Site-Directed Spin-Labeling of the Catalytic Sites Yields Insight into Structural Changes within the F_oF_1-ATP Synthase of *Escherichia coli*†

Michael V. Kersten,‡ Stanley D. Dunn,§ John G. Wise,‡ and Pia D. Vogel*,‡

Fachbereich Chemie/Abteilung Biochemie, Universität Kaiserslautern, 67663 Kaiserslautern, Germany, and Department of Biochemistry, University of Western Ontario, London, Ontario, N6A 5C1, Canada

Received November 24, 1999

ABSTRACT: Electron spin resonance (ESR) spectroscopy using site-specific cysteine spin-labeling of the catalytic nucleotide binding sites of F_1-ATPase was employed to investigate conformational changes within the nucleotide binding sites of the enzyme. Mutant *Escherichia coli* F_1 that had been modified at position β-Y331C with a spin label showed almost normal catalytic activity and enabled us to study the effects of binding of different nucleotides and of the F_o subunit b on the conformation of the catalytic binding sites. The ESR spectra of the spin-labeled, nucleotide-depleted F_1 indicate asymmetry within the sites as is expected from the structural models of the enzyme. Nucleotide binding to the enzyme clearly affects the conformation of the sites; the most pronounced feature upon nucleotide binding is the formation of catalytic site(s) in a very open conformation. Using the same β-331 spin-labeled F_1 and a truncated form of F_o subunit b, b_{24-156}, we found that binding of b_{24-156} to spin-labeled F_1 significantly changes the conformation of the catalytic sites. In this paper we present data that for the first time directly show that a conformational binding change takes place upon binding of nucleotides to the nucleotide binding sites and that also show that binding of b_{24-156} strongly affects the conformation of the catalytic sites, most likely by increasing the population of binding sites that are in the open conformation.

F_oF_1-ATP synthase catalyzes the synthesis of ATP from ADP and inorganic phosphate by using a proton gradient across energy-coupling membranes. The *Escherichia coli* ATP synthase consists of a membrane integral proton channel called F_o that is composed of three different subunits with the stoichiometry ab_2c_{10-12} and a hydrophilic F_1 part ($\alpha_3\beta_3\gamma\delta\epsilon$) (*1*). Six nucleotide binding sites are located on the major subunits α and β (*2*−*4*); three of these sites are thought to be directly involved in the catalytic processes and are termed catalytic sites (*2*). The function of the three remaining binding sites on the α-subunits is not clear. They are referred to as noncatalytic sites. Further insight into this complicated enzyme was obtained by the X-ray crystallographic model of beef heart mitochondrial F_1-ATPase by Abrahams et al. (*5*) and more recently from Amzel and co-workers (*6*). One of the most striking features of the structure of the beef heart enzyme is the asymmetry within the conformation of the catalytic nucleotide binding sites, which nicely correlates with the binding change mechanism that was postulated by Boyer some 20 years ago (reviewed in ref *7*). Earlier data from our group using spin-labeled nucleotide substrates showed that asymmetry in the catalytic sites was observed also in solution and in fully active F_1 (*8*).

The possible function of F_o subunit b as a stator during the rotation of parts of F_o and γ relative to the $\alpha_3\beta_3$ substructure has drastically increased the interest of researchers in this protein. Little is known about the overall structure of this 156 amino acid long protein that consists of a short hydrophobic segment at the N-terminus, which is embedded in the membrane, and a highly hydrophilic portion that extends from the membrane (*9*−*11*) and is most likely involved in the binding of the F_1-ATPase to F_o. Earlier studies using a soluble form of the b-subunit, b_{24-156}, did not show any effect of the binding of b to F_1 on the ATP hydrolysis activity of the ATPase (*11*), and it was therefore assumed that binding of b did not directly affect the nucleotide binding sites.

