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Minireview

Catalytic Sites of *Escherichia coli* F_1-ATPase

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The catalytic site of *Escherichia coli* F_1-ATPase is reviewed in terms of structure and function. Structural prediction, biochemical analyses, and mutagenesis experiments suggest that the catalytic site is formed primarily by residues 137–335 of β-subunit. Subdomains of the site involved in phosphate-bond cleavage/synthesis and adenine-ring binding are discussed. Ambiguities inherent in steady-state catalytic measurements due to catalytic site cooperativity are discussed, and the advantages of pre-steady-state ("unisite") techniques are emphasized. The emergence of a single high-affinity catalytic site occurs as a result of F_1-oligomer assembly. Measurements of unisite catalysis rate and equilibrium constants, and their modulation by varied pH, dimethylsulfoxide, and mutations, are described and conclusions regarding the nature of the high-affinity catalytic site and mechanism of catalysis are presented.

STRUCTURE: MOST OR ALL OF THE CATALYTIC SITE IS LOCATED ON BETA-SUBUNIT

The α and β subunits of F_1 each contain both "Homology A" and "Homology B" consensus sequences (Walker *et al.*, 1982) which are diagnostic of nucleotide-binding proteins. The α subunit contains 20513 residues, the β subunit contains 459 residues,[2] and, for each subunit, secondary structure prediction using the Chou–Fasman approach suggests the presence of a nucleotide-binding fold ~ 200 residues long in the central part of the sequence (Duncan *et al.*, 1986; Senior, 1988; Maggio *et al.*, 1987; Pagan and Senior, 1990a), consisting of a series of six β-strands, with intervening α-helices and appropriate turns. Speculative tertiary folding models of α and β nucleotide sites have been presented (Duncan *et al.*, 1986; Rao, 1988).

Generally, these models resemble known structures, e.g., for *ras* p21, EF-Tu, adenylate kinase, and *recA* protein. Each of these proteins contains both Homology A and B sequences, component residues of which are directly involved in interactions with phosphates of the bound nucleotide. In all four cases, the interactions between Homology A residues and phosphates of the bound nucleotide were shown to be essentially the same, and it was suggested that the terminal aspartate of the Homology B sequence also has similar geometry in all four cases (Story and Steitz, 1992). It is not unreasonable to suppose that a similar arrangement of Homology A and B residues occurs around the α, β, and γ phosphates of bound ATP and ADP in F_1-α and β subunits.

The minimum subunit composition that supports ATPase activity is $(\alpha\beta)$-oligomer, which has a maximal ATPase turnover of $0.05\,s^{-1}$, as compared to $\geqslant 50\,s^{-1}$ in F_1 ($\alpha_3\beta_3\gamma\delta_\varepsilon$) or $\alpha_3\beta_3\gamma$. Isolated α or β alone did not have significant catalytic activity (Al-Shawi *et al.*, 1990b).[3] Table I describes the properties of nucleotide-binding sites in F_1, isolated α, and isolated β

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[2]All statements refer to *Escherichia coli* F_1-ATPase, unless specific reference to another species is made. All residue numbers refer to *E. coli* F_1-subunits.

[3]In thermophilic *Bacillus* PS3 (TF_1), the $\alpha_3\beta_3$ oligomer was shown to be catalytically active (Miwa and Yoshida, 1989; Yoshida and Allison, 1990).

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Table I. Nucleotide-Binding Sites in *E. coli* F_1, Isolated α-, and Isolated β-Subunits

Parameter	F_1	Isolated α	Isolated β
Hydrolysis substrates	ATP, GTP, ITP	None	None
Synthesis substrates	ADP, GDP, IDP	None	None
Number of binding sites for ATP, ADP	6 (3 exchangeable; 3 nonexchangeable)	1	1
Number of binding sites for GTP, ITP	3 (exchangeable)	0	1
Sites involved in catalysis	1. 10^{-10} M (K_d) 2. ~4 μM (K_M) 3. ~250 μM (K_M)	0	0
k_{off} ATP	$\leq 10^{-5}$ s^{-1} (3 nonexchangeable sites)	0.003 s^{-1}	≥ 0.1 s^{-1}
K_d ATP	—	100 nM	71 μM

