The Subunit b Dimer of the F0F1-ATP Synthase

INTERACTION WITH F1-ATPase AS DEDUCED BY SITE-SPECIFIC SPIN-LABELING*

Christian Motz‡‡§§, Tassilo Hornung‡‡, Michael Kersten‡‡, Derek T. McLachlin**‡‡††, Stanley D. Dunn**, John G. Wise‡‡, and Pia D. Vogel‡‡§§

From the ‡Department of Biological Sciences, Southern Methodist University, Dallas Texas 75275, §Pfachbereich Chemie der Universität Kaiserslautern, Erwin-Schödinger Strasse, D-67663 Kaiserslautern, Germany, and **Department of Biochemistry, University of Western Ontario, London, Ontario N6A 5C1, Canada

We have used site-specific spin-labeling of single cysteine mutations within a water-soluble mutant of subunit b of the ATP synthase and employed electron spin resonance (ESR) spectroscopy to obtain information about the binding interactions of the b dimer with F1-ATPase. Interaction of $b_2$ with a $\delta$-depleted F1 ($F_1+\delta$) was also studied. The cysteine mutations used for spin-labeling were distributed throughout the cytosolic domain of the b subunit. In addition, each position between residues 101 and 114 of b was individually mutated to cysteine. All mutants were modified with a cysteine-reactive spin label. The room temperature ESR spectra of spin-labeled $b_2$ in the presence of $F_1$ or $F_1-\delta$ when compared with the spectra of free $b_2$ indicate a tight binding interaction between $b_2$ and $F_1$. The data suggest that $b_2$ packs tightly to $F_1$, between residues 80 and the C terminus but that there are segments of $b_2$ within that region where packing interactions are quite loose. Two-dimensional gel electrophoresis confirmed binding of the modified b mutants to F1-ATPase as well as to F1-$\delta$. Subsequent addition of $\delta$ to $F_1-\delta b_2$ complex resulted in changes in the ESR spectra, indicating different binding interactions of b to F1 in the presence or absence of $\delta$. The data also suggest that the reconstitution of the ATP synthase is not ordered with respect to these subunits. Additional spectral components observed in b preparations that were spin-labeled between amino acid position 101 and 114 are indicative of either two populations of b subunits with different packing interactions or to helical bending within this region.

The F$_0$F$_1$-ATP synthase, an enzyme found in similar forms in all organisms, catalyzes the formation of ATP from ADP and inorganic phosphate, P$_i$. It uses the energy inherent in a proton gradient across energy-coupling membranes in bacteria, chloroplasts, and mitochondria to drive this endergonic chemical reaction. Like all related ATP synthases, the enzyme from Escherichia coli is highly asymmetric and can be divided into two sections. The membrane-associated F$_1$-part contains the nucleotide binding sites and has a subunit stoichiometry $\alpha_3\beta_3\gamma_5\delta\epsilon$. The membrane-integral F$_0$ part contains the proton-translocating unit and consists of subunits $ab\epsilon_{10-12}$.

Proton translocation appears to be catalyzed by a concerted action of subunits $c$ and $\epsilon$ and results in a rotary motion of subunits $c$. Rotary movement of subunits $c$ then drives rotation of the internal stalk of F$_1$, which consists of subunits $\gamma$ and $\epsilon$. This in turn drives the conformational transitions within the catalytic sites to enable product release from the binding sites (for reviews, see Refs. 1–3). Movement of the internal stalk of F$_1$ together with subunits $c$ of F$_0$ relative to the ring formed of subunits $\alpha$ and $\beta$ implies the necessity of a second, external stalk that functions as a stator to stabilize the F$_0$F$_1$ structure. Convincing evidence has been provided that supports a second stalk consisting of the dimer of subunit $b$ of F$_0$ and the $\delta$ subunit of F$_1$ (4–8). Subunit $a$ of F$_0$, in addition to functioning in proton translocation, appears also to be part of the stator. Subunit $a$ is positioned externally to the ring of c subunits, interacting with $c$ and the dimer of subunits $b$ (9–12). It has been discussed that rotation of subunits $c$, $\gamma$, and $\epsilon$ generates elastic torque within the two stalks that needs to either be stored or dissipated into conformational energy (13, 14). The knowledge of the structure of the stator subunits and their protein-protein interaction is, therefore, of great importance to the understanding of the mechanism of energy transduction within the F$_0$F$_1$-ATP synthase.

