Journal of Bioenergetics and Biomembranes, Vol. 24, No. 5, 1992

Minireview

Functional Sites in F_1-ATPases: Location and Interactions[1]

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Received April 22, 1992; accepted May 20, 1992

This review focuses on the location and interaction of three functional sites in F_1-ATPases. These are catalytic sites which are located in β subunits, noncatalytic nucleotide-binding sites which are located at interfaces of α and β subunits and modulate the hydrolytic activity of the enzyme, and a site that binds inhibitory amphipathic cations which is at an interface of α and β subunits. The latter site may participate in transmission of conformational signals between catalytic sites in F_1 and the proton-conducting apparatus of F_0 in the intact ATP synthases.

KEY WORDS: Catalytic sites; regulatory sites; inhibitory amphipathic cations; signal transmission.

INTRODUCTION

The current working hypothesis adopted by most laboratories studying the molecular mechanism of the F_0F_1-ATP synthases accommodates the structural model proposed by Capaldi's laboratory (Gogol *et al.*, 1989; Lücken, *et al.*, 1990) for the *E. coli* ATP synthase with the binding change mechanism for ATP synthesis developed in Boyer's laboratory (Boyer, 1989). To account for the coupling process, it is postulated that proton flow through F_0 initiates a conformational change that is relayed through the stalk subunits to F_1 where the propagated signal affects the affinities of two catalytic sites for substrates and products in reciprocal fashion. At the level of F_1, the propagated conformational signal is postulated to promote release of ATP, formed spontaneously at a high affinity catalytic site, while simultaneously trapping ADP and Pi, bound initially with low affinity at another catalytic site, in a tight conformation in which spontaneous ATP synthesis occurs. The ATP formed spontaneously from ADP and Pi at the newly configured tight site is released in the next catalytic cycle.

The remainder of this brief review is focused on the location of three functional sites in the F_1-ATPases and interaction between these sites which have been detected during chemical modification studies. The three interacting sites are catalytic sites, noncatalytic nucleotide binding sites which have a regulatory role, and sites that bind inhibitory amphipathic cations. The latter sites appear to be in conformational equilibrium with catalytic sites and also might be at, near, or perhaps in communication with the aurovertin and OSCP binding sites.

RESIDUES COMPRISING THE CATALYTIC NUCLEOTIDE-BINDING DOMAIN

Two lines of evidence suggest that catalytic sites of the F_1-ATPases are for the most part, if not completely contained in β subunits. Chemical modifications which alter catalytic sites invariably derivatize residues in the β subunit (Penefsky and Cross, 1991). Furthermore, the difference circular dichroism spectrum generated when the TF_1-ATPase[2] binds ADP, which is committed to form ATP in the presence of Mg^{2+}, Pi, and dimethyl sulfoxide, closely resembles the difference spectrum generated when the isolated β

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[2]Abbreviations used: TF_1, MF_1, EF_1, CF_1, and YF_1, the F_1-ATPases from the thermophilic bacterium PS3, mammalian mitochondria, *Escherichia coli*, spinach chloroplasts, and yeast mitochondria, respectively; Nbf-Cl, 7-chloro-4-nitrobenzofurazan; DCCD, dicyclohexylcarbodiimide; FSBI, 5′-*p*-fluorosulfonylbenzoylinosine; FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; 8-N_3-FSBA, 5′-*p*-fluorosulfonylbenzoyl-8-azidoadenosine, and OSCP, the oligomycin sensitivity conferring protein.

0145-479X/92/1000-0469$06.50/0

subunit of TF_1 binds ADP (Yoshida and Allison, 1986). From homology of the β subunit of F_1 with the ATP binding domain of adenylate kinase, Fry *et al.*, (1986) and Duncan *et al.*, (1986) suggested independently that the middle 1/3 of the β subunit of F_1 is folded similarly to the ATP binding domain of adenylate kinase, the structure of which has been deduced from x-ray crystallography (Schulz *et al.*, 1990). Substitution of residues by *in vitro* mutagenesis (Ohtsubo *et al.*, 1987; Hsu *et al.*, 1987; Parsonage *et al.*, 1981; 1982; Esch *et al.*, 1981), Nbf-Cl (Ferguson *et al.*, 1975a, b; Andrews *et al.*, 1984a, b), or ADP-pyridoxal (Tagaya *et al.*, 1988) in this region support the contention that this part of the primary structure contributes to the catalytic site. Garboczi *et al.*, (1988) reported that a synthetic peptide corresponding to residues 141–190 of the β subunit of rat liver F_1 is precipitated by ATP, other nucleoside triphosphates, ADP, PPi, but not by AMP. This suggests that this segment of the β subunit is part of the subdomain which interacts with the pyrophosphoryl moieties of nucleotides bound to catalytic sites. Comparison of the sequences of the α and β subunits and the catalytic subunits of the V-ATPases in the segment corresponding to residues 141–190 of rat liver β subunit reveals two positions which are invariably occupied by Lys or Arg. Using the residue numbers of MF_1, one invariant basic residue is Lys-β162, the residue to which the Nbf-group migrates from Tyr-β311 under alkaline conditions. Lys-β162 is present in Walker homology A (Walker *et al.*, 1982), the so called "glycine-rich loop", a consensus sequence found in most enzymes that bind purine nucleotides. The other invariant basic residue is Arg-β189, which is immediately preceded by an invariant Glu in the β subunits of all F_1's and catalytic subunits of V-ATPases which have been sequenced.