References: Senior (1990); Al-Shawi *et al.* (1990b); Rao *et al.* (1988a); Pagan and Senior (1990b).

subunits. F_1 has six total nucleotide sites: three are exchangeable, three are nonexchangeable. The latter sites are clearly noncatalytic. A comparison of nucleotide preference (ATP vs. GTP/ITP), binding affinity, and "exchangeability" (k_{off} rates) supports the idea that the β sites are potentially catalytic. An important feature is apparent, which is that on formation of F_1-oligomer a high-affinity catalytic site emerges which is not present on isolated α or β. The two other sites involved in catalysis show K_M ATP 4 μM and 250 μM, and correspond more closely to isolated β-subunit in properties. The three exchangeable sites also show negative binding cooperativity for the analogs AMPPNP or *lin*-benzo-ADP (Wise *et al.*, 1983; Weber *et al.*, 1992). The minimum-size subunit oligomer ($\alpha_3\beta_3$?, $\alpha_2\beta_2$?) which can form the high-affinity catalytic site is not yet known.

Numerous point mutations which impair catalysis without substantially affecting F_1 structure have been found in both α and β subunits. A map of the currently known mutations in α-subunit is given in Pagan and Senior (1990a); the mutations in β subunit studied by our laboratory are discussed in Senior (1990) and Lee *et al.*, (1991). Our general conclusions from these studies may be summarized thus: (1) Mutations in α may inhibit steady-state ("multisite") ATP hydrolysis and ATP synthesis to varying degree. They do so primarily by impairing cooperativity between catalytic sites (i.e., β–α–β signal transmission). So far, they have not been seen to significantly affect intrinsic activity of the high-affinity catalytic site ("unisite catalysis"). Mutations of αK175 in the Homology A sequence inhibit steady-state ATPase only partially (Rao *et al.*, 1988b).[4] (2) In contrast, β mutations invariably affect both multisite and unisite catalysis. Major effects of β mutations on unisite catalysis have been noted (Senior and Al-Shawi, 1992; Al-Shawi *et al.*, 1989, 1990a). Mutations βK155E and βK155Q (Homology A) reduce multisite ATPase to 0.02% of normal, and βD242N (Homology B) reduces it to 0.04%.[5] (3) The inhibitory β mutations obtained so far fall predominantly in the region β137–β251 (Lee *et al.*, 1991), suggesting that this region is involved directly in β–γ phosphate bond cleavage and formation. (4) Residue βY331 has been shown to interact directly with the adenine ring of bound substrate, forming part of an obligatorily hydrophobic subdomain of the catalytic site. This interaction facilitates catalytic turnover (Weber *et al.*, 1992).

In summary, work from our laboratory using *E. coli* F_1 suggests that as a result of oligomerization of subunits into F_1, a high-affinity hydrophobic catalytic site appears on β-subunit, formed by residues β137–β335 approximately. We hypothesize that it has the features of a typical nucleotide-binding fold, with probably six parallel β-strands. Residues involved in β–γ phosphate-bond cleavage occur in the region β137–β251, and residues involved in adenine-ring binding occur around β331 (and possibly β297; see

[4] As also did the mutation αD26IN of Homology B sequence of TF_1 (Yohda *et al.*, 1988).

[5] These values are revised downward from previously published work (Senior, 1990) and will be described fully elsewhere (Senior and Al-Shawi, 1992).

Weber *et al.*, 1992). Whether the α-subunit abuts directly on the catalytic site is well worth considering although as yet uncertain. It appears plausible since β–α–β intersubunit conformational signal transmission is critical for physiological catalysis rates.