X-ray structural models of some substructures of the ATP synthase have been available for some time now (15–20). There are also NMR and/or x-ray structural data available for the smaller subunits $\epsilon$ and $\delta$ (21–23). Structural information on subunit $a$ has so far been limited to mutational analysis, chemical modification, and limited proteolysis (for example, see Ref. 24). Subunit $b$ forms a dimeric structure and contains a highly hydrophobic N-terminal region of 33 amino acids that functions as a membrane anchor (25). The remaining 121 amino acids are predicted to form an extended, mostly $\alpha$-helical structure that interacts with F$_1$. Ultracentrifugation and CD spectroscopic analysis indicate coiled coil packing interaction of $b_2$ (26–28). Recent CD spectroscopic studies of isolated subunit $b$ reconstituted into E. coli lipid vesicles further suggest ~14% $\beta$-turns in the secondary structure composition of $b$ (29). The relative length of the b dimer does not seem to be very crucial for function as was shown by mutational deletions and insertions into b (30, 31). Deletions of up to 11 and insertions of up to 14 amino acids were possible, while biological activity was retained.

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(residues 1–34) has been solved using NMR spectroscopy (32). The expression of an N-terminal truncated version of subunit \( b \) has allowed extensive studies of the dimer in a water-soluble form (Refs. 26–28 and 33; for reviews see Refs. 34 and 35). An x-ray crystallographic model of a monomeric version of a 60-residue part of subunit \( b \) was recently published (36).

Clearly one of the main functions of the subunit \( b \) dimer is to tether the \( a_{\beta_2} \) subunit ring to the \( F_0 \) part of the synthase. Recent results from Senior and co-workers (37) show that the binding affinity of subunit \( b \) to \( F_1 \) strongly influenced the conformation of the catalytic sites, giving most of the binding energy of more than 15 kJ/mol.

Previous work from our laboratory further showed that connecting \( F_1 \) to \( F_0 \) is not the only function of the \( b \) dimer. In studies where we specifically spin-labeled the catalytic sites of \( F_1 \), we demonstrated that binding of subunits \( b \) to the modified \( F_1 \) strongly influenced the conformation of the catalytic sites, rendering a larger population of catalytic nucleotide sites in a more open conformation (38).

ESR\(^{2}\) spectroscopy with the use of stable nitroxide radicals has been used extensively to study proteins and enzymes, including F\(_1\)-ATPases from different sources. The stable radicals can be either linked to enzyme substrates (Refs. 39–47; for reviews, see Refs 48 and 49), or they may be introduced by cysteine-specific spin-labeling (Refs. 50 and 51; for review, see Ref. 52). The use of these probes allows the observation of conformational transitions upon protein-protein interactions during catalytic turnover as well as of protein-dynamic events.

In the work described here we have introduced single cysteines along the amino acid chain of subunit \( b \) of the ATP synthase and chemically modified them with a cysteine-reactive spin-label. In addition to introducing spin labels throughout most of the \( b \) chain, we also inserted radicals at every position between residues 101 and 114. Observing the ESR spectra at room temperature in the presence and absence of \( F_1 \), dependent on the presence and absence of subunit \( b \), gave further information about protein-protein interactions between \( F_1 \) and the \( b_2 \).

MATERIALS AND METHODS

Protein—The \( E. \ coli \) strain used to express normal F\(_1\)-ATPase (SWM1) was a gift from A. E. Senior, University of Rochester, Rochester, NY. The cells were grown on supplemented minimal medium (+2.5% LB) containing chloramphenicol. Cultures of 12 liters were harvested in the late exponential phase, and F\(_1\)-ATPase was isolated by a modification of (53) that substituted a DEAE-cellulose anion exchange column (Whatman, 23 × 150 mm) for the high performance liquid chromatography column. The ATP hydrolysis activities were in the range of 20–30 units/mg. Protein samples were prepared for ESR experiments by concentrating the F\(_1\) solutions in Centricon 30 concentrators (Amicon) and desalting the F\(_1\) by two passages through centrifuge columns (54) Sephadex G-50 (Amerham Biosciences), equilibrated with 50 mM Tris-HCl at pH 8.0.