In the crystal structures of the Ha-Ras p 21 protein (Schlichting, *et al.*, 1990) and elongation factor Tu (Clark *et al.*, 1990) the phosphoanhydride moieties of nucleotides bound to catalytic sites interact with the polyamide backbone of the "glycine-rich loop." In the structure deduced for the p 21 protein, the protonated ε-amino group of the equivalent of Lys-β162 of MF_1 interacts with the γ-phosphate of bound GTP. The catalytic properties of site-directed mutants of F_1-ATPases with substitutions at Lys-β162 and other residues in the "glycine-rich loop" suggest that analogous interactions exist at the catalytic site of F_1-ATPases (33–37). Chemical modification studies also suggest that the protonated ε-amino group of Lys-β162 interacts with the γ-phosphate of ATP or Pi bound to the catalytic site. The F_1-ATPases are inactivated when Nbf-Cl derivatizes Tyr-311 in a single β subunit. The observation that the Nbf-group migrates from Tyr-β311 to Lys-β162 under alkaline conditions suggests that these residues are close to each other in the catalytic binding domain (Andrews *et al.*, 1984a, b). This contention is supported by the observation that Pi, which presumably binds to Lys-β162, protects *Rhodospirillum rubrum* F_1 (Cortez *et al.*, 1983) and MF_1 (Perez *et al.*, 1986) against inactivation by Nbf-Cl. Garin *et al.*, (1989) have shown that Tyr-β311 is derivatized when MF_1 is photoinactivated with 4-azido-2-nitrophenylphosphate in a reaction that is also attenuated by Pi. From these observations, Garin *et al.*, presented a model which suggests that the nitro- or phosphate moiety of these inactivators interacts with the protonated ε-amino group of Lys-β162 during derivatization of Tyr-β311. Since it was reported (Hollemans *et al.*, 1983) that Tyr-β311, Ile-β304, and Lys-β301 are derivatized when MF_1 is photoinactivated with 8-N_3-ATP, Garin *et al.*, (1989) placed the side chain of Tyr-β311 adjacent to the adenine moiety of ATP in thier model of the catalytic site. Inconsistent with this arrangement, Weber *et al.*, (1992) failed to detect changes in catalytic or fluorescent properties when comparing the interaction of *lin*-benzo-ADP bound to cataltyic sites of wild-type EF_1 and a site-directed mutant in which the tyrosine residue in question was replaced with phenylalanine.

That the side chain of Tyr-β345 is in the proximity of the adenine moiety of ATP or ADP bound to catalytic sites is supported by the results of both chemical modification and *in vitro* mutagenesis studies. Garin *et al.*, (1986) were the first to show that Tyr-β345 is derivatized when MF_1 is photoinactivated with 2-N_3-ADP generated from 2-N_3-ATP at catalytic sites. It was shown that the same residue is derivatized in a single β subunit when MF_1 is inactivated with FSBI (Bullough and Allison, 1986b). Evidence has been provided that the 5′-*p*-fluorosulfonylbenzoylnucleosides are foled in aqueous solution with the benzene and purine rings stacked (Jacobson and Colman, 1984). Therefore, the observation that both FSBI and 2-N_3-ADP derivatize Tyr-β345 suggests that FSBI remains in a folded conformation when bound to the catalytic site of MF_1. Tyr-β345 of MF_1 (Aloise *et al.*, 1991), and its equivalent in CF_1 (Adman and Hammes, 1987) is also derivatized when

the ATPases are photoinactivated with 3′-*O*-(benzoyl)benzoyl-ATP. This suggests that the photoaffinity label may also be folded in a manner which places the 3′-*O*-(benzoyl)benzoyl moiety in proximity of the adenine moiety.