FUNCTION: STUDIES OF THE HIGH-AFFINITY CATALYTIC SITE OF *E. COLI* F_1 YIELD INSIGHTS INTO MECHANISM

Experiments conducted under unisite conditions, in which F_1/ATP concentration ratio was 3–10 or higher, demonstrated the presence of a high-affinity catalytic site, with reaction K_{eq} around unity, but with slow ATP hydrolysis, and release of Pi and ADP at rates well below steady-state turnover rates (Wise *et al.*, 1984; Duncan and Senior, 1985). K_d ATP at this site is six orders of magnitude smaller that K_M ATP for maximal steady-state ATPase. Promotion of catalysis at this site occurs when ATP binds to second and/or third site(s), accelerating the rate of ATP hydrolysis at the first (high-affinity) site up to steady-state turnover rate (Duncan and Senior, 1985). ^{18}O-isotope exchange measurements confirmed the presence of a reversible catalytic site and its modulation in the presence of higher ATP concentrations (Wood *et al.*, 1987).

It is apparent, therefore, that interpretation of steady-state F_1-ATPase measurements presents serious difficulties. The parameter K_M applies to substrate interactions at a different sites(s) from the parameter k_{cat}. K_M reflects in all probability a compounded, possibly synergistic effect of interactions at two or three different sites, and therefore the parameter k_{cat}/K_M has as yet only qualitative value when applied to studies of F_1. This has seriously impeded studies of reaction mechanism and regulation.

Recent progress in understanding enzyme mechanisms has derived largely from measurements of individual reaction rate and equilibrium constants of steps of reaction, thermodynamic interpretation of these data, and perturbation of the system by mutagenesis (Fersht, 1988; Knowles, 1987; Wells, 1990). With F_1 one would like to do similar analyses. For reasons noted above, steady-state measurements are too complex to yield appropriate data, thus directing our attention to pre-steady-state measurements. The major experimental advances have so far come from application of unisite techniques to obtain values of rate and equilibrium constants at the high-affinity catalytic site (Penefsky and Cross, 1991).

A.

$$F_1 + ATP \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} F_1{<}ATP \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} F_1{<}ADP.Pi \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} F_1{<}ADP + Pi \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} F_1 + ADP$$

B.

k_{+1} ($M^{-1}s^{-1}$)	1.1×10^5	K_aATP (M^{-1})	4.4×10^9
k_{-1} (s^{-1})	2.5×10^{-5}		
k_{+2} (s^{-1})	0.12	K_2 (K_{eq})	2.9
k_{-2} (s^{-1})	0.043		
k_{+3} (s^{-1})	1.2×10^{-3}	K_dPi (M)	2.4
k_{-3} ($M^{-1}s^{-1}$)	4.8×10^{-4}		
k_{+4} (s^{-1})	1.6×10^{-3}	K_dADP (M)	8.8×10^{-6}
k_{-4} ($M^{-1}s^{-1}$)	1.8×10^2		

C.

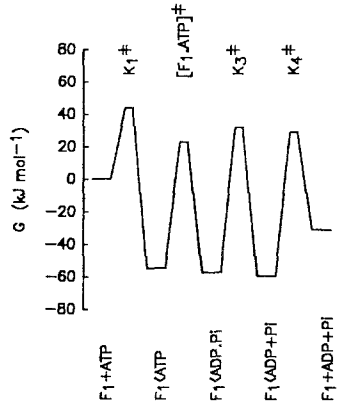

Fig. 1(A) Reaction steps of unisite catalysis. (B) Unisite rate and equilibrium constants at *p*H 7.5. (C) Gibbs free energy diagram of unisite catalysis, *p*H 7.5 (ATP, ADP, Pi = 1 M; free F_1 ($G_{F_1} = 0$)).

Unisite Rate Constants in *E. coli* F_1

There are four reaction steps to consider in unisite catalysis, namely (1) ATP binding/release, (2) ATP hydrolysis/resynthesis, (3) Pi release/binding, and (4) ADP release/binding (Fig. 1A). Methods for obtaining each of these rate constants in *E. coli* F_1 have been described and discussed in detail (Al-Shawi and Senior, 1992a).