ESR[1] spectroscopy is a valuable tool for investigating structural differences and conformational changes within proteins. Spin-labeled substrates have been successfully employed by our group to obtain information about structural changes in or near active sites of enzymes (*8*, *12*−*16*). In this paper we describe the use of site-directed spin-labeling, a technique that has been successfully applied in recent years (for review see ref *17*), to further study the mechanism of F_1-ATPase. We specifically modified β-Y331C (*18*) of the *E. coli* F_1-ATPase, which is located in the catalytic site of the ATPase, using an iodooxopropylidene spin label (IOPI), which then acts as an "integrated reporter group" to show conformational transitions within the catalytic sites.

MATERIALS AND METHODS

Enzyme Preparations. The β-$Tyr_{331}Cys$ mutant of *E. coli* F_1-ATPase was isolated from strain JP17/pJW1β-Y331C

† This work was supported by a grant from the Deutsche Forschungsgemeinschaft (P.D.V.) and a grant from the Medical Research Counsel of Canada, MT-10237 (S.D.D.).

* To whom correspondence should be addressed: Phone 49-631-205-3801; FAX 49-631-205-3419; Email vogel@chemie.uni-kl.de.

‡ Universität Kaiserslautern.

§ University of Western Ontario.

[1] Abbreviations: ESR, electron spin resonance; IOPI, 4-(3-iodo-2-oxo-1-propylidenyl)-2,2,3,5,5-pentamethylimidazolidine-1-oxyl.

10.1021/bi992699v CCC: $19.00
Published on Web 03/07/2000

Table 1

	specific ATPase activity[a] (units/mg)		SH groups detectable/F_1 (mol/mol)
unmodified β-Y331C F_1	14.6	6.8	8.6 ± 0.2
F_1 after treatment with NEM in the presence of MgATP and after nucleotide depletion	16.6	9.5	3.1 ± 0.1
F_1 after labeling with IOPI-SL	11.5	6.3	0.2 ± 0.05

[a] The data of two independent F_1 preparations are shown as examples.

using a modified procedure described in ref *18*. To specifically label the three cysteine thiol groups within the catalytic binding sites (β-Cys_{331}), the mutant F_1-ATPase was incubated with 6 mM Mg^{2+}/ATP to protect the nucleotide binding sites and was then treated for 25 min with a 190-fold molar excess of *N*-ethylmaleimide (NEM) over F_1 (10-fold excess over the naturally occurring 19 cysteines) to modify the accessible cysteine residues of the enzyme. The modified F_1-ATPase was depleted of excess and intrinsic nucleotides and excess NEM as described by Garrett and Penefsky (*19*), except that 0.5 mM dithiothreitol (DTT) was added to the buffer to stabilize the catalytic-site cysteine residues and react with unreacted NEM. The determination of the nucleotide content of the resulting F_1-ATPase using HPLC techniques (*20*) showed that about 1−1.5 mol of adenine nucleotide had remained bound after the nucleotide depletion column, presumably at noncatalytic nucleotide binding sites. The F_1-ATPase was then concentrated in an argon atmosphere using a Centricon 30 microconcentrator (Amicon) and could be stored at −80 °C. To spin-label the cysteine residues within the nucleotide binding sites, DTT was removed by passage of the sample through a Sephadex G50 fine column in 50 mM Tris-HCl, 1 mM $MgCl_2$, and 10% glycerol, pH 7.0 (ESR buffer). A 30-fold molar excess of iodooxopropylidene spin label [4-(3-iodo-2-oxo-1-propylidenyl)-2,2,3,5,5-pentamethylimidazolidine-1-oxyl, IOPI-SL; 10-fold excess over the three remaining SH groups in position β-331] was added to the eluted protein and the mixture was incubated for 10 min at 0 °C in an ice/water bath. The spin-labeled F_1-ATPase was then concentrated for ESR experiments by use of Centricon 30 microconcentrators (Amicon). Multiple dilution/concentration steps resulted in modified F_1-ATPase that contained only a very small fraction of unreacted IOPI spin label. All modification steps were monitored by determining the number of unreacted thiol groups with Ellman's reagent (*21*). To control for the specificity of the modification of β-Tyr331, wild-type F_1-ATPase was treated as described above for the mutant enzyme. The ESR spectra of such treated F_1 did not show the signals for enzyme-bound radicals, therefore indicating that no nonspecific labeling had taken place.