\( \delta \)-Depletion of F\(_1\)-ATPase (Smith et al. (55) with Some Modifications)—10 mg of \( E. \ coli \) F\(_1\)-ATPase were precipitated using 67% ammonium sulfate. The precipitate was dissolved in 100–150 \( \mu \)l of a buffer consisting of 50 mM glycine (NaOH), 2 mM EDTA, 1 mM ATP, and 10% glycerol, pH 9.4. The protein solution was applied to a Sephacryl S-300R column (1.5 × 95 cm) and was eluted with the same buffer. The main fraction eluted after 80 ml and was detected by its UV absorption at 277 nm. The main peak contained \( \delta \)-depleted F\(_1\) (F\(_{1-\delta}\)) and was concentrated in a Centricon 30 to about 50 \( \mu \)l. 2 ml of a buffer containing 50 mM Tris-HCl and 10% glycerol, pH 7.5, were added, and the sample was again concentrated to about 60 \( \mu \)l and stored at –80 °C.

Mutagenesis and Expression of Subunit \( b \)—The single cysteine mutations of subunit \( b \) were obtained by standard molecular biology techniques and as described (33, 34). Cysteine mutants of the truncated subunit \( b \) dimer were expressed from \( E. \ coli \) strain JM 109.

Purification and Chemical Modification of Subunit \( b \)—Truncated single cysteine mutants were purified as described (33). The protein-containing fractions were assayed for protein content using the Bradford technique (56) and were pooled and concentrated to 3–5 ml using Amicon ultrafilters equipped with PM-10 membranes. The protein was then aliquoted and stored at –80 °C. For chemical modification the proteins were transferred into a buffer consisting of 50 mM Tris-HCl, 5 mM MgSO\(_4\), and 300 mM KCl, pH 7.5, by passage through a Sephadex G-25 column equilibrated with the same buffer. The protein-containing fractions were pooled and concentrated to 2–3 ml using Centricon-10 microconcentrators. The samples were then incubated for 30–60 min at 20 °C with IAAT (4-(2-iodoacetamido)-TEMPO) spin label (100 mM in acetonitrile or Me\(_2\)SO) at a 10-fold molar excess of IAAT over \( b \) dimer. Excess spin label was removed with the same column system as in the previous step. The modified \( b \) was concentrated by Centricon-10 microconcentrators and stored at –80 °C. Protein purity was verified using SDS gel electrophoresis (57). IAAT spin label was purchased from Sigma. The degree of spin-labeling was usually between 25 and 75% depending on the position of the amino acids and the time of incubation.

ESR Spectroscopy—ESR spectra were acquired at a Bruker ESP-300 E equipped with a dielectric cavity TE001 or a Bruker EMX 6/1, also equipped with a dielectric cavity TE001. All spectra were acquired in the X-band mode with a peak-to-peak modulation amplitude of 1 G. The receiver gain was adjusted to the protein concentrations and the labeling degrees of the samples. The microwave power was 12.5 milliwatts, and the spectra were acquired at 293 K. The buffer used for ESR spectroscopy was 50 mM Tris-HCl, 5 mM MgSO\(_4\), and 300 mM KCl, pH 7.5.

The rotational correlation times were calculated directly from the spectra using the line width of the middle field signal and the signal amplitudes of the low, middle, and high field signals according to Xu et al. (58). The formula used was,

\[
t_\beta = 0.6 \times 10^{-19} \left( \frac{1}{t_1} + \frac{1}{t_2} \right) = \left( \frac{1}{t_1} + \frac{1}{t_2} \right)^{-1} - 2
\]

where \( \beta_0 \) is the reciprocal line width (1/T2) of the middle field signal.

Routine Analysis—Protein concentrations were determined according to the method of Bradford (56) using defatted bovine serum albumin (Roche Applied Science) as standard. ATP hydrolisis was assayed at 30 °C and pH 8.0 with 2–3 \( \mu \)g of enzyme as described by Wise et al. (59). The production of inorganic phosphate was detected by the method of Tausk and Shorr (60). The purity of the protein was routinely assayed using SDS-PAGE electrophoresis (57). Two-dimensional gel electrophoresis was accomplished by running a non-denaturing PAGE according to Laemmli (61) upon incubation of the solubilized protein with F\(_1\) in the presence or absence of subunit \( b \). The corresponding lanes were cut out and loaded onto a 10% SDS gel and fixed with melted agarose (1%) or SDS gel, sample buffer was added, and the gel was run according to Laemmli (57).