Figure 1B gives the rate and equilibrium constants obtained for unisite catalysis in normal *E. coli* F_1 at physiological pH 7.5. The affinity for ATP ($K_a = 4.4 \times 10^9\ M^{-1}$) is high and the equilibrium constant for the catalytic step is 2.9. The K_d Pi is 2.4 M, i.e., there is effectively zero Pi binding at normal cellular Pi concentrations (6 mM; Kashket, 1982), and the Pi association rate (k_{-3}) must change by a minimum of 7–8 orders of magnitude under the influence of $\Delta\mu H^+$ in order to yield rates of 10–100 s^{-1} for ATP synthesis in cells. ADP binding affinity ($K_d \sim 9\mu M$) appears significantly lower than ATP binding affinity.

A Gibbs free energy profile of the reaction steps (Fig. 1C) shows that the enzyme has achieved a high

degree of efficiency, with relatively equal stabilization of different ground and transition states. The catalytic step transition state $[F_1\text{-ATP}]^{\ddagger}$ is most effectively stabilized. ATP release is, of course, an energy-requiring step, as others have already emphasized.

It is interesting to compare the unisite parameters of *E. coli* F_1 and bovine mitochondrial F_1 (MF_1). K_a ATP is smaller in *E. coli* by 2 orders of magnitude, implying that the catalytic site in mitochondrial F_1 makes more, or more effective, interactions with the substrate. Similar arguments hold for each of the transition states and for K_d Pi, implying that MF_1 is the more evolved enzyme (Knowles, 1987). Originally, Grubmeyer *et al.*, (1982) reported a K_d ADP of 0.3 μM for MF_1 , but recently Cunningham and Cross (1988) revised this value downward to 1.0 nM. This is considerably tighter than we have seen for *E. coli* F_1 (K_d ADP = 8.8 μM at pH 7.5; Fig. 1B). Values for *E. coli* F_1 K_d ADP in the micromolar range were also measured by equilibrium dialysis or centrifuge column technique (Wise *et al.*, 1981; Issartel *et al.*, 1986). Nevertheless, there is the possibility that this parameter is sensitive to environmental conditions (e.g., Pi ions) and we are currently reinvestigating this issue experimentally because it has interesting ramifications, as follows.

First, a tight ADP-binding (species $F_1 <$ ADP + Pi in Fig. 1C) would appear to put the enzyme into a "thermodynamic pit," which would disfavor ATP synthesis driven by $\Delta\mu H^+$. Second, lower-affinity ADP binding may be a requirement specifically for *E. coli* F_1, where ATP hydrolysis linked to proton extrusion is a physiological necessity which might be precluded by a tight (inhibitory) binding of ADP. Possibly, the requirement for a "loose" ADP site constrains the effectiveness with which the *E. coli* enzyme can bind ATP and the internal transition states, leading to an apparently less well-evolved enzyme when compared to MF_1.

Modulations of Unisite Catalysis by pH, Dimethylsulfoxide, and Mutations

pH Effects

Each of the eight rate constants was obtained at pH varied from 5.5 to 9.5 (Al-Shawi and Senior, 1992a). A salient finding was that neither the forward nor backward catalytic rate constant (k_{+2}, k_{-2}) was changed significantly over the whole pH range, implying that the catalytic site is effectively shielded from the medium. If a catalytic base side-chain is involved, a pK_a below 5 or above 10 is indicated, and the result implies, but does not prove, that protons *per se* are not reactants. ATP dissociation (k_{-1}) accelerated at higher pH, implying the presence of a side-chain with $pK_a \sim 8.0$ in the catalytic site, yielding increased net negativity. Pi binding (k_{-3}) slowed markedly at higher pH, implying the presence of a similar side-chain with $pK_a \sim 8.4$. It is tempting to consider that the same enzyme group is affecting both ATP dissociation and Pi binding, and to speculate that this group may be the ε-amino of residue βK155 in the Homology A sequence. The data showed that $H_2PO_4^-$ is likely to be the actual Pi species bound into the catalytic site. The effects of pH on ADP binding (k_{-4}) were much more gradual than on Pi binding or ATP release, suggesting that two different enzyme conformations occur, one for ATP and ADP · Pi binding, and one for ADP binding.