The ATPase activities were measured at 30 °C by the detection of P_i released (*22*).

The soluble portion of subunit b, b_{24-156}, was isolated as described (*11*, *23*). Incubation of b_{24-156} with F_1-ATPase was carried out in 10 mM HEPES(KOH), 5 mM $MgCl_2$, and 300 mM KCl, pH 7.0.

Spin Label. 4-(3-Iodo-2-oxo-1-propylidenyl)-2,2,3,5,5-pentamethylimidazolidine-1-oxyl was prepared as described in ref *24*.

ESR Spectroscopy. The ESR spectra were recorded on a Bruker ESP 300 E spectrometer operating in the X-band mode. The resonance cavity used was a dielectric cavity TE_{011} (ER 4118). The experiments were carried out at 295 K in quartz capillary tubes with a total volume of 20 μL. The spectra were recorded at 6.3 mW microwave power and a peak-to-peak modulation amplitude of 1 G. If needed, the signal gain was increased for better visualization of the signals. The experimental conditions are given in the figure legends.

RESULTS AND DISCUSSION

To further investigate the effects of binding of nucleotides and of subunit b on the relative conformation of the catalytic nucleotide binding sites of F_1-ATPase, we specifically spin-labeled a site-specific mutant of F_1, where the catalytic site βY331 had been substituted for a more reactive cysteine residue (*18*). Investigations had shown that the cysteine in position 331 did not drastically alter the kinetic characteristics of the enzyme (*18*). Earlier studies using photoaffinity labeling had shown that βY331of the *E. coli* F_1 was in close contact with the adenine of bound nucleotide (*25*). Also, the X-ray structural model of the beef heart mitochondrial F_1 by Abrahams et al. (*5*) places the corresponding amino acid of the mitochondrial enzyme in direct contact with the catalytic site bound nucleotides. Position 331 was therefore a very promising candidate for site-specific spin-labeling.

To specifically label the three cysteines in position 331 of the three β subunits in the presence of the other 19 naturally occurring cysteines in F_1 (*26*, *27*), we first modified all accessible cysteines in F_1 with *N*-ethylmaleimide (NEM) in the presence of a large excess of MgATP that protected the site-specifically introduced cysteines within the catalytic sites from modification. Table 1 shows two different but typical sets of modification experiments of site-specific spin-labeling of F_1 that were chosen to show the upper and lower limit of the ATPase activities that were obtained in a variety of different enzyme preparations. The ATPase activity as well as the number of sulfhydryl groups was monitored after every important modification step. Neither normal F_1-ATPase nor the β331C mutant is sensitive to NEM (data not shown). This finding was somewhat surprising, since a variety of V-type ATPases are sensitive to NEM (*28*) and it was thought that a cysteine residue in the vicinity of the binding sites is responsible for this inactivation. However, the modified cysteine of the V-ATPases is not in a homologous position to β331 of *E. coli* F_1 and its modification may affect nucleotide binding in a quite different way than labeling of position 331.

