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Insights into ATP Synthase Structure and Function Using Affinity and Site-Specific Spin Labeling

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A variety of different approaches has been used during the last couple of decades to investigate structure and function relationships within the catalytic portion of the F_0F_1-ATP synthase and of its interactions with the proton-translocator F_0. In our group, we employ ESR spectroscopy with the use of stable organic radicals, so-called spin labels, as reporter groups. The radicals are either attached to substrates/ligands or specifically inserted into the protein structure by "site-specific spin labeling." Both approaches bear intrinsic advantages for their special uses and result in the specific information that is available through ESR, e.g., structural changes due to binding of effector molecules (e.g., Mg^{2+} ions), conformational transitions during catalytic turnover, distance information on radicals bound at 20 Å or less, and information on the binding characteristics of labeled substrates. This review summarizes the results of a variety of different approaches we have used during the last years to study, with the help of ESR spectroscopy, the structure of the nucleotide binding sites of F_1-ATPases of different origins as well as interactions with F_0 subunits.

KEY WORDS: ESR spectroscopy; spin-labeled nucleotides; site-specific spin labeling; nucleotide analogs; conformational changes; photoaffinity spin labeling.

INTRODUCTION

Clearly, within the last decade of the 20th century, important findings in bioenergetics research brought a breakthrough in the understanding of nature's elegant design to solve the problem of energy transduction in biological systems. The finding that F_1-ATPase is, indeed, a molecular motor (Duncan *et al.*, 1995; Sabbert *et al.*, 1996; Noji *et al.*, 1997), as Boyer had already proposed in the 1970s and 1980s (reviewed in Boyer, 1993), working with almost 100% efficiency (Kinosita *et al.*, 2000) gave a further push to the field that far exceeded all expectations. What was known and often cited up to the middle of the last decade was that F_1-ATPases contain six nucleotide binding sites on the α and β subunits that are located at the interface between these major subunits (Cross and Nalin, 1982; Wise *et al.*, 1983; Xue *et al.*, 1987a; Bullough and Allison, 1988). Three of the sites are thought to be catalytic in nature, whereas no consensus has been reached on the function of the remaining three sites, although some researchers have reported evidence for a regulatory function of the so-called noncatalytic sites (Di Pietro *et al.*; 1980; Milgrom *et al.*, 1990, 1991). The use of the photoaffinity derivative of ATP, 2-N_3-ATP, showed that specific labeling of the catalytic sites resulted in modification of β-Tyr345 of the beef heart mitochondrial enzyme, while β-Tyr368 was specifically derivatized under conditions where preferably noncatalytic sites were occupied (Cross *et al.*, 1987). Similar observations were made for the specific modification of F_1-ATPases from other sources (Garin *et al.*, 1986; Xue *et al.*, 1987, Wise *et al.*, 1987; Guerrero and Boyer, 1989). The combination of the specific modification of either the catalytic or the noncatalytic nucleotide binding sites of F_1 using a probe that, in addition, is able to report about the environment of

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the nucleotide-binding site, i.e., that can sense conformational changes was and therefore, remains, a powerful tool for further investigating ATPase.

Electron-spin resonance spectroscopy (ESR) was shown earlier to be an important method for the investigation of structure–function relationships in biological systems. Through the introduction of stable organic radicals or "spin labels" by Harden McConnell in the 1960s (McConnell and McFarland, 1970), the method became applicable also to systems that do not contain an intrinsic paramagnetic center. With a specific reactive group, the radicals can be either linked to substrate or cofactor molecules and then report directly about the environment of the active sites within the enzymes (for examples, see reviews: Trommer, 1987; Park and Trommer, 1989; Trommer and Vogel, 1992, or original articles Neuhofen *et al.*, 1996, Haller *et al.*, 1997; Scheibel *et al.*, 1997). More recently, with the common usage of site-specific mutagenesis, a technique was introduced where cysteine residues are inserted into the protein structure at specific, mechanistically or structurally important positions by mutagenesis techniques and the reactive sulfhydryl group is then covalently modified with respective spin labels. The method is referred to as "site-specific spin labeling" and is broadly applicable for a variety of different systems (for review, see Feix and Klug, 1998).

