmol ATP $min^{-1} mg^{-1}$)	5.15 ± 0.10	0.46 ± 0.004	0.090
Reverse electron transport (+0.25 μg oligomycin mg^{-1}) (μmol NADH $min^{-1} mg^{-1}$)	29.4	2.78	0.095

Table IV. ATP Hydrolysis and Oxidative Phosphorylation by Chemically Modified SMP at 30°C

Measurement	F_1-ASU	O-β'-NBD-F_1-ASU	r
ATPase (μmol ATP $min^{-1} mg^{-1}$)	3.51 ± 0.09	0.43 ± 0.08	0.12
Oxidative phosphorylation (μmol ATP $min^{-1} mg^{-1}$)	111 ± 5	48 ± 2	0.42

activity of submitochondrial particles (SMP), before a valid comparison could be made between the catalytic efficiency for ATP hydrolysis and that for ATP synthesis by chemically modified SMP. For this purpose, (O-β'-NBD)$_n$$F_1$ with $n = 1.03$ and $r = 0.03$ was used to reconstitute with ASU (Wang *et al.*, 1987). The steady-state rate of catalytic ATP hydrolysis by the reconstituted SMP was measured both directly in uncoupled SMP in the presence of 12.5 μM FCCP and indirectly in energized SMP through the coupled reduction of NAD^+ by succinate. The results summarized in Table III show that the ratio r of the specific ATPase activity of the labeled enzyme complex to that of the unlabeled control is essentially independent of the state of energization, and is determined only by the state of specific labeling of F_1.

The observed r values for catalytic ATP hydrolysis and oxidative phosphorylation by another pair of labeled and unlabeled SMP samples are summarized in Table IV. The observed value of r for oxidative phosphorylation is clearly much higher than that for ATP hydrolysis. Since the respiration of F_1-ASU and O-β'-NBD-F_1-ASU are essentially the same (Steinmeier and Wang, 1979), these data suggest that steady-state oxidative phosphorylation may involve more catalytic sites of F_1 than steady-state ATP hydrolysis.

One may speculate on the possibility that the labeled SMP O-β'-NBD-F_1-ASU is less leaky than the control SMP F_1-ASU. This could make the labeled SMP more efficient in utilizing the proton flux for ATP synthesis than the control SMP, which could lead to a higher value of r for oxidative phosphorylation than that for ATP hydrolysis. But this possibility cannot be true, because the value of r observed for ATP-driven reverse electron

Table V. Catalytic Activities of SMP Reconstituted from (Tyr-β''311-[^{14}C]NBD)$_{1.3}$F$_1$ and ASU at 30°C

Measurement	Control F$_1$-ASU	(O-β''-[^{14}C]NBD)$_{1.3}$F$_1$-ASU	r
ATP hydrolysis (μmol ATP min^{-1}mg^{-1})	1.88	1.84	0.98
Oxidative phosphorylation (nmol ATP min^{-1}mg^{-1})	269	160	0.61

transport, which also involves utilization of the proton flux, is the same as that for ATP hydrolysis (Table III).

It is not clear how more β subunits in the F_0F_1 complex can catalyze ATP synthesis, whereas only a single β' subunit catalyzes efficient steady-state ATP hydrolysis. One possibility is that due to its unique interaction with the smaller subunits, only β' can undergo the proper conformation changes for catalyzing the rapid formation or decomposition of certain intermediates along the reaction path or for the rapid release of products during the promoted steady-state ATP hydrolysis (Gresser *et al.*, 1982; Fellous *et al.*, 1984). But during ATP synthesis by SMP, the same formation or decomposition or release could be affected by conformation changes driven by the proton flux so that all three β subunits could catalyze effectively.

A decisive test of this possibility that all three β subunits catalyze oxidative phosphorylation could be conducted if we could use F$_1$-ATPase with [^{14}C]NBD-labeled Tyr-β''311 and unlabeled Tyr-β'311 to reconstitute the SMP. Because of their much lower reactivity, the Tyr-β''311 residues in F$_1$ cannot be labeled directly by [^{14}C]NBD chloride with satisfactory specificity. But by using three cycles of the label–scramble–relabel process similar to that illustrated in Fig. 2, the labeled enzyme (Tyr-β''311-[^{14}C]NBD)$_{1.3}$-F$_1$ was finally prepared and used to reconstitute with ASU particles (Wang *et al.*, 1987). The observed catalytic efficiencies for the hydrolysis and synthesis of ATP by those reconstituted SMP are listed in Table V. The values of r in Table V show that the NBD-label attached to Tyr-β''311 inhibits oxidative phosphorylation but does not inhibit ATP hydrolysis. This observation confirms the speculation that although only the β' subunit in F$_1$ catalyzes steady-state ATP hydrolysis, both β' and β'' catalyze oxidative phosphorylation.

One may also wonder whether the catalysis by all three β subunits during oxidative phosphorylation could be due to the scrambling of the β subunits by the proton flux. We could assume that only β' catalyzes both the hydrolysis and synthesis of ATP, but that during oxidative phosphorylation the β subunits were scrambled by the proton flux so that both the labeled and unlabeled β subunits had their turn in the catalytic β' position. If the

functional differentiation of β' and β'' is due to a closer interaction of β' with the smaller subunits, such a picture would be equivalent to assuming that the proton flux causes internal rotation of the $\alpha_3\beta_3$ or β_3 moiety relative to the remainder of the F_0F_1 complex during oxidative phosphorylation. In this way, the value of r for oxidative phosphorylation would be higher than that for ATP hydrolysis.

In order to test this possibility, the reconstituted SMP samples were preincubated under simulated oxidative phosphorylation conditions for 12 min and subsequently assayed for ATPase activity. Had the β subunits indeed switched roles in each catalytic cycle, we would expect the observed value of r for ATP hydrolysis to be approximately the same as that found for oxidative phosphorylation, i.e., $r = 0.42$ (Table IV), because the F_0F_1 complex turned over 40,000 times in the preincubation period. But the observed value of r was only 0.23, which is not significantly different from the value $r = 0.16$ for the same pair of samples before preincubation. Therefore, we have to conclude that β' and β'' did not switch roles and hence internal rotation of $\alpha_3\beta_3$ relative to the remainder of the F_0F_1 complex did not take place during oxidative phosphorylation. Recent cross-linking studies also indicate that internal rotation is not required for ATP hydrolysis catalyzed by CF_1-ATPase from spinach chloroplasts (Musier and Hammes, 1987).

New Conclusions

A great deal of valuable information on F_1-ATPase has been obtained since the enzyme was first isolated (Pullman *et al.*, 1960; Penefsky *et al.*, 1960). Although we still do not fully understand this vital enzyme, recent chemical modification studies in many laboratories enabled us to draw the following new conclusions concerning its catalytic mechanism:

1. The six catalytic and regulatory sites of bovine heart F_1 are all on the three β subunits. The catalytic and regulatory sites of the same β subunit are probably sufficiently close to each other and the interactions between the various catalytic and regulatory sites are probably sufficiently strong to raise the uni-site rate of ATP hydrolysis by several orders of magnitude to that of promoted (multi-site) ATP hydrolysis.

2. Although all three β subunits in F_1 possess weak uni-site ATPase activity, only one of them (β') catalyzes promoted ATP hydrolysis. It does not alternate with the other two β subunits in function during each catalytic cycle.

3. All three β subunits catalyze ATP synthesis driven by the proton flux.

4. Internal rotation of the $\alpha_3\beta_3$ or β_3 moiety relative to the remainder of the F_0F_1 complex did not occur during oxidative phosphorylation by reconstituted submitochondrial particles.

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