The presence of MgATP during NEM incubation successfully protected the positions β-Y331C of the F_1-ATPase from modification by NEM as can be seen in Table 1. The specific ATPase activity of the NEM-treated enzyme after passage through the glycerol column was even slightly higher than before NEM treatment (Table 1). This, however, is most likely due either to a further purification of the protein or to

removal of inhibitory nucleotides (*29*) in this step. Three cysteines were accessible in the F_1-ATPase after modification with NEM as compared to about nine accessible cysteines that were detected prior to the NEM modification step. The resulting F_1-ATPase contained approximately 1−1.5 mol of nucleotides as was determined by HPLC techniques. Prior to modification of β-Cys331 with the IOPI-SL, the modified F_1-ATPase was passed through a second Sephadex G-50 column, now eluted with ESR buffer containing 50 mM Tris-HCl, 1 mM $MgSO_4$, and 10% glycerol. Previous experiments showed that the modification of β-Cys331 was more efficient in this buffer than in the high-glycerol buffer used for nucleotide depletion; also, DTT that had been added during the nucleotide depletion step to protect the catalytic-site cysteines was removed by this column. A 30-fold molar excess of IOPI-SL over F_1 (10-fold excess over the remaining three SH groups) was then added and the reaction mixture was incubated for 10 min in an ice/water bath. Excess, unreacted IOPI-SL was removed by repeated passage through Centricon microconcentrators. Table 1 shows the ATP hydrolyis activity remaining after this treatment. The rather hydrophobic spin-label residue at position 331 did not alter the ATP hydrolysis activity drastically; 80−90% of the control unmodified specific activity was observed. Similar results had been obtained in earlier studies where a variety of amino acids was substituted for the normally occurring tyrosine at position β-Y331 (*18, 30*). Table 1 also shows that no SH groups were detectable after incubation of the nucleotide-depleted, NEM-modified F_1-ATPase with IOPI-SL, indicating that all three site-specifically introduced cysteine residues in position 331 of the three β-subunits were modified by IOPI spin label. This result also indicates that the residual nucleotide present after the glycerol chromatography step was most likely bound to noncatalytic sites. As a further control to show that the modification was specific for the catalytic-site residue Cys331, wild-type F_1-ATPase was treated as described for the mutant enzyme. Upon removal of excess NEM and nucleotide, F_1 was incubated with a 30-fold molar excess of IOPI under conditions as described above. The resulting ESR spectra does not show the signals that result from enzyme-bound radicals; only a very minor amount of freely mobile radical (unbound, not reacted IOPI spin label) could be detected that had not been fully removed by the dilution/concentration steps (data not shown). This strongly supports the conclusion that, under the conditions used, the IOPI spin label only reacted with Cys331 residues of the β-Y331C mutant F_1.

Figure 1A shows the ESR spectrum of F_1-ATPase that was site-specifically spin-labeled at position β-Y331C. For better visualization, the high- and low-field areas are mathematically enlarged in Figure 1B. One signal of a highly immobilized radical component (indicated by dotted lines in the spectra A and B of Figure 1, with a $2A_{zz}$ value of 63 G) is observed. In addition, a very undefined signal with a smaller $2A_{zz}$ value can be seen as a shoulder to signal 1 (arrows). The latter seems to be the result of overlapping spectra of a variety of radicals that are bound in slightly different environments. This indicates strong conformational differences within the environments of the protein-bound spin label, suggestive of at least two significantly different conformations of the catalytic binding sites. The data are in accordance with the strong structural asymmetry of F_1 that