Such ESR experiments employing spin labels, either attached to ligands or cofactors or directly to the proteins, serve several purposes: (1) Binding studies are possible under equilibrium conditions and even in turbid solutions and at concentrations up to about millimolar substrate, because the spectral line shape of the freely tumbling, not protein-bound spin label or spin-labeled substrate is distinctly different from the line shape of enzyme-bound and, therefore, immobilized radicals; (2) changes in the environment of the radical very strongly effect the shape of the ESR spectra, therefore making ESR spectroscopy a most conformationally sensitive technique; and (3) distances between two radicals up to 20 Å can be determined due to line broadening that results from dipolar interaction of the radicals. If the radicals are rigidly fixed within the protein, e.g., in substrate binding sites, dipolar interactions are obvious in the ESR spectra when the outermost splitting of the high-and low-field extrema is more than 80 G (for example, see Wilder *et al.*, 1989; Karim *et al.*, 1989; Hartmann *et al.*, 1991). Labeling of two amino acid side chains at close distance, however, leads to broadening of the spectral lines in the immobilized samples (i.e., in frozen solutions) because of the different and partially random orientations that the spin labels may take relative to each other. In 1995, Rabenstein and Shin published a paper where they correlate the line-broadening of the ESR signals to the distance of two spin labels that were attached to an α-helix-forming peptide at specific positions. The method became known as the "molecular ruler" and is employed in our laboratory, as well, for measuring intermolecular distances between proteins, e.g., the dimer structure of subunit *b* of F_0.

PHOTOAFFINITY SPIN LABELING OF F_1-ATPases

To study the relative environments of the nucleotide-binding sites of F_1-ATPase, a first analog of ATP, 8-N_3-2′,3′-SL-ATP, that both carries a stable radical and a photoactivatable azido function was introduced in 1988 (Vogel-Claude *et al.*, 1988). This analog carries an azido function that, upon irradiation, forms a reactive nitrene at the C8-position of the adenine ring. A stable radical is attached at the 2′,3′ position of the ribose. Photoaffinity analogs of substrates have the advantage that they can be covalently incorporated into the protein. Free spin-labeled substrate, that would otherwise dominate the ESR-spectra, because of the much narrower and stronger signals, can then be conveniently removed using gel filtration techniques (e.g., Penefsky, 1977). After such treatment, only the signal of the enzyme-bound component is observable in the spectra. The 8-N_3-2′,3′-SL-analog of ATP can be covalently incorporated into nucleotide depleted F_1-ATPase from beef heart mitochondria. Binding of the analog in the dark was rather weak, however, with apparent binding constants almost in the millimolar range. The low affinity of F_1-ATPase for this analog apparently depends on the equilibrium in solution between the normal "anti"-conformation of the adenine ring with respect to the ribose versus the "syn" conformation that is favored when the adenine is modified at the C8-position of the ring (Garin *et al.*, 1988). Such syn-predominated analogs are not bound by the ATPase with high affinity.

The use of a substrate analog that is better suited for specific high-affinity binding to and modification of the nucleotide binding sites of F_1-ATPases was introduced by the authors in 1992 (Vogel *et al.*, 1992). It carries the azido function at the 2 position of the adenine, 2-N_3-2′, 3′-SL-ATP, thereby favoring the normal "anti" conformation of the adenine with respect to the

ribose. The analog binds readily to F_1, as was shown in experiments like those described above, for using the 2-azido-ATP to photoaffinity label the enzyme. Like in the 8-N_3-SL-ATP analog, a spin label is attached to the ribose hydroxyl groups through an ester bond of a spin-label-carboxylic acid (2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid). Because of the *cis*-configuration of the 2′- and 3′-hydroxyl groups of the ribose, a transesterification between the 2′ and 3′ position is possible, resulting in a mixture of approximately 70% of the 3′ and 30% of the 2′ modification (Steckenbach *et al.*, 1980).

The Mg^{2+}-ion complex of 2-N_3-2′, 3′-SL-ATP is hydrolyzed by beef heart mitochondrial·ATPase (MF_1) at about 10 % of the rate of normal Mg^{2+}-ATP and up to about 5 mol of the analog can be bound to nucleotide-depleted enzyme in the dark (Vogel *et al.*, 1992). Upon irradiation, the equivalent amino acid residues of MF_1 are modified as with 2-N_3-ATP, indicating that the radical at the ribose does not strongly alter the binding of the substrate analog to F_1. ESR spectra of the corresponding complexes of nucleotide-depleted mitochondrial F_1-ATPase reveal additional peaks, with a distance of the outermost maxima of 80 G. This 80 G distance between the maxima that cannot be attained by immobilization effects of the radical and can, therefore, only be due to dipolar interactions of two radicals at rather close vicinity. In addition, increase in the power of the microwave in the experiment did not lead to saturation of the additional signals, which is an indication that the signals result from specific dipolar interaction of two radicals at close distance (Vogel *et al.*, 1992; Burgard *et al.*, 1994). The possibility that the 80 G signals result from hydrogen bonding of the radical to protein amino acids (Damerau and Fittkau, 1983) was also ruled out by the power saturation experiments.