The contention that the purine moiety of nucleotides bound to catalytic sites interacts with the aromatic ring of Tyr-β345 is supported by the effects of substitutions at this position on catalytic properties of the enzyme and on the fluorescence properties of *lin*-benzo-ATP bound to catalytic sites (Wise, 1990; Weber *et al.*, 1992). It was shown (Wise, 1990) that the V_{max}/K_m value of a mutant form of EF_1 with Tyr substituted by Phe in the position corresponding to Tyr-β345 of MF_1 was about 50% that observed for the wild-type enzyme. However, site-directed mutants in which the Tyr was substituted with Cys, Ser, Gly, or Ala exhibited V_{max}/K_m values which were at least 10-fold lower than that determined for the wild-type enzyme. This study was extended (Weber *et al.*, 1992) to include other substitutions and also compared the fluorescence excitation and emission spectra generated when the mutant forms bound *lin*-benzo-ATP with those generated when the wild-type enzyme bound the substrate analog. From the results of these studies, it was concluded (Weber *et al.*, 1992) that: (a) the side chain of Tyr-β345 is part of the catalytic subdomain which binds adenine; (b) an aromatic residue is not essential at this position for catalytic function, but increase in polarity of the side chain impairs function; and (c) when tyrosine is present in this position and *lin*-benzo-ATP is bound to catalytic sites, the phenolic hydroxyl interacts directly with the fluorophore.

NONCATALYTIC NUCLEOTIDE-BINDING SITES SPAN α AND β SUBUNITS

Isolated α and β subunits of bacterial F_1-ATPases each bind 1 mol of adenine nucleotide per mol (Ohta *et al.*, 1980; Dunn and Futai, 1980), suggesting that noncatalytic sites are present in α subunits. However, it was reported the the β subunit of the *Rodospirillum rubrum* F_1-ATPase binds 2 mol of adenine nucleotide per mol (Gromet-Elhanan and Khananshvili, 1984). Consistent with this observation, affinity labeling and mutagenesis studies have shown that noncatalytic nucleotide binding sites span α and β subunits. These studies suggest that the phosphoanhydride moiety of a noncatalytic nucleotide interacts with a subdomain in the α subunit, whereas the subdomain that binds the adenine moiety is in the β subunit. Evidence supporting this conclusion is the following. Whereas maximal inactivation of MF_1 by FSBI is observed when Tyr-β345 is derivatized in a single catalytic site (Bullough and Allison, 1986b), maximal inactivation of the enzyme by FSBA is observed when either Tyr-368 or His-427 are derivatized in mutually exclusive reactions in three copies of the β subunit (Bullough and Allison, 1986a). It was shown that Tyr-β368 is also labeled on irradiating MF_1 after specifically loading noncatalytic sites with 2-N_3-ADP (Cross *et al.*, 1987). The observation that both 2-N_3-ADP and FSBA derivatize Tyr-β368 suggests that the phenolic side chain of this residue is near the adenine moiety of nucleotides bound to noncatalytic sites. Although His-β427 is predominantly labeled during inactivation of MF_1 by FSBA at pH 6.5, a histidine is found in the corresponding position only in other mammalian F_1-ATPases and that from the plasma membranes of the alkaliphilic bacterium, *Bacillus firmus* OF4 (Ivey and Krulwich, 1991). His-427 is predicted to be in a β-turn defined by Gly-β426 and Gly-β429. Since glycines occupy the corresponding positions in the β subunits of all F_1ATPases that have been sequenced to date, it has been suggested that the structural element in this region of the β subunit that interacts with the adenine moiety of nucleotides bound to noncatalytic sites is the β-turn (Bullough and Allison, 1986a). It is serendipitous that histidine is present in the β-turn in MF_1, the only enzyme probed in detail with FSBA and 8-N_3-FSBA. It is interesting that a double mutant of EF_1 has been described with deficient ATPase activity carrying substitutions at positions corresponding to Gly-β429 and Pro-β417 of MF_1 (Kironde *et al.*, 1989).

That part of the noncatalytic nucleotide binding site which is contributed by the α subunit has been explored by chemical modification and mutagenesis studies. Inactivation of the intact EF_1ATPase with ADP-pyridoxal is accompanied by derivatization of Lys-α201 (Tagays *et al.*, 1988). The same residue was modified when the isolated α subunit from EF_1 was treated with ADP-pyridoxal (Rao *et al.*, 1988a). It is interesting that Lys-α201 of EF_1 corresponds to Arg-β189 of MF_1, a residue conserved in all F_1-ATPases which is adjacent to the glutamic acid residue that reacts with DCCD in TF_1 (Yoshida *et al.*, 1981). Lys-α175 of EF_1 is a residue by the "glycine-rich loop." Substitution of this residue with Ile or Glu by site-directed mutagenesis led to a 2.5-fold and 3-fold decrease, respectively, in ATPase activity compared to that of the wild-type enzyme (Rao *et al.*, 1988b).