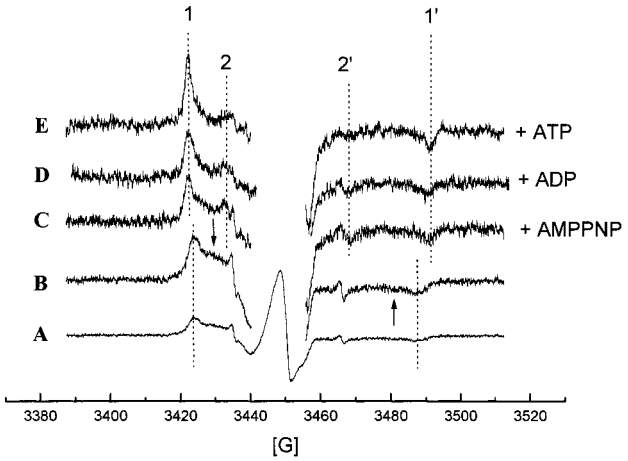

FIGURE 1: Effect of binding of various nucleotides on the conformation of the catalytic nucleotide binding sites of F_1. (A) ESR spectrum of 41 μM nucleotide-depleted F_1-ATPase that was labeled specifically in the catalytic sites with IOPI spin label. (B) The signal was mathematically enlarged for better visualization of the signals. (C) Conditions were as described for spectrum A, except that 10 mM AMPPNP was included in the sample. (D) Conditions were as described for spectrum A, except that 10 mM ADP was included in the sample. (E) Conditions were as described for spectrum A, except that 10 mM ATP was included in the sample. The experiments were carried out in 50 mM Tris-HCl, 1 mM $MgCl_2$, and 10% glycerol, pH 7.0 (ESR buffer).

was observed in the X-ray structural model (*5*). The structural model of the $\alpha_3\beta_3$ substructure of the thermophilic bacterium PS 3 in the absence of nucleotides shows three structurally identical catalytic sites in open conformations, and it was suggested that the catalytic sites took the open conformation when no nucleotides were bound (*31*). The ESR spectra shown here clearly demonstrate that even in the absence of nucleotides at the catalytic sites most of the catalytic sites of the *E. coli* F_1 are in a rather closed conformation that does not allow much rotational freedom to the radical. The second spectral component, seen as a broad shoulder on the inner side of the immobilized component, however, indicates that some of the catalytic binding sites are in a distinctly different, relatively open but diverse conformation and that there is an a priori asymmetry of the catalytic sites even in the absence of bound nucleotides. The data suggest that the presence of the minor subunits, most likely of γ, already induces asymmetry to the F_1 complex, even in the absence of nucleotides at the catalytic sites. It should also be added that the F_1 used in these experiments contained 1−1.5 mol of adenine nucleotides in the noncatalytic sites, which may also induce asymmetry within the catalytic sites.

Addition of 10 mM nucleotide in the presence of 1 mM Mg^{2+}, like the nonhydrolyzable analogue AMPPNP (Figure 1C), ADP (Figure 1D), or ATP (Figure 1E), led to a drastic change in the shape of the spectra. The $2A_{zz}$ values of the signals of the more immobilized spectral components (dotted lines, now also indicated as 1 and 1′) increased to 67 G (in the presence of AMPPNP), 68 G (in the presence of ADP), and 68.5 G (after incubation with ATP). A very sharp and defined spectral component (indicated as signals 2 and 2′) that is suggestive of a very mobile radical species is pronounced in the presence of AMPPNP and ADP and is also visible but not as pronounced after incubation with ATP (or ADP and P_i after turnover).

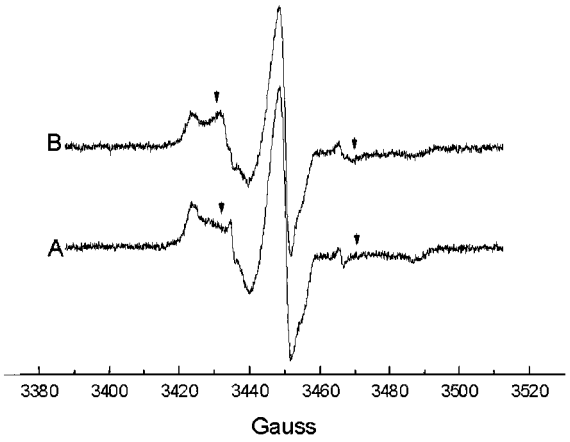

FIGURE 2: Effect of binding of b_{24-156} on the conformation of the catalytic nucleotide binding sites of F_1. (A) ESR spectrum of 43 μM nucleotide-depleted F_1-ATPase that was labeled specifically in the catalytic sites with IOPI spin label. (B) b_{24-156} dimer (130 μM) was added to the solution of spectrum A. The acquisition conditions for both spectra are identical.