Line-shape simulation analysis of the spectra suggest a distance of the radicals of 15 to 20 Å. A catalytic chase with normal unlabeled ATP resulted in the total loss of the additional signals, suggesting that at least one of the interacting radicals was bound to a catalytic site. The relative area of the "interaction peaks" and the peaks resulting from "normal" binding of the nucleotide analogs to MF_1, suggests that only a small population of binding sites is in a conformation that allows for interactions of the bound radicals. Observations of ATP analogs binding to MF_1-ATPase in solution at less than 20 Å were also made using fluorescence energy transfer (Divita *et al.*, 1993). However, the X-ray crystal structural models (Abrahams, *et al.*, 1994; Bianchet *et al*, 1998; Stock *et al.*, 1999) place the nucleotide binding sites at least 27 Å apart from each other. This seeming contradiction between X-ray and ESR or fluorescence data may be explained by the strong conformational changes that take place in F_1-ATPase during nucleotide binding and catalysis, possibly only transiently bringing two binding sites into closer distance. Alternatively, the small population of binding sites that are in the appropriate conformation for dipolar interactions to occur may possibly not be in a form that allows crystallization and may, therefore, not be visible in the crystal structure.

In addition to the signals that suggest dipolar interactions (80 G), and the signals that result from binding of the nucleotide analog to a nucleotide binding site in a rather rigid manner (68 G splitting, called the $2A_{zz}$ value), there is a second type of binding site that allows a high mobility for the protein-bound radical ($2A_{zz}$ = 57 G), indicating a binding site in a rather open conformation, as will be discussed later. Binding of SL-ADP does not result in dipolar interactions, indicating a different binding conformation of the spin-labeled ADP analog or a different conformation of the binding sites when ADP is bound instead of ATP or ADP + P_i.

The presence of Mg^{2+} ions is required for dipolar interactions to be visible in the ESR spectrum of mitochondrial F_1-ATPase in complex with 2-N_3-SL-ATP in the dark, as was described by Burgard *et al.* (1994). Binding of the analog is much weaker in the absence of Mg^{2+} and subsequent addition of $MgSO_4$ does not result in a significant change in the shape of the spectra or in additional binding. Clearly visible, however, are two sets of ESR signals with a $2A_{zz}$ of 68 G for the more rigidly bound radical and a $2A_{zz}$ of 57 G for the more mobile form, indicating a strong asymmetry of the binding sites of mitochondrial F_1-ATPase, even in the absence of Mg^{2+} ions.

F_1-ATPase from the thermophilic bacterium PS3 (TF1) also reveals nucleotide-binding sites that differ significantly in conformation, as can be seen from the different components of the corresponding ESR spectra. In the absence of excess Mg^{2+} ions, two signals that are almost equal in amplitude are observed that indicate two distinctly different environments of the spin labels, e.g., two distinct conformations of the binding sites. In the absence of Mg^{2+} ions, binding of the analog is weak, with maximum binding at about 3 mol/mol TF_1. Subsequent addition of Mg^{2+} ions brings both the binding stoichiometry and the shape of the ESR spectra to the same level (5 mol/mol) and

line shape as if Mg^{2+} ions had been present before addition of the nucleotide analog. This indicated that binding of Mg^{2+} ions and nucleotides is not occurring in an ordered mechanism. Dipolar interactions of spin-labeled nucleotides are not or only very weakly observed for the TF_1.

Further investigations revealed that such dipolar interactions of two radicals at a close vicinity are only observed for the beef heart mitochondrial enzyme. Neither in the case of TF_1, nor with the F_1-ATPases from chloroplasts or *Escherichia coli* were we able to observe ESR signals that suggested such a close vicinity of some of the nucleotide binding sites.

NUCLEOTIDE-INDUCED ASYMMETRY OF THE CATALYTIC SITES

A question that remained to be answered was concerned with the character of the nucleotide binding sites that show such strong structural differences, i.e., result in the ESR signals with $2A_{zz}$ values of 68 G and 57 G. A first guess was that the intrinsic structures of catalytic and noncatalytic sites may differ significantly and would, therefore, lead to the two different spectral components. A second hypothesis was that one type of sites, either the catalytic or the noncatalytic sites, are intrinsically heterogeneous in structure. The latter was favored due to Walker's X-ray model of the catalytic binding sites (Abrahams *et al.*, 1994), however, it was not clear whether the observed asymmetry in the catalytic sites shown in the structural model was induced by the presence of the different nucleotide during crystallization or whether an *a priori* asymmetric structure existed. An X-ray crystallographic model of a nucleotide-free $\alpha_3\beta_3$ substructure of the TF_1-ATPase suggested symmetric catalytic sites (Shirakihara *et al.*, 1997).