RESULTS

Site-specific Spin-labeling of \( b_2 \)—We have investigated the interactions of 33 single cysteine mutations of a truncated, water-soluble version of the subunit \( b \), \( b_{24-156} \), of the F\(_{1}\)-ATPase synthase with respect to their interaction with the soluble F\(_1\)-ATPase. 20 of the single cysteine mutations in subunits \( b \) were more or less evenly distributed between the N-terminal and the C terminus (at the top of the F\(_1\) structure). The engineered cysteine residues in \( b \) were modified with a cysteine reactive spin label, iodoacetamide-TEMPO (IAAT). The labeling efficiencies we obtained were between 25 and 75% and depended on the relative position of the modified amino acid. We were, however, always able to obtain sufficient labeling to perform the ESR experiments.

The ESR spectra of 20 of the spin-labeled \( b \) subunits are shown in Fig. 1, A and B, left panel. All of the spectra show a significant decrease in the high field signal amplitute, indicative of lower mobility of the radical when compared with unreacted IAAT (data not shown). The relatively sharp overall signals, however, suggest that the radical was attached to a macromolecule in a manner that retained a rather high degree

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1 The abbreviations used are: ESR, electron spin resonance; \( b_{24-156} \) a water-soluble truncated form of subunit \( b \) that contains amino acid residues 24–156 with an N-terminal leader sequence consisting of MTMITNSH; IAAT, 4-(2-iodoacetamido)-TEMPO.
of rotational mobility. The high field to middle field ratios of the spectra were between 0.6 and about 0.2, where the larger ratio indicates higher mobility of the spin probe (the high field to middle field ratio of unreacted IAAT is almost equal to 1). Because of the high concentrations of \( b \) subunits employed in the ESR experiments (usually between 10 and 100 \( \mu \)M), it can be assumed that the \( b \) subunits were present as dimers in our experimental set-up. The \( K_D \) for \( b \) dimerization was determined to be about 2 \( \mu \)M (62).

**Interaction of Spin-labeled Subunit b with \( \delta \)-Depleted F\(_1\)**—The addition of the spin-labeled \( b \) dimer to F\(_1\)-ATPase that had been depleted of its \( \delta \) subunit did not result in significant differences in the overall shape of the spectra or in variations in the high field to middle field ratio; see Fig. 1, A and B, middle panel. We initially interpreted these results as supportive of earlier publications that presented evidence that binding of \( b \) to F\(_1\) (or F\(_1\) to F\(_\delta\)) was conferred by and dependent on the presence of subunit \( \delta \) (4, 63). To test this potential interpretation, we performed two-dimensional gel electrophoresis, where the first dimension consisted of a non-denaturing PAGE (61), and the second dimension was a denaturing SDS-PAGE (57). Fig. 2 shows the second dimension gels (denaturing conditions) of spin-labeled mutants \( b_{A107C} \) and \( b_{I126C} \) in complex with F\(_1\)-\( \delta \) (lanes 1 and 3). The traces are compared with the spin-labeled mutants \( b_{A107C} \) and \( b_{I126C} \) in complex with the whole five-subunit F\(_1\) (lanes 2 and 4, Fig. 2). The legends on the side of the gels identify the corresponding subunits. The fact that the water-soluble, truncated subunit \( b \) co-migrated with \( \delta \)-depleted F\(_1\) under non-denaturing conditions strongly suggests that complex formation between F\(_1\)-\( \delta \) and \( b \) had occurred. In control experiments we showed that \( b \) migrated differently from the \( b\)-F\(_1\) complex on the non-denaturing gel in the absence of F\(_1\) or when \( b \) was added in super-stoichiometric amounts over F\(_1\); see also lane 4 of Fig. 2. The presence of subunits \( b \) was further confirmed by Western blot analysis of the second dimension gel (data not shown).