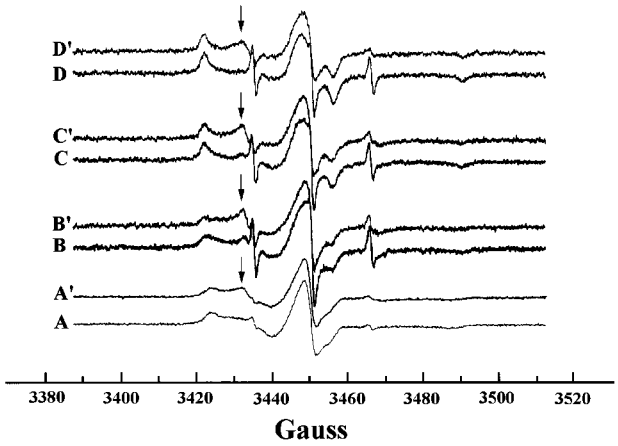

FIGURE 3: Effect of binding of b_{24-156} on the conformation of the catalytic nucleotide binding sites of F_1 in the presence of nucleotides. (A) ESR spectrum of 43 μM nucleotide-depleted F_1-ATPase that was labeled specifically in the catalytic sites with IOPI spin label. (A′) b_{24-156} dimer (130 μM) was added to the solution of spectrum A. Spectra A and A′ are similar to Figure 2 spectra A and B and were added for better comparison. (B) ESR spectrum of 43 μM of IOPI-labeled F_1-ATPase in the presence of 10 mM AMPPNP and 1 mM Mg^{2+}. (B′) b_{24-156} (130 mM) was added. (C) ESR spectrum of 43 μM IOPI-labeled F_1-ATPase in the presence of 10 mM ADP and 1 mM Mg^{2+}. (C′) b_{24-156} (130 mM) was added. (D) ESR spectrum of 43 μM IOPI-labeled F_1-ATPase in the presence of 10 mM ATP and 1 mM Mg^{2+}. (D′) b_{24-156} (130 mM) 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 to the right 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.

The data indicate that the asymmetry of the catalytic sites of F_1 that was observed by our technique even in the absence of nucleotides bound to the catalytic sites is further enhanced when nucleotides are added. The increase of the $2A_{zz}$ value of the more immobile radical component upon incubation with nucleotides can be readily explained by binding of the nucleotides to the binding sites. Direct interaction of the adenine moiety of the bound nucleotides with the radical at position 331 should decrease the mobility of the radical within the binding sites, resulting in higher $2A_{zz}$ values. The small differences within the $2A_{zz}$ values upon addition of the three adenine nucleotides used (AMPPNP, ADP, or ATP) may indicate small differences on the positioning of the nucleotides within the binding sites and therefore slightly different interactions with the amino acid side chains (or here the spin label). Alternatively, or in addition to this effect, nucleotide binding to the catalytic sites may make this closed site even more rigid than in the absence of nucleotide.

What is striking, however, is that a second type of binding site, in which the radical has much higher relative mobility, is formed upon binding of the nucleotides. This can only be explained by a conformational transition of some of the binding sites to a very open structure. Comparing the relative areas of the signals, we estimate that approximately 0.5 to maximally 1 out of the 3 sites is in the open conformation. Due to the overlap of the different types of signals and the rather high noise of the spectra, an accurate integration of the peak areas seems not feasible.

This is the first biophysical demonstration of a conformational transition within the nucleotide binding sites upon nucleotide binding and therefore the first direct physical demonstration of the binding change mechanism taking place upon binding of nucleotides to the catalytic sites.