To investigate the possibilities, we made use of the photoaffinity group of our probes and the characteristic of TF_1 to readily dissociate into subunits that can then be reassociated into functional ATPase (Harada *et al.*, 1991 and references therein). We used isolated β subunits from TF_1 that were labeled with 2-N_3-SL-ATP and acquired the corresponding ESR spectra (Erbse *et al.*, unpublished results; see Fig. 1). As we expected, the spectra showed, after removal of nonreacted, unbound ANP analog, one single component for protein-immobilized radicals (Fig. 1, middle spectrum). Upon addition of stoichiometric amounts of unmodified α subunits, the line shape of the ESR

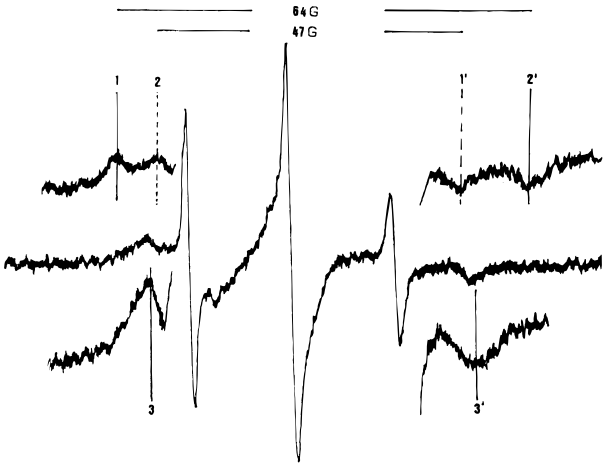

Fig. 1. Nucleotide-induced asymmetry of catalytic sites. Isolated subunits β of the TF_1-ATPase were covalently modified with the photoaffinity spin-label analog of ATP, 2-N_3-SL-ATP; the corresponding ESR spectrum is shown in the middle. The sharp signals in the middle portion of the spectrum result from a small amount of unlabeled, free 2-N_3-SL-ATP or from nonspecific binding of the analog to the surface of the protein. Signals 3,3′ show the signals of the protein-bound spectral component. Addition of unlabeled subunit α leads to the signals 1, 1′ and 2, 2′. The signal 3, 3′ is no longer observed under these conditions. The data show that upon interaction of labeled β with unlabeled α, the catalytic sites on β change conformation to form a closed and an open environment for the bound spin label.

spectra changed significantly. The originally observed signal of labeled β subunit was no longer visible. Two new signals were observed: one that indicates a stronger immobilization of the radical and, surprisingly, one signal that indicates a higher motional freedom for the protein-bound radical (compare signals 3 and 3′ to 1,1′ and 2,2′). Since binding of the ATP analog to the β subunits was covalent, the changes in the spectra can only be due to conformational changes within the β subunit upon interaction with the α subunits.

As was mentioned above, the X-ray crystallographic model of the $\alpha_3\beta_3$ ring in the absence of nucleotide shows high symmetry with respect to the nucleotide-binding site structure (Shirakihara, *et al.*, 1997), with all catalytic sites being in an "open" conformation. Our data suggest, that even without the asymmetry introduced by γ, binding of nucleotides results in conformational effects that are consistent with the "binding change mechanism" by Boyer (Boyer *et al.*, 1973; Cross, 1981), namely, a change in conformation within one catalytic site when nucleotides are bound to the remaining catalytic sites. The data might even suggest that α-bound nucleotides do

not play a role in these transitions, therefore, supporting findings by Senior's group that showed no effect of nucleotide binding to the noncatalytic sites on the mechanistic characteristics of the enzyme (Wise and Senior, 1985; Weber *et al.*, 1994).

CATALYTIC SITES ON THE CHLOROPLAST F_1-ATPase

As was mentioned before, dipolar interactions due to the presence of two nucleotide-binding sites at close vicinity seems to be a characteristic for beef heart mitochondrial F_1-ATPase. The two quite different sets of signals for enzyme-bound spin-labeled nucleotides, however, are visible in F_1-ATPases from a variety of different sources. Our investigations using F_1-ATPase from spinach chloroplasts (CF_1) gave some very interesting further suggestions about the characteristics/function of said conformational differences within the catalytic sites.