Table I. Homology of α and β Subunits of MF_1 and EF_1 in the Region Surrounding Tyr-β345 of MF_1[a]

EF_1-α	$_{341}$VP*TNVI*SITD	G*IFLETNLF	NAGIRPAVN*P	GI*SV*S*RVGAA$_{380}$
MF_1-α	$_{335}$IPTNVISITD	GQIFLETELF	YKGIRPAINV	GLSVSRVGSA$_{374}$
MF_1-β	$_{321}$APAVTFAHLD	ATTVLSRAIA	ELGIYPAVDP	LDSTSRIMDP$_{360}$
EF_1-β	$_{307}$SPATTFAHLD	ATVVLSRQIA	SLGIYPAVDP	LDSTSRQLDP$_{346}$

[a] Residues marked with asterisks represent those substituted by mutation resolution in defective ATPase activity described by Senior's laboratory (Maggio *et al.*, 1987; Pagan and Senior, 1990.

There is an intriguing segment of primary structure near the C-terminus of the α subunit in which several random mutations which affect the activity of EF_1 are found. Table I illustrates the sequences of this segment in the α subunit of EF_1 and MF_1 along with a comparison of the corresponding segments of the β subunits of the two ATPases. It is interesting that two clusters of mutations (Maggio *et al.*, 1987; Pagan and Senior, 1990), which are marked with asterisks, flank the position equivalent to that occupied by Tyr-β345 of MF_1. Arginine is present in the position equivalent to that occupied by Tyr-β345 of MF_1 in all α subunits which have been sequenced to date. From secondary structure predictions, homology of α and β subunits, and the homology of β subunits with the ATP binding domain of adenylate kinase, it was proposed that residues 160–340 comprise the nucleotide binding domain of the α subunit of EF_1 (Maggio *et al.*, 1987). It was also suggested that residues 343–375, which contain the two clusters of mutations illustrated in Table I that affect cooperativity, "may be intimately involved in β—α—β intersubunit conformational interaction which mediates positive catalytic site cooperativity". The binding of nucleotides to the isolated F_1-ATPases carrying these mutations has not been studied. However, the affinity of the mutant α subunits for ATP was estimated to be 50–1000-fold less than that of the wild-type α subunit using an indirect binding assay (Rao *et al.*, 1987). It was argued that since the ATP concentration in *E. coli* cells is 3 mM and the K_d of the complex of isolated α subunit with ATP is 0.1 μM, the decreased binding affinity noted for the isolated mutant α subunits was of no physiological significance. Given that the noncatalytic nucleotide-binding domain spans α and β subunits, it is possible that these mutations might affect the arrangement of α and β subunits which could have a large effect on the affinity of the assembled enzyme for noncatalytic nucleotides. Thus, it will remain an open question whether or not the impaired activity of these mutants is caused by decreased affinity of noncatalytic sites for nucleotides until direct binding studies are carried out with the assembled enzymes.

ARRANGEMENT OF NUCLEOTIDES BOUND TO CATALYTIC AND NONCATALYTIC SITES

A cross-linking study using the bifunctional affinity label, 8-N_3-FSBA, has provided evidence that the purine moieties of nucleotides bound simultaneously to the noncatalytic and catalytic sites of an interacting $\alpha\beta$ pair are adjacent (Zhuo *et al.*, 1992a). Inactivation of MF_1 with 8-N_3-FSBA in the absence of light derivatizes Tyr-368 or His-427 in mutually exclusive reactions in three copies of the β subunit. Subsequent irradiation of the derivatized enzyme cross-linked that part of the reagent tethered to Tyr-β368, in shot gun fashion, to several sites in the α subunit. On the other hand, on irradiation, that part of the reagent attached to His-β427 is cross-linked in high yield to Tyr-345 in the same β subunit. The nitrene scavenger, β-mercaptoethanol, virtually eliminated photo-induced α–β cross-links, but had no effect on cross linking of His-β427 to Tyr-β345. Therefore, it appears that the 8-N_3-adenine moiety of reagent tethered to the side chain of Tyr-β368 is exposed to solvent and is oriented near the surface of an adjacent α subunit, whereas the 8-N_3-adenine moiety of reagent covalently attached to the side chain of His-β427 is shielded from solvent and is oriented close to the side chain of Tyr-β345 in the subunit. The observation that 8-N_3-FSBA labels either Tyr-368 or His-427 in a given β subunit suggests that both residues are part of the subdomain in the β subunit that interacts with the adenine moiety of nucleotides bound to noncatalytic sites. As pointed out in the previous section, if this is indeed the case, the putative β turn defined by Gly-β426 and Gly-β429 would probably be the structural element involved in this interaction. From the cross linking results, it has been suggested that when the catalytic and noncatalytic binding domains on the same β subunit are occupied simultaneously, the purine rings of catalytic and noncatalytic nucleotides are adjacent (Zhuo *et al.*,

1992a). To conform with chemical modification and mutagenesis studies presented in the previous section, we suggest that the phosphoanhydride moieties of nucleotides bound to catalytic and noncatalytic sites on an interacting $\alpha\beta$ pair are distant from each other.