In a second approach presented here, we investigated the effects of binding of a soluble portion of F_o subunit b, b_{24-156}, on the conformation of the catalytic sites of F_1-ATPase. We again employed F_1-ATPase where IOPI-SL was reacted at position β-Y331C of the catalytic sites. Figure 2 shows the ESR spectra of a typical experiment where a 3-fold excess of b_{24-156} (calculated for the dimer) was incubated with β-331-spin-labeled F_1. Figure 2A shows the spectra of nucleotide-depleted, spin-labeled F_1-ATPase similar to the spectrum in Figure 1A, showing at least two different relative environments of the radicals (two different conformations of the catalytic sites), one where only little motion of the radical is possible, and a second broad, but more mobile component as a shoulder to the immobilized species (arrows). A very small amount (1% or less) of unbound, unreacted IOPI is also visible as a rather sharp peak to the right of the low-field arrow in spectrum A of Figure 2. Addition of b_{24-156} results in a drastic change of the spectra as can be seen in Figure 2B. While the outer signal of the more immobilized spin label does not change significantly in position, the size of the inner signal of the more mobile spin label increases significantly and becomes more defined in shape. This indicates that the population of the two different conformations changes in favor of the more open conformational state, which may be attributed to the open conformation of the catalytic binding site. Due to dilution of the sample by adding b_{24-156}, the signals of the unreacted IOPI are hardly visible at all in spectrum 2B.

The effect of b binding to F_1 is independent of the occupation of the nucleotide binding sites and can also be seen in the presence of adenine and guanine nucleotides. As an example for the effects of binding of subunit b on the conformation of the catalytic sites in the presence of nucleotides, Figure 3 shows corresponding experiments that were performed in the presence of either no nucleotide (Figure 3, spectrum A without and spectrum A′ with b_{24-156}), or 10 mM AMPPNP, ADP, or ATP. Figure 3 spectrum B corresponds to the spectrum of labeled F_1 in the presence of

AMPPNP, and B′ is the respective spectrum after addition of b_{24-156}. Figure 3 spectrum C corresponds to the presence of 10 mM ADP, spectrum D results from the presence of ATP, and the corresponding spectra C′ and D′ are the spectra that result when b_{24-156} was added to the solutions of C or D, respectively. The signal of the enzyme-bound but mobile spin-label component (open conformation of the catalytic site) is in this experiment overlaid by a very small amount (less than 3%) of unreacted IOPI spin label that can be identified by the very sharp peaks in spectra B, C, and D. Upon addition of soluble subunit b (Figure 3, spectra A′, B′, C′, and D′), the signal that corresponds to the open conformation increases significantly (marked by arrows). Due to dilution effects upon the addition of subunit b, the signals of the unreacted IOPI spin label decrease and are no longer easily visible. Comparison of the relative areas of the two different enzyme-bound components (restricted versus open environments of the bound radicals) indicates that the population of open catalytic sites changes significantly from a ratio of approximately 0.5 open out of the total 3 catalytic sites to about 1.5 or possibly 2 open sites (Figure 3, spectra B′ and C′) upon addition of subunit b. Again, due to the different overlapping signals, a more accurate estimation on the number of catalytic sites in the respective different conformations by peak integration methods seems not feasible.

In earlier studies, no effect of the soluble domain of subunit b on the ATP hydrolysis activity of F_1 was detected (*11*). Nevertheless, the results presented here clearly demonstrate for the first time that binding of subunit b to F_1 strongly influences the conformation of the catalytic sites. The data may suggest that, in addition to the possible stator function of b, this subunit of F_o also introduces a permanent asymmetry into the F_oF_1 complex that is independent of nucleotide binding and may very well have implications on the mechanism of the whole F_oF_1 complex, especially since it changes the population of catalytic sites in the various conformational states.