Native F_1-ATPase from chloroplasts has, upon purification, only very weak ATP hydrolysis activity relative to the highly active homologous enzymes from mitochondria and bacteria (Lien and Racker, 1971; for review, see Boyer, 1993). This is most likely because of an energy-conserving mechanism in plants to avoid futile ATP hydrolysis, e.g., in the dark when ATP synthesis is quiescent. *In vivo*, the ATP hydrolysis is modulated enzymatically, while *in vitro* researchers stimulate ATPase by various techniques that involve treatment with dithiothreitol (DTT), depletion of the inhibitory ε subunit, or so-called heat treatment, where the enzyme is brought to 60°C for a short period of time (Lien and Racker, 1971). We have studied the effects of such different treatments on the structure of the nucleotide-binding sites of CF_1, essentially as described above for F_1-ATPases from other species (Lösel *et al.*, 1996). CF_1 that was not activated by any of the treatments named above, showed, at low concentrations of the nucleotide analog, mainly one signal for protein-immobilized radicals. The outermost splitting ($2A_{zz}$ = 68 G) indicates that the radicals are rather immobilized. Only a very weak shoulder on the inner side of the main signal indicates a second, more open type of binding environment for the radical. At higher concentrations of spin-labeled nucleotides, the latter signal becomes more pronounced, indicating that the nucleotides bind to this second type of binding site with a slightly lower affinity. Similar results were obtained when CF_1-ATPase is used that was activated in the presence of DTT or that was depleted of the ε subunit. DTT activation has been correlated to a reduction of a disulfide within the γ subunit of the enzyme and it is thought that this results in conformational changes that bring on higher ATP hydrolysis rates (Dann and McCarty, 1992 and references therein). The results from our ESR investigations, however, show that the reduction of the disulfide bond does not change the relative conformations and affinity characteristics within the nucleotide-binding sites.

A strong change of the conformation of the nucleotide-binding sites of CF_1 is brought on, however, when heat treatment is used to activate the enzyme. After CF_1 was incubated at 60°C in the presence or absence of DTT, the corresponding ESR spectra in complex with $2N_3$-SL-ANP show, even at low nucleotide analog concentrations, a second signal indicative of a very open environment of the radical, i.e., a nucleotide-binding site in an open conformation. The relative ratio of the different conformations is close to one, suggesting that during the heat-activation procedure (with or without DTT), a conformational change occurs upon which binding of the nucleotide analog to an "open" conformation of the binding sites is favored. Increasing the concentration of 2-N_3-SL-ATP leads to filling of the sites of the more closed structure. Additional effects by DTT are not visible by ESR, although protein that was heat activated in the presence of DTT shows the highest ATP hydrolysis activities.

NUCLEOTIDE BINDING SITES ON THE *E. coli* F_1-ATPase

Using a variety of different spin-labeled nucleotides, we further investigated the structure of the catalytic and noncatalytic binding sites of the F_1-ATPase of *Escherichia coli* (Lösel *et al.*, 1997). A question we wanted to answer was whether the different conformational states of the binding sites that we observed for MF_1, TF_1, and CF_1 may have been caused by different phosphorylation states of the nucleotides bound, i.e., does spin-labeled ADP or SL-ADP + P_1 bound to the nucleotide sites result in ESR spectra that differ from binding of the corresponding ATP analog. To answer this question, we used in addition to 2-N_3-SL-ATP, a spin-labeled derivative of the nonhydrolyzable ATP analog, AMP–PNP. Binding of the nonhydrolyzable SL-AMP–PNP to EF_1 is weaker than the binding of analogs of hydrolyzable ATP. However, the two spectral components that were observed before are also

visible in the corresponding spectra (Fig. 2). The data therefore show that the different structures of the binding sites are not induced by hydrolysis of the nucleotide but are intrinsic to the structure of the enzyme or happen upon binding of the nucleotide. A chase of F_1-bound 2-N_3-SL-ANP using MgATP or MgGDP, which was shown not to bind to the noncatalytic sites (Weber *et al.*, 1994), results in ESR spectra that only contain the signal for the highly immobilized component, indicating that the more mobile component is due to interaction of nucleotide analogs with catalytic sites in a very distinct, open conformation. Finally, comparing the spectral shapes of nucleotide-depleted EF_1-ATPase that was fully occupied with the 2-N_3-SL-ATP-analog to the spectra of native F_1-ATPase that had retained some of the intrinsic adenine nucleotides bound to high-affinity sites, we were able to show that the noncatalytic sites are equivalent in structure and resemble, at least as far as measurable with the ESR technique, the structure of the catalytic sites in the closed conformation. There is, however, in both cases, a significant population of catalytic nucleotide-binding sites that retain a rather open conformation and that can be correlated to Boyer's "open" sites of the binding-change mechanism.