Penefsky and Cross (Penefsky and Cross, 1991) have suggested a different arrangement of nucleotides on noncatalytic and catalytic sites in an interacting $\alpha\beta$ pair. The model that they have proposed is based on the results of a study (Vogel and Cross, 1991) which showed that MF_1 is inhibited by certain 5′, 5′-bis-adenosine oligophosphates and on homology of segments of primary structure of the β subunit of MF_1 with residues in both the AMP and ATP binding sites of adenylate kinase. In the model proposed (Penesky and Cross, 1991) the phosphoanhydride moieties of nucleotides bound to the catalytic and noncatalytic sites of an interacting $\alpha\beta$ pair are in an orientation that would allow transphosphorylation. There are two problems with this model. First it does not account for considerable evidence cited in the previous section which suggests that the subdomain for the phosphoanhydride moiety of noncatalytic site is in the α subunit. Furthermore, the proposed homology between the binding domain for AMP in adenylate kinase and the domain for noncatalytic nucleotide is weak. For instance, in the proposed model, Arg-β189 of MF_1 is placed with Glu-β188 in the noncatalytic domain to conform with homology to Arg-44 of adenylate kinase which interacts with AMP. The equivalent of Glu-β188 is derivatized when TF_1 is inactivated with DCCD (Yoshida *et al.*, 1981). Substantial evidence suggest that this residue is at the catalytic site (Yoshida *et al.*, 1981; Ohtsubo *et al.*, 1987).

MODULATION OF CATALYTIC ACTIVITY BY LIGANDS BINDING TO NONCATALYTIC SITES

Enthusiasm for early interpretations of the kinetic behavior of isolated F_1-ATPases in terms of regulatory site to catalytic site interaction (Schuster *et al*,. 1975; DiPietro *et al.*, 1980, 1981) were dampened considerably when it was reported (Wise and Senior, 1985) that nucleotides bound at noncatalytic sites are not involved in oxidative phosphorylation. They observed that EF_1, presumably freed of endogenous adenine nucleotides by gel permeation chromatography in the presence of 50% glycerol, catalyzed GTP synthesis when reconstituted with plasma membrane vesicles depleted of EF_1. That the preparations of EF_1 were free of endogenous nucleotides was based on an A_{280}/A_{260} ratio of 1.90–1.95. This ratio was shown previously (Garrett and Penefsky, 1975) to indicate that MF_1 is free of endogenous nucleotides. This might not be the case for EF_1. It was reported (Hanada *et al.*, 1989) that gel permeation chromatography in the presence of 50% glycerol does not completely remove endogenous nucleotides from EF_1. Moreover, preparations of EF_1 submitted to gel permeation chromatography in the presence of 50% glycerol which exhibited anA_{280}/A_{260} ratio of 2.0 were shown to contain 2 mol of adenine nucleotide per mol (Issartel *et al.*, 1986). The difference in the number of tryptophan and tyrosine residues present in EF_1 (8 Trp; 109 Tyr) as opposed to the number present in MF_1 (1 Trp; 97 Tyr) (Walker *et al.*, 1985) is consistent with the results of Issartel *et al.* (1986). From these considerations, it remains an open question whether or not nucleotides bound to noncatalytic sites modulate oxidative phosphorylation.

Although a defined physiological role has not been elucidated for noncatalytic nucleotide binding sites, it is clear that the hydrolytic activity of the enzyme responds differently, depending on the nature of the nucleotide bound to these sites. It was shown in Boyer's laboratory (Milgrom *et al.*, 1990, 1991) that CF_1 is hydrolytically inactive unless ATPase not GTPase activity. A study recently completed in our laboratory with MF_1 depleted of endogenous nucleotides has shown that the hydrolytic activity of the mitochondrial ATPase also depends on the nature of ligands bound to noncatalytic sites. The filling of these sites with ATP, as opposed to ADP, is required to achieve optimal rates of ATP hydrolysis.

The steady-state kinetics of MF_1 have been examined over a large range of substrate concentration. From Eadie–Hofstee plots of kinetic data, Wong *et al.*, (1984) determined three K_m with associated V_{max} values for MF_1 containing endogenous nucleotides in the absence of bicarbonate and other activating anions ($K_{m1} = 1\,\mu M$; $V_{max1} = 26\,s^{-1}$; $K_{m2} = 160\,\mu M$, $V_{max2} = 310\,s^{-1}$; $K_{m3} = 1\,mM$; $V_{max3} = 191\,s^{-1}$). In the presence of bicarbonate, the highest K_m disappeared with its associated V_{max}. On the other hand, only two K_m and associated V_{max} values ($K_{m1} = 1\,\mu M$; $V_{max1} = 20\,s^{-1}$; $K_{m2} = 260\,\mu M$; $V_{max2} = 680\,s^{-1}$) were observed for MF_1 depleted of endogenous nucleotides. Curiously, ATP hydrolysis

