**F** \(_{1}\)F\(_{0}\)-ATP synthase: development of direct optical probes of the catalytic mechanism

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Abstract

Using strategically-placed tryptophan (Trp) residues as optical probes to monitor nucleotide binding and hydrolysis, we demonstrate that all three catalytic nucleotide binding sites in F\(_{1}\)-ATPase must be filled to obtain physiological (\(V_{\text{max}}\)) MgATP hydrolysis rates. At \(V_{\text{max}}\) hydrolysis rates, the predominant enzyme species has one of the three catalytic sites filled with unhydrolyzed substrate MgATP, the other two sites are filled with product MgADP. A specifically-inserted Trp probe was also developed to characterize nucleotide binding to the noncatalytic sites, and a model to explain the specificity of these sites is shown. These sites appear to play no role in ATP hydrolysis.

Keywords: ATP synthase, F\(_{1}\)-F\(_{0}\); ATPase, F\(_{1}\); Catalytic mechanism; Nucleotide binding site; Tryptophan fluorescence

1. Introduction

F\(_{1}\)F\(_{0}\)-ATP synthase catalyzes the final step in oxidative phosphorylation, the synthesis of ATP from ADP and P\(_{i}\) (for reviews, see Refs. [1–3]). The nucleotide binding sites are located on the \(\alpha\)- and \(\beta\)-subunits of the F\(_{1}\)-sector, which has a subunit stoichiometry of \(\alpha_{3}\beta_{3}\gamma\delta\epsilon\). The total number of nucleotide binding sites is six [4,5]. Three of the sites, located primarily on the three \(\beta\)-subunits, are catalytic; the other three sites are noncatalytic and are located primarily on the \(\alpha\)-subunits [6]. Efforts to assign a function to the noncatalytic sites have so far failed.

Earlier attempts to study the catalytic mechanism, the role of noncatalytic sites, and the cooperative interactions within one type or between both types of sites, were seriously hampered by lack of direct probes to determine the degree of occupancy of each type of site under given experimental conditions. Conventional techniques were either too slow (e.g. equilibrium dialysis) or not true equilibrium methods (e.g. centrifuge columns [7]), and were unable to distinguish precisely between catalytic-site bound and noncatalytic-site bound nucleotides. Thus, there has been no accurate experimental measurement of the number of catalytic sites occupied, nor of the actual ligands present in the catalytic sites, during steady-state turnover. This means that all previous 'models' for the F\(_{1}\) mechanism were in effect really hypotheses (see, e.g., Refs. [3,8,9]). Here we describe how we have overcome this obstacle by generating fluorescence probes in the form of tryptophan residues specifically-inserted into both catalytic and noncatalytic nucleotide binding sites. Because of the ease of genetic manipulations, we are using the ATP synthase from *Escherichia coli*. All fluorescence experiments described below were performed on isolated F\(_{1}\), which can be prepared with high yield and high purity.

2. Specific placement of tryptophan residues as optical probes

It was first necessary to identify amino acid residues close to the nucleotide binding sites, where substitution by tryptophan (Trp) might result in an active enzyme with a fluorescence signal responding to nucleotide binding. For the catalytic sites, photolabeling with 2-azido-AMP [10,11] had suggested that \(\beta\)Tyr331 might be a suitable candidate. Using a combination of site-directed mutagenesis and fluorescence spectroscopy with the *ln*-benzoadenine analog of ADP, we were able to demonstrate conclusively that this residue forms a significant component of the adenine-binding subdomain [12]. Replacement of \(\beta\)Tyr331 by Trp in...
BY331W mutant F₁ gave an active enzyme, and an ideal probe to measure occupancy of the catalytic sites: the fluorescence of residue βTrp331 was substantial, and was quenched completely upon nucleotide binding to the catalytic sites [13,14]. This response is due to direct interaction of the residue with the adenine moiety of bound nucleotide [6]. The same quenching of fluorescence was seen with MgATP, MgADP, free ATP, free ADP, and a variety of nucleotide analogs [13,15,16].

To obtain a probe able to differentiate between catalytic site-bound ATP and ADP, Trp residues were introduced in several positions close to the P-loop (‘Homology A’) motif [17]. One of these, namely βTrp148, turned out to have the desired properties; the results are described below.

As to the noncatalytic sites, the case was less straightforward. Photolabeling experiments with 2-azido-ATP had identified βTyr354 as close to the noncatalytic binding site [10,11]. Nevertheless, a body of sound evidence was available, mostly from work on bacterial F₁, to show that the noncatalytic sites resided mainly on the α-subunits (reviewed in Ref. [18]). From the amino acid sequence, the residue in α-subunit corresponding to catalytic site residue βTyr331 is αArg365. Taking into account the preference of arylazido photolabel compounds for amino acids with nucleophilic side chains [19], we introduced a Tyr into position α365, and indeed, photolysis of 2-azido-ATP in presence of αR365Y mutant F₁ resulted in dramatically increased incorporation of photolabel into the α-subunit. Further experiments, using 2-azido-ATP photolabeling and lin-benzo-ATP fluorescence, confirmed that residues αArg365 and βTyr354 are both located in the adenine-binding subdomain of the noncatalytic sites [18]. A Trp residue was introduced at either position, and each gave a substantial fluorescence signal. On binding of nucleotides to the noncatalytic sites the fluorescence of residue αTrp365 was virtually completely quenched [20]. The crystal structure [6] confirms that the hydrophobic aliphatic middle portion of the αArg365 side chain makes close contact with the adenine moiety of MgATP.