Dimethylsulfoxide Effects

Dimethylsulfoxide (40% v/v) decreased K_a ATP by 1500-fold and increased K_d ADP only 3-fold, supporting the idea of two different conformations of the catalytic site mentioned above, and implying that the ATP-binding conformation is normally the more hydrophobic (Al-Shawi and Senior, 1992b). The catalytic interconversion steps (k_{+2}, k_{-2}) were slowed ~10-fold, although the reaction equilibrium constant was little changed. The reduction in catalysis rates may well be related to the large decrease in K_a ATP (i.e., the substrate is now less constrained and the catalytic transition state is likely less stabilized also). We had initiated these experiments because we hoped to make actual experimental measurement of k_{-3} (Pi binding). However, significant Pi binding was not measurable (using centrifuge column technique) either in the presence or absence of dimethylsulfoxide.

Effects of Mutations

We found previously that mutations in β subunit can have large effects on the steps of unisite catalysis (Duncan and Senior, 1985; Al-Shawi and Senior, 1988; Al-Shawi *et al.*, 1989). We demonstrated that catalysis derives largely from use of binding energy consequent upon a large number of interactions between the catalytic site binding surface and bound substrates and transition states, and we proposed a mechanism for catalysis (Al-Shawi and Senior, 1990a). In recent work we have re-examined several mutants in light of the finding that in earlier work we

mistakenly used hybrid enzymes, containing both mutant and normal β subunits, in certain cases (βK155Q, E; βE181Q; βE192Q; βD242N, V). When we examined the homogeneous mutant F_1 in these cases, even greater effects on unisite catalysis were seen (Senior and Al-Shawi, 1992). However, the essential conclusions remain unchanged. Strong, correlated effects are seen on ATP binding/release (K_a ATP), internal catalysis (k_{+2}, k_{-2}), and Pi binding/release (K_d Pi). Much weaker effects are seen on ADP binding/release (K_d ADP), again supporting the idea of two major conformations of the catalytic site for ATP (and ADP · Pi) vs. ADP binding.

CONCLUSIONS

1. Arguments are presented for the proposal that the catalytic site in F_1 is formed from the central part of the β subunit, and specific functions are ascribed to regions of this domain.

2. A high-affinity catalytic site for ATP is formed on one β subunit when F_1-oligomer forms. This site has ATP hydrolysis rate $\sim 0.1\,s^{-1}$ and Pi and ADP release rates $\sim 10^{-3}\,s^{-1}$ in unisite catalysis, rising to an overall steady-state turnover of $\geqslant 50\,s^{-1}$ in ATP-loaded enzyme. Apparent K_M values of $\sim 4\,\mu M$ and $250\,\mu M$ suggest two sites bind ATP to promote catalysis.

3. Measurements of unisite catalysis parameters give insights into mechanism. Effects of varied pH, dimethylsulfoxide, and mutations give additional information. The catalytic site is hydrophobic and highly sequestered in one conformation ("ATP binding"). It contains an ionizable group (or groups), $pK_a \sim 8$, which appears to affect ATP release and Pi binding. In a second conformation ("ADP binding") the catalytic site is more hydrophilic. Pi binding is greatly disfavored at pH 7.5, and $\Delta\mu H^+$ must produce a very large change in order to allow Pi to bind for ATP synthesis. Catalysis derives in large part from binding energy derived from multiple interactions distributed over a catalytic binding surface.

4. The mechanism of rate enhancement due to positive catalytic site cooperativity is not discussed here. It may well derive from substantial changes in the conformation of the high-affinity catalytic site, resulting in further stabilization of the catalytic transition state; or perhaps from propulsion into the catalytic site, into the vicinity of the β–γ phosphates, of a specific catalytic side-chain. The number of actual catalytic sites capable of hydrolysis is also not discussed here. Our working hypothesis is that, at any one moment, catalysis occurs at one site only, with progression of this catalysis-competent site around the three β-subunits in a cyclical fashion.

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