by TF_1, which does not contain endogenous nucleotides, exhibited three K_m and associated V_{max} values. Gresser *et al.*, (1982) reported two K_m and associated V_{max} values for their preparations of MF_1 ($K_{m1} = 1.7\,\mu M$; $V_{max1} = 15\,s^{-1}$; $K_{m2} = 250\,\mu M$; $V_{max2} = 356$). Given that the K_{m3} and V_{max3} determined by Wong *et al.*, (1984) disappear when endogenous nucleotides are removed and were not observed with the preparation of Gresser *et al.*, (1982), it is possible that these values may be caused by heterogeneity of occupancy of noncatalytic sites.

Owing to its complicated characteristics, the steady-state kinetics of MF_1 has been variously interpreted. For instance, Cross and colleagues (Cross *et al.*, 1982; Cross, 1988; Cunningham and Cross, 1988) have suggested that three catalytic sites participate equivalently in hydolyzing ATP under saturating conditions. They propose that the first catalytic site is filled at much less than micromolar concentrations of ATP and suggest that the first K_m noted by the Boyer and Hatefi laboratories (Gresser *et al.*, 1982; Wong *et al.*, 1984) represents bisite catalysis. However, they have reported that the K_m and V_{max} values for bisite catalysis are $30\,\mu M$ and $300\,s^{-1}$, respectively, on the basis of Lineweaver–Burk plots (Cross *et al.*, 1982). Also from the Lineweaver–Burk plots, they suggest that the K_m and V_{max} values for trisite catalysis are $150\,\mu M$ and $600\,s^{-1}$, respectively. With an opposing point of view, Berden *et al.*,(1991) suggest that one of the two nonexchangable nucleotides in MF_1 is bound to a catalytic site, thus removing one of the potential catalytic sites from participation in catalysis. According to the two catalytic site model that they proposed, the K_m of about $1\,\mu M$ associated with V_{max} of $10–20\,s^{-1}$ observed (Gresser *et al.*, 1982; Wong *et al.*, 1984) represents ATP hydrolysis at a single catalytic site with ADP release stimulated by ATP binding to the second noncatalytic site to be filled. Two phases of bisite catalysis are thought to occur, one unaugmented and the other augmented by ATP binding to the third noncatalytic site. Unaugmented bisite catalysis is thought to procede with K_m and V_{max} values of 20–$50\,\mu M$ and $300\,s^{-1}$, respectively. In augmented bisite catalysis, it is proposed that ATP binding to the third noncatalytic site accelerates the rate of ADP release form catalytic sites. The apparent K_m and V_{max} values associated with ATP binding to the third noncatalytic site are $200–500\,\mu M$ and $660\,s^{-1}$, respectively. Each model has a major weakness. That favored by Cross and colleagues (Cross *et al.*, 1982; Cross, 1988; Cunningham and Cross, 1988) does not take into account participation of noncatalytic sites in modulating catalytic activity. On the other hand, the hypothesis of Berden *et al.*, (1991) that one of the potential catalytic sites participates in a noncatalytic role is not supported by substantial experimental evidence. Obviously, more experimental information is required before we have a full understanding of the kinetic mechanism of ATP hydrolysis catalyzed by the isolated F_1-ATPase and its control by adenine nucleotides binding to noncatalytic sites.

The differential sensitivity of ITP hydrolysis, opposed to ATP hydrolysis, to inactivation by FSBA or 8-N_3-FSBA suggests that binding of ATP to noncatalytic sites affects the global conformation of the enzyme. Whereas three noncatalytic sites of MF_1 must be modified with FSBA or 8-N_3-FSBA to observe maximal loss of ATPase activity, only a single noncatalytic site is modified when maximal loss of ITPase activity is attained (Bullough and Allison, 1986b; Zhuo *et al.*, 1992a). Endogenous nucleotides dissociate as the enzyme is derivatized. To explain these observations, it has been suggested that modification of a single noncatalytic site with either reagent is sufficient to distort the enzyme into an inactive conformation. During assay of ATP hydrolysis, it is thought that ATP binding to an open, unmodified noncatalytic site partially restores the active conformation. However, since ITP apparently does not bind to noncatalytic sites, no restoration takes place during assay of ITP hydrolysis.

Three kinetic modulations associated with binding of adenide nucleotides to noncatalytic sites of MF_1 have been described (Bullough *et al.*, 1988), which disappear as the third noncatalytic site is derivatized with FSBA. The modulations affected are: ADP-induced hysteretic inhibition which is associated with ADP binding to three noncatalytic sites; the Hill coefficient which increases from 0.6 to 1.0; and an augmented rate observed at pH 8.0 over that observed at pH 6.5.