**Fig. 1. Effect of nucleotides on tryptophan fluorescence spectra of βF148W mutant F₁.** βF148W is the sole Trp in the enzyme; all wild-type Trp residues had been previously substituted by Phe, Tyr, or Leu [26]. Dotted line, no nucleotide added. Solid line, (A) plus 2 mM MgADP; (B) plus 2 mM MgAMPPNP; (C) plus 0.5 mM ADP and beryllium fluoride; (D) plus 2 mM MgATP.
contact to the adenine moiety of noncatalytic site-bound nucleotide. βTrp354 gave no response on binding of nucleotide.

3. Catalytic sites: mechanism of ATP hydrolysis

Using the fluorescence signal of βTrp331, we determined thermodynamic and kinetic parameters for binding of nucleotides and nucleotide analogs to the catalytic sites [13–16]. With MgATP as the ligand, pronounced binding cooperativity was obvious. The first catalytic site exhibited a very high affinity and $K_{d1}$ could not be determined by the fluorescence method. $K_{d2}$ was 1 μM, $K_{d3}$ 30 μM. When tested under identical conditions, the substrate concentration dependence of the MgATPase activity could be described by a single $K_m$ value of 40 μM [13]. These results answered the important question as to how many of the three catalytic sites must be filled to obtain $V_{max}$ catalysis rates. It was known that MgATP bound at the first, highest-affinity site is hydrolyzed only very slowly, unless additional catalytic site(s) are filled with substrate [22]. Here we showed that filling of the second site was not sufficient to obtain significant hydrolysis rates; virtually all activity was due to enzyme molecules with all three catalytic sites filled (see Fig. 4C in Ref. [13], Fig. 3 in Ref. [23]).

Another important result obtained using the βTrp331 fluorescence was the demonstration that in absence of Mg$^{2+}$ all three catalytic sites exhibit the same affinity for ATP ($K_{d1,2,3} = 71 \mu$M) [14,15]. The pronounced binding cooperativity between the catalytic sites, as well as the catalytic competency, is critically dependent on Mg$^{2+}$ [15,23].

To further dissect the catalytic mechanism, it was necessary to determine which fraction of the catalytic sites is occupied by (unhydrolyzed) substrate MgATP during steady-state catalysis, and which fraction by product MgADP. This required a probe able to differentiate between the two nucleotides. As shown in Fig. 1, βTrp148 provided such a probe. With MgADP as the ligand, the fluorescence of βF148W mutant F1 at 350 nm is quenched (Fig. 1A). With MgAMPPNP the fluorescence at 325 nm increased (Fig. 1B), and very similar results were obtained with MgATP·BeF$_3$ complex (Fig. 1C), which is also isoelcetronic and probably isosteric with MgATP (see Ref. [24]). Binding of MgATP resulted in both a fluorescence increase at 325 nm and quenching at 350 nm (Fig. 1D). Detailed analysis revealed that during steady-state hydrolysis at mM concentrations of MgATP ($V_{max}$ conditions) one of the three catalytic sites was filled with substrate MgATP, the two other sites were filled with product MgADP. A model for MgATP hydrolysis encompassing these findings is shown in Fig. 2 (see legend for details). The fluorescence signal under $V_{max}$ conditions demonstrates that enzyme molecules in state C of the model are the predominant population. The crystal structure of bovine mitochondrial F$_1$ [6] could correspond to state D, which is, under substrate saturation, a short-lived transient conformational stage of the proposed cycle.

4. Noncatalytic sites: nucleotide binding and function

The fluorescence signal of residue αTrp365 allowed determination of thermodynamic and kinetic parameters for binding of nucleotides to the noncatalytic sites. There were pronounced differences in ligand binding behavior between noncatalytic and catalytic sites, in particular the noncatalytic sites exhibited no binding cooperativity [14,20]. Another difference was the specificity and surprisingly high affinity of the noncatalytic sites for pyrophosphate [14]. $K_d$ for MgPP, was about 20 μM, similar to the values found for MgATP, MgADP, and MgAMPPNP. We were able to use pyrophosphate as a trap to prevent nucleotide rebinding in experiments to determine rates of
dissociation of MgATP and MgGTP from noncatalytic sites. For MgATP, an off-rate of about \(1 \cdot 10^{-3} \text{ s}^{-1}\) was found when the dissociation reaction was initiated 20 sec after start of the association reaction, but the off-rate decreased considerably with time. In contrast, the MgGTP off-rate was found to be much faster, > 0.1 s\(^{-1}\); moreover, prolonged preincubation of the enzyme with MgGTP did not decrease the off-rate.

These findings resulted in the model for noncatalytic site nucleotide binding shown in Fig. 3. We propose that in the initial binding phase it is the 'pyrophosphate' end of the nucleotide molecule that is recognized, leading to 'state I', in which the site is occupied, but the bound nucleotide has a relatively high off-rate. Then, if the nucleotide contains adenine as the base, a conformational change is induced which sequesters the nucleotide in 'state II', rendering it virtually 'non-exchangeable' by reducing \(k_{\text{off}}\). With guanine nucleotide, this conformational change does not occur. This model explains the apparent preference of noncatalytic sites for adenine nucleotide, and the fact that the noncatalytic sites are usually filled with 'endogenous' ATP and ADP.

Further studies showed that rapid hydrolysis of MgATP at catalytic sites was obtained when not even a significant fraction of the noncatalytic sites was filled by nucleotide [20,25]. Thus, occupancy of the noncatalytic sites is not necessary for hydrolysis, and a physiologically relevant regulatory role for these sites appears unlikely [20]. As to the ATP synthesis reaction, we found that the mutation \(\alpha D261N\), which decreased the affinity of the noncatalytic sites for MgATP and MgADP drastically, caused no measurable impairment of oxidative phosphorylation in vivo [25].

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