SPIN-LABELED NUCLEOTIDE ANALOGS: PREFERENCES OF F_1-ATPase

As was mentioned in the beginning of this review, the esterification of the ribose's 2′-and 3′-hydroxyl groups of the nucleotides with the spin label results in a dynamic mixture of two different analogs, one where the 3′-OH is modified (70 %) and one where the label is attached to the 2′-position of the sugar (30 %) (Streckenbach *et al.*, 1980). To understand the potential influence of the radicals bound at the different positions of the nucleotide, we synthesized a variety of different spin-labeled adenine nucleotides where the radicals are in defined positions and investigated the effects on binding and the shape of the corresponding ESR spectra (Motz *et al.*, unpublished results). In the SL-analogs, 3′-aminodeoxy-3′-SL-ATP and 2′-deoxy-3′-SL-ATP, the radical is permanently and specifically bound to the 3′position, while in the 2′-SL-3′-deoxy-ATP, the spin label is positioned at the 2′-hydroxyl of the ribose. Interestingly, except for the 2′-desoxy analog, all nucleotide analogs showed similar binding characteristics when added to native (not nucleotide-depleted) EF_1. This is consistent with the observed lower binding affinity of the ATPase for 2′-deoxy-ATP (Boos, 1976). All of the nucleotide analogs tested, however, gave rise to the two distinct spectral components that we have attributed to the different conformations of the catalytic sites.

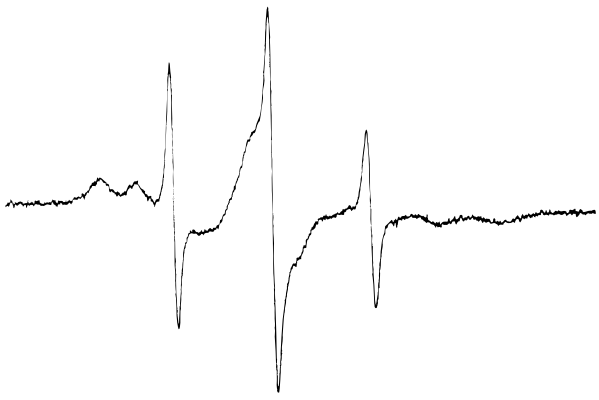

Fig. 2. ESR spectrum of SL-AMP–PNP in complex with EF_1-ATPase. The nonhydrolyzable spin-labeled ATP-analog, SL-AMP–PNP, was incubated with EF_1. Excess nucleotide analog was removed by passage through a centrifuge column. A remaining, very small amount of free SL-AMP–PNP (less than 5%) is visible as sharp signals in the center of the spectrum. The two distinctly different environments of enzyme-bound SL-AMP–PNP can be seen as the two signals in the low- and high-field region of the spectrum.

SITE-SPECIFIC SPIN-LABELING OF THE F_1-ATPase

As a further approach to study the structure of the nucleotide-binding sites and to be able to give information as to conformational changes that occur upon nucleotide binding or upon interaction with further components of the ATP-synthase, we employed the powerful technique of site-specific spin labeling (Kersten *et al.*, 2000). In these experiments, we used a mutant EF_1-ATPase, where β-tyrosine 331 of the catalytic sites was exchanged for a reactive cysteine residue (Wise, 1990). The mutant enzyme retains about 50% of normal catalytic activity. Specific labeling of β-Cys331 can be achieved by first modifying all accessible cysteine residues with *N*-ethylmaleimide (NEM), while the β-Cys331 of the catalytic sites are protected by excessive MgATP. The degree of modification was monitored by determining the amounts of accessible SH groups. Upon removal of excess NEM and MgATP, the enzyme was incubated with a stable, SH-specific spin label IOPI [4-(3-iodo-2-oxo-1-propylidenyl)-2,2,3,5,5-pentamethylimidazolidine-1-oxyl[(Volodarsky, 1988). The modified enzyme retains a specific activity of about 10 U/mg, no residual SH groups can

be determined. The ESR spectra (Fig. 3) of the β-Cys331 spin-labeled *E. coli*- F_1-ATPase consist of one signal of a highly immobilized radical component (closed conformation of the catalytic) and a very undefined signal component that can be seen as a shoulder to the defined signal (most likely due to radicals in an environment that is more open, but less clear in its structure and thereby resulting in an overlay of a variety of spectral components). The spectra are shown in Fig. 3A and B.

Addition of nucleotides, either AMP–PNP, ADP or ATP in the presence of Mg^{2+}-ions (see Fig. 3, spectra C, D, and E, respectively), for the first time directly shows the conformational changes that occur within the binding sites upon interaction with substrate, i.e., the "binding change" that was proposed by Boyer in the early 1970s. While a subset of binding sites change the conformation into a more rigid, closed form (the spectral peaks 1 and 1′ are at a greater distance), a second population of sites opens up (peaks 2 and 2′) to form the site that most likely is able to release product in the catalytic cycle. From the relative area of the signals, we estimate, that under these conditions more than two sites are in the closed conformation (comprising the "loose" and the "tight" form in binding change terminology) while the population of sites in the "open" conformation (binding change terminology) is less than 1.