LOCATION AND FUNCTION OF THE SITE THAT BINDS INHIBITORY AMPHIPATHIC CATIONS

A large number of amphipathic cations inhibit the ATPase activity of isolated and membrane-bound F_1. The inhibitors include substituted alkyl guanidines (Tuena de Gómez Puyou *et al.*, 1977, Feldman and Sigman, 1985); substituted phenothiazines

(Chazotte *et al.*, 1982, Laikind *et al.*, 1982); substituted xanthenes (Emaus *et al.*, 1986; Wieker *et al.*, 1987; Bullough *et al.*, 1989a); substituted acridines (Bullough, 1989a, b); dequalinium (Zhuo and Allison, 1988; Zhou *et al.*, 1992b); the bee venom peptide, melittin; and synthetic peptides corresponding to the presequence of yeast cytochrome oxidase subunit IV (Bullough *et al.*, 1989a). Steady-state kinetic analyses have shown that the amphipathic cations are noncompetitive, mixed, or uncompetitive inhibitors (Wiecker *et al.*, 1987; Bullough *et al.*, 1989a) Although quinacrine is a weak inhibitor (K_d = 580 μM), the aziridinium generated from quinacrine mustard is an effective inactivator of MF_1 (K_d = 27 μM). It has been shown that inactivation of the ATPase with the aziridinium is accompanied by modification of one or any one of the carboxylic acid side chains in the segment of the β subunit with the sequence DELSEED (Bullough *et al.*, 1986b). Several amphipathic cations protect MF_1 against inactivation by the aziridinium with an order of effectiveness roughly in accord with their experimentally determined K_I's. When irradiated at 350 nm in the presence of dequalinium, an effective protector against inactivation by quinacrine mustard, MF_1 is rapidly inactivated (Zhuo and Allison, 1988). With the use of [^{14}C] dequalinium, it has been shown that complete photoinactivation of the enzyme is accompanied by incorporation of about 1.5 mol of reagent per mol of enzyme (Zhuo *et al.*, 1992b). Photoinactivation of the enzyme is accompanied by mutually exclusive and equal derivatization of Phe-403 and Phe-406 in a single copy of the α subunit. Other amphipathic cations, particularly rhodamine 6 G, protect the enzyme against photoinactivation by dequalinium.

Chemical modification of MF_1 with quinacrine mustard and dequalinium, which appears to occur mutually exclusively, suggests that the binding site for inhibitory amphipathic cations includes the DELSEED segment of the β subunit and the segment of the α subunit surrounding Phe-403 and Phe-406. The sequences of the α and β subunits containing these segments from representative F_1-ATPases are compared in Table II. Whereas the sequence surrounding and including the DELSEED segment is highly conserved within β subunits, there is only weak homology, if any, between α and β subunits in this region. The α subunits are also highly conserved in the region including and surrounding Phe-α403 and Phe-α406 of MF_1. Both the Chou–Fasman (Chou and Fasman, 1974) and Garnier (Garnier *et al.*, 1978) algorithms predict that the DELSEED segment of β is in a short α-helix. As illustrated in Table II, the Chou–Fasman algorithm predicts that residues 378–405 of the α subunit are α-helical and that Phe-406 is the first residue of a β-turn. In contrast, the Garnier algorithm predicts that residues 377–406 are α-helical, thus placing Phe-406 at the end of the helix. The observation that photoinactivation by dequalinium is accompanied by equal and mutually exclusive derivatization of Phe-α403 and Phe-α406 is more consistent with the helical structure predicted by the Garnier algorithm. Energy minimization of the helical structure predicted by the Garnier algorithm places the aromatic rings of the two phenylalanine residues within 4.67 Å of each other in a position which would allow an aromatic ring of dequalinium to stack between them. In contrast, energy minimization of the helix-turn structure predicted by the Chou–Fasman algorithm places the phenylalanine side chains 6.27 Å apart with the *para*-position of Phe-α406 pointing toward the aromatic ring of Phe-α403.

Since amphipathic cationic peptides including melittin bind to this site with high affinity, studies have been carried out to determine if other subunits of the F_0F_1 complex protect MF_1 against inactivation by dequalinium and quinacrine mustard. The inhibitor protein (Pullman and Monroy, 1963) has no effect on inactivation by either reagent. Furthermore, inhibitory amphiphilic cations do not interfere with binding of the inhibitor protein. However, unpublished experiments have shown that OSCP protects MF_1 against inactivation by both quinacrine mustard and dequalinium.