REFERENCES

1. Foster, D. L., and Fillingame, R. H. (1982) *J. Biol. Chem. 257*, 2009−2015.
2. Cross, R. L., and Nalin, C. M. (1982) *J. Biol. Chem. 257*, 2874−2881.
3. Wise, J. G., Duncan, T. M., Latchney, L. R., Cox, D. N., and Senior, A. E. (1983) *Biochem. J. 215*, 343−50.
4. Xue, Z., Zhou, J.-M., Melese, T., Cross, R. L., and Boyer, P. D. (1987) *Biochemistry 26*, 3749−3753.
5. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature 370*, 621−628.
6. Bianchet, M. A., Hullihen, J., Pedersen, P. L., and Amzel, L. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 11065−11070.
7. Boyer, P. D. (1993) *Biochim. Biophys. Acta 1140*, 215−250.
8. Lösel, R. M., Wise, J. G., and Vogel, P. D. (1997) *Biochemistry 35*, 1188−1193.
9. Senior and Wise (1983) *J. Membr. Biol. 73*, 105−124.
10. Dunn, S. D., and Chandler, J. (1998) *J. Biol. Chem. 273*, 8646−8651.
11. Dunn, S. D. (1992) *J. Biol. Chem. 267*, 7630−7636.
12. Vogel, P. D., Nett, J. H., Sauer, H. E., Schmadel, K., Cross, R. L., and Trommer, W. E. (1992) *J. Biol. Chem. 267*, 11982−11986.
13. Burgard, S., Nett, J. H., Kagawa, Y., Schäfer, H.-J., Wise, J. G., Vogel, P. D., and Trommer, W. E. (1994) *J. Biol. Chem. 269*, 17815−17819.
14. Lösel, R. M., Erbse, A. H., Nett, J. H., Wise, J. G., Berger, G., Girault, G., and Vogel, P. D. (1996) *Spectrochim. Acta, Part A 52*, 73−83.
15. Neuhofen, S., Theyssen, H., Reinstein, J., Trommer, W. E., and Vogel, P. D. (1996) *Eur. J. Biochem. 240*, 78−82.
16. Haller, M., Hoffmann, U., Schanding, T., Goody, R. S., and Vogel, P. D. (1997) *J. Biol. Chem. 272*, 30103−30107.
17. Feix, J. B., and Klug, C. S. (1998) in *Biological Magnetic Resonance, Spin Labeling, The Next Millennium* (Berliner, L. J., Ed.) Vol. 14, pp 251−279, Plenum Press, New York.
18. Wise, J. G. (1990) *J. Biol. Chem. 265*, 10403−10409.
19. Garrett, N. E., and Penefsky, H. S. (1975) *J. Biol. Chem. 250*, 6640−6647.
20. Vogel, P. D., and Cross, R. L. (1991) *J. Biol. Chem. 266*, 6101−6105.
21. Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.
22. Taussky, H. H., and Schorr E. (1953) *J. Biol. Chem. 202*, 675−685.
23. McLachlin, D. T., and Dunn, S. D. (1997) *J. Biol. Chem. 272*, 21233−21239.
24. Volodarsky, L. B. (1988) in *Imidazoline Nitroxides*, Vol. 1, CRC Press Inc., Boca Raton, FL.
25. Wise, J. G., Hicke, B. J., and Boyer, P. D. (1987) *FEBS Lett. 223*, 395−401.
26. Walker, J. E., Fearnley, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M., and Tybulewicz, V. L. (1985) *J. Mol. Biol. 184*, 677−701.
27. Stan-Lotter, H., Clarke, D. M., and Bragg, P. D. (1986) *FEBS Lett. 197*, 121−124.
28. Bowman, B. J., and Bowman, E. J. (1986) *J. Membr. Biol. 94*, 83−97.
29. Hyndman, D. J., Milgrom, Y. M., Bramhall, E. A., and Cross, R. L. (1994) *J. Biol. Chem. 269*, 28871−28877.
30. Weber, J., Lee, R. S., Grell, E., Wise, J. G., and Senior, A. E. (1992) *J. Biol. Chem. 267*, 1712−1718.
31. Shirakihara, Y., Leslie, A. G., Abrahams, J. P., Walker, J. E., Ueda, T., Sekimoto, Y., Kambara, M., Saika, K., Kagawa, Y., and Yoshida, M. (1997) *Structure 5*, 825−836.

BI992699V