Interestingly, this population of binding sites in the open conformation increases significantly when F_1 is in complex with subunits of the membrane–complex F_0 (see Fig 4, arrows). We studied this effect using a truncated form of the subunit *b* of F_0 (Dunn, S.D., 1992) and the specifically β-331-labeled EF_1. Independent of the presence of nucleotides, a drastic conformational change takes place within the catalytic sites when soluble subunit *b* is added to the modified F_1 (compare spectra A to A′ and B to B′, of Fig. 4). The signal for the mobile radical, i.e., the spin label bound to the "open" site, increases significantly to a ratio of 1.5 to 2 open sites compared the 0.5 to 1 sites that were open in the absence of *b* (cf. Figs. 3 and 4). The data suggest that subunit *b* of F_0 may not simply be the "hook" that holds F_1 to the membrane and that functions as a stator during proton translocation and energy transduction. One functional implication of this data is that the pathway of ATP hydrolysis by F_1-ATPase and the reverse pathway of ATP synthesis by the F_0F_1-complex may not be identical.

STRUCTURE AND INTERACTIONS OF SUBUNIT *b* OF F_0

We are presently studying the structure of the "stator–subunits *b*" of the F_0F_1-ATPase from *E. coli*,

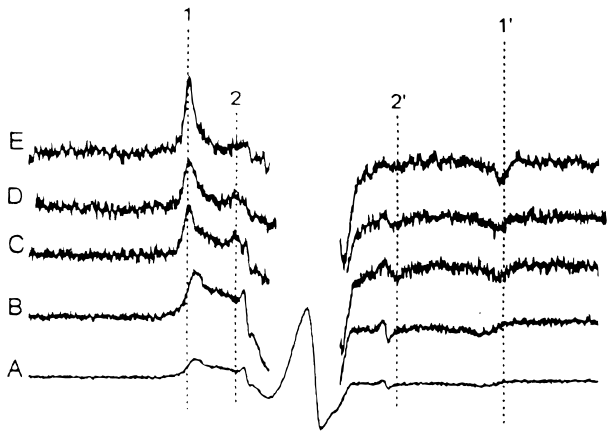

Fig. 3. Change of the catalytic site conformation upon nucleotide binding. (A) ESR spectra of *E. coli* F_1-ATPase that was site-specifically labeled at β-331 using an IOPI spin label (see text) in the absence of nucleotides; (B) spectrum as in (A), acquired at higher signal gain for better visualizing the signal; (C) AMP–PNP is added; (D) ADP is added; (E) ATP is added. All added nucleotides were 10 mM and in the presence of 1 mM $MgSO_4$. In all cases, the interaction with the nucleotides lead to a larger splitting (higher immobilization of the radical) of signal 1,1′, while a new signal 2, 2′ (indicative of an open site) appears.

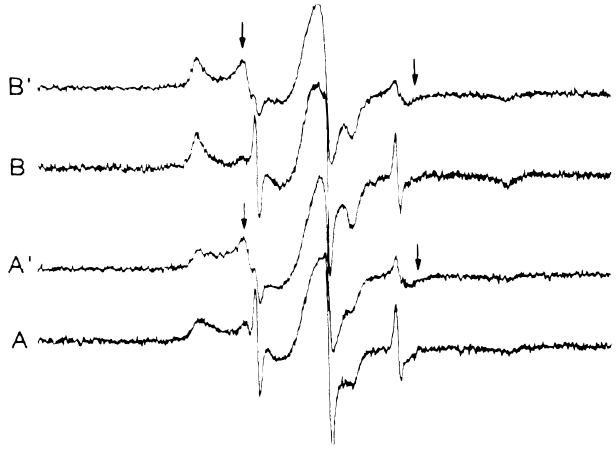

Fig. 4. Effect of binding of subunit *b*. (A) ESR spectrum of β-331 IOPI-labeled F_1-ATPase in the presence of 10 mM AMP–PNP and 1 mM Mg^{2+}; (A′) soluble subunit *b* was added; (B) ESR spectrum of β-331 IOPI-labeled F_1-ATPase in the presence of 10 mM ADP and 1 mM Mg^{2+}; (B′) soluble subunit *b* was added. The ESR signal that corresponds to the open catalytic site is marked by arrows. A small fraction (< 3 %) of unreacted IOPI spin label is visible as sharp signals at the side of the signals marked by arrows in the low field and also in the high field. Due to the dilution upon addition of subunit *b*, the signal amplitude of the unreacted IOPI decreased.

as well as interactions of the *b* dimer with F_1-ATPase (Kraft *et al.*, unpublished). We have used the specific cysteine mutations of soluble subunit *b* that were introduced by Dunn's group (see McLachlin and Dunn, 1997; McLachlin *et al.*, 2000; Dunn *et al.*, 2000 and references therein) and labeled them with appropriate spin labels. Using the method of Rabenstein and Shin (1995), we obtained structural data that indicate that the subunits *b* form parallel helices in the middle portion of the *b*-subunits. A structural model of the *b*-dimer (amino acids 100–145) is shown in Fig. 5. The model was obtained using the relative distances between spin labels that we introduced into identical positions within each *b* subunit in the dimer and molecular modeling using the programs Quanta 97 CharmM from Molecular Simulations.