From these preliminary results, it is tempting to suggest that the site that binds inhibitory cations is an interaction site for stalk subunits in the intact F_0F_1 complex. Three observations are consistent with this suggestion. (1) Although the amphipathic cations are not competitive inhibitors of F_1, ATP, ADP, ITP, and IDP are effective in protecting MF_1 against photoinactivation by dequalinium and inactivation by quinacrine mustard (Zhuo and Allison, 1988; and unpublished experiments). This suggests that the binding site for amphipathic cations is in conformational equilibrium with catalytic sites. (2) Membrane-bound MF_1 is more sensitive to a variety of amphipathic cations than soluble MF_1 (Mai and Allison, 1983; Wiecker *et al.*, 1987; Bullough *et al.*, 1989a; Bârzu *et al.*, 1989). Furthermore, it was reported that inhibition of membrane-bound F_1 by cetyltrimethylammonium is potentiated by oligomycin (Bârzu *et al.*, 1989). (3) Unpub-

Table II. Primary and Predicted Secondary Structures around Residues in α and β Subunits Labeled with Inhibitory Amphipathic Cations[a]

CF_1-β	Y K E L Q D I I A I	L G L D E L S E E D	R L L V A R A R K I	E R F L S
EF_1-β	$_{367}$Y Q E L Q D I I A I	L G M D E L S E E D	K L V V A R A R K I	Q R F L S_{401}
TF_1-β	Y K E L Q D I I A I	L G M D E L S D E D	K V L V A R A R K I	Q F F L S
YF_1-β	Y K S L Q D I I A I	L G M D E L S E Q D	K L T V E R S R K I	Q R F L S
Chou–Fasman	[[[[[[∧ ∧ ∧ ∧	∧ ∧ ∧ ∼ ∼ ∼ ∼ ∼ ∼ ∼ ∼	∼ ∼ ∼ ∼ ∼ ∼ ∧ ∧ ∧ ∧	∧ ∧ ∧ ∧ ∧
Garnier	[[∼ ∧ ∧ ∧ ∧ ∧ ∧	∧ ∼ ∼ ∼ ∼ ∼ ∼ ∼ ∼	∼ ∼ ∼ ∼ ∼ ∼ ∼ ∼ [∧	∧ ∧ [
MF_1-β	$_{381}$Y K S L Q D I A A I	L G M D E L S E E D	K L T V S K R A R K I	Q R F L S_{415}
MF_1α	$_{398}$R E V A A F A Q F G	S D L D A A T Q Q L	L S R G V R L T E L	L K Q G Q_{432}
Chou–Fasman	∼ ∼ ∼ ∼ ∼ ∼ ∼ ∼ [[	[[∼ ∼ ∼ ∼ ∼ ∼ ∼ ∼	[[[[∼ ∼ ∼ ∼ ∼ ∼	∼ ∼ [[[
Garnier	∼ ∼ ∼ ∼ ∼ ∼ ∼ ∼ ∼ [	∼ ∼ ∼ ∼ ∼ ∼ ∼ ∧	∧ [∧ ∧ ∧ ∼ Q	∼ [[[
YF_1-α	R E V A A F A Q F G	S D L D A S T K Q T	L V R G Q R L T Q L	L K Q N Q
TF_1-α	R E L A A F S Q F A	S D L D K A T Q A N	V A R G A R T V E V	L K Q D L
EF_1-α	$_{401}$R E L A A F S Q F A	S D L D D A T R K Q	L D H G Q K V T E L	L K Q K Q_{435}
CF_1-α	A E L E A F A Q F A	S D L D K A T Q N Q	L A R G Q R L R E L	L K Q S Q

[a] Symbols used to designate secondary structures predicted by algoriths of the (Chou and Fasman (1974) and Garnier *et al.* (1978): ∼, alpha helix; ∧, beta structure; [, beta turn; and no symbol, no preferred structure.

lished experiments have shown that aurovertin, the binding site of which is in conformational equilibrium with nucleotide binding sites in F_1 (Chang and Penefsky, 1974) and the oligomycin site in F_0 (Matsuno-Yagi and Hatefi, 1985), protects MF_1 against inactivation by quinacrine mustard. This protection is potentiated by ATP. Given these results, it is interesting that the DELSEED segment of the β subunit is 12 residues upstream from the position occupied by Arg-β398 of EF_1 which is underlined in Table II. Senior's laboratory has shown that substitution of this residue by His, Cys, or Trp reduces the sensitivity of EF_1 to inhibition by aurovertin (Lee *et al.*, 1989, 1991).

These preliminary observations suggest that the site that binds inhibitory amphipathic cations might be connected to one or more components of the stalk in the F_0F_1 complex. This raises several interesting questions. For instance, in isolated F_1, does this site undergo conformational changes during catalysis? Is a sluggish hydrolytic activity of prokaryotic and photosynthetic F_1-ATPases compared to that of mitochondrial F_1-ATPases related to a differences in the δ and ε subunits in the different enzymes (Walker *et al.*, 1985)? Certainly, further investigation of this intriguing site is warranted.

ACKNOWLEDMENT

This work was supported by U.S. Public Health Service grant GM16,974.

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