Our data also indicate that the structure of the soluble *b* dimer changes slightly upon interaction with F_1, resulting in a movement of the subunits relative to each other. In addition, the data show that under the conditions used for the ESR experiments binding of the *b* dimer also occurs to δ-depleted F_1, indicating that δ is not absolutely necessary for interaction of *b* and F_1.

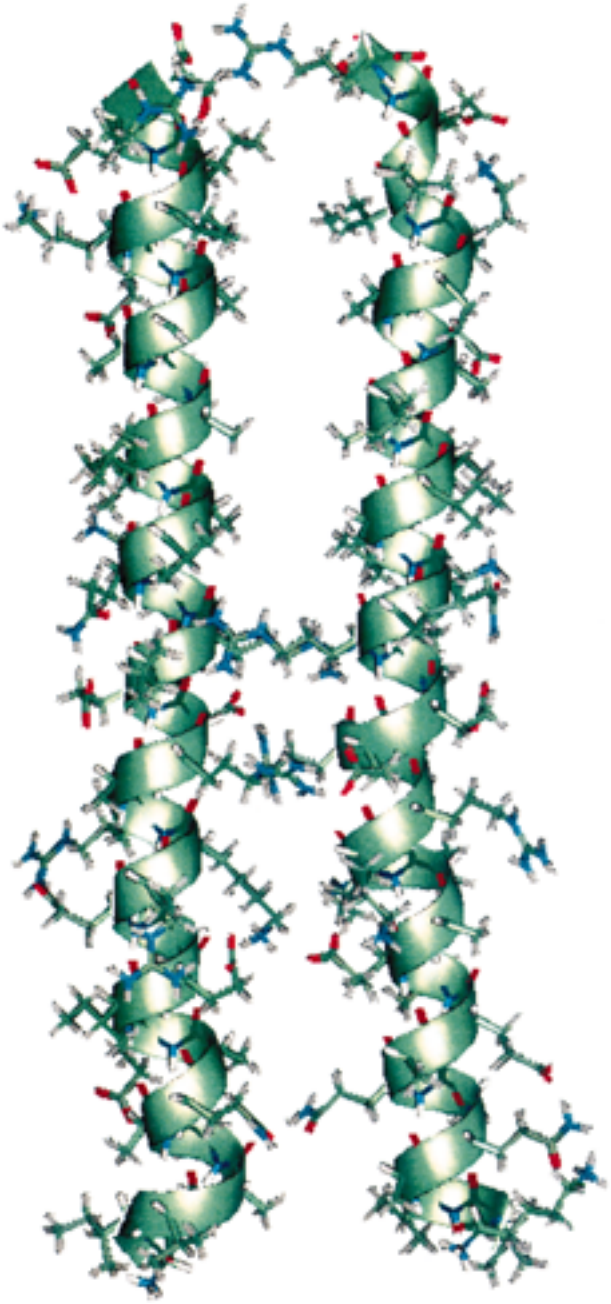

Fig. 5. A structural model of the subunit *b* dimer. The model of the *b* dimer was derived from ESR investigations involving site-specific spin labeling of a variety of mutants of soluble subunits *b*. The relative distances between the spin labels attached to both chains of the *b* dimer are consistent with parallel α helixes. The model was obtained using molecular modeling and the programs Quanta 97 and CharmM (Molecular Simulations).

OUTLOOK

The use of ESR spectroscopy in determining the structure and function within complicated biochemical systems has shown the potential power of this technique over the last years. For studies of the ATP synthase, the availability of a cysteine-free enzyme (Kuo *et al.*, 1998) will enable scientists to specifically modify important areas of the enzyme, which will then allow much more detailed information on mechanistically important conformations and conformational transitions. Using multiple labeling of substructures, we will be able to map the whole enzyme and substructures carefully to further our understanding of the structure and mechanism of this complicated and elegant enzyme. Furthermore, the development of newer and more powerful ESR techniques will allow us an even deeper insight into the details of dynamic protein structures that will clearly complement the information that is obtained by other biophysical techniques.

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