**Interaction of \( b \) with F\(_1\)-ATPase**—When the spin-labeled \( b \) dimers were incubated with the whole F\(_1\)-ATPase, differences in the ESR spectra of the labeled \( b \) in the absence and presence of F\(_1\) became obvious at amino acid positions 62 and 84 and especially toward the C-terminal region (compare Fig. 1, A and B, right panel to left panel). The spectra in presence of F\(_1\) are considerably broader, which is especially noticeable in the pronounced decrease of the high field signal amplitude (right...
panel) when compared with the spectra of $b_2$ in the absence of $F_1$ (left panel). The data clearly localize tighter packing interactions between $F_1$ and $b_2$ in the upper, C-terminal half of the $b$-subunit.

The results become clearer when the rotational correlation times of the radicals in the presence or absence of $F_1$, and $F_1$-$b$ are compared with the $b$ dimer alone. In Fig. 3A the rotational correlation times calculated from the corresponding ESR spectra are compared for $b_2$ (front), $b_2$ in complex with $F_1$ (middle), and $b_2$ in complex with $F_1$ (back). Although no significant difference between $b_2$ alone and in complex with $F_1$ was observed, a significant increase in the rotational correlation times was prominent for $b_2$ in complex with whole $F_1$ from about amino acid position 84 and higher (toward the C terminus), indicative of a tight protein-protein binding interaction occurring in this region.

The addition of isolated subunit $b$ to $F_1$-ATPase in complex with spin-labeled $b_{24-156}$ resulted in ESR spectra identical to those obtained when labeled $b_{24-156}$ was added to the whole $F_1$. Fig. 4 shows a corresponding ESR experiment where isolated subunit $b$ was added (spectrum B) to spin-labeled $b_{130}$-1340 in complex with $F_1$ (spectrum A).

**Site-specific Spin-labeling of b Positions 101–114**—The spectra that result from a spin-labeled cysteine at amino acid position 109 indicated further interesting characteristics. In addition to the three typical, rather sharp signals that we observed for most of the amino acid positions and that are shown in Fig. 1A and B, a second set of much broader signals can be seen in the ESR spectra for spin-labeled residue 109. These signals indicate at least one further spectral component that likely stems from a population of spin labels that is significantly more motion-restricted than the population of spin labels that give rise to the three sharp lines observed for the other mutations. The primary sequence of the amino acid stretch between position 100 and about 120 shows an unusually high content of charged amino acids. To further investigate this specific region of subunit $b$, we mutated every amino acid between residue 101 and 114 to cysteine (−4 helical turns) and subsequently modified the cysteines with IAAT. The resulting ESR spectra of the spin-labeled 101–114 proteins in the absence and presence of $F_1$ are shown in Fig. 5. Especially at amino acid positions 102, 109, 112, and 113, additional spectral components were observed in the corresponding ESR spectra both in the presence and absence of $F_1$. The corresponding enlarged ESR spectra are shown for better clarity in Fig. 6. The outermost splitting (2$A_{zz}$ values) of the less mobile components of the different mutants ranges between 50 and 60 G, differing significantly from the splitting of the more mobile components that range between 37 and 39 G. The plus and minus $F_1$ spectra are overlaid in the figure and show no significant differences in the spectral shape resulting from binding of the spin-labeled $b$ to $F_1$. These results indicate a lack of tight binding interactions between $b_2$ and $F_i$ at these amino acid residues.

The ESR spectrum of $b_2$ that was spin-labeled at position 108 shows unusually broadened, but still rather symmetrical line shapes when compared with the rest of the spectra shown in Figs. 1, A and B, and 5, i.e. no shoulder is observed, especially at the low field peak of the spectrum. The line shape of the spectrum indicates that at this particular position the spin labels in each $b$ subunit are similarly immobilized, probably through interaction with neighboring amino acids.

The high to middle field ratios of the sharper components of the spin-labeled $b_2$ in the absence of $F_i$ range mostly between 0.16 and 0.34, indicating stronger immobilization of the radicals between amino acid positions 101 and 114 than those positioned near the N-terminal or C-terminal positions. Binding of these labeled $b_2$ to $F_i$ did not alter the shape of the ESR spectra significantly (compare left and right panels in Fig. 5). Comparison of the calculated rotational correlation times of labeled $b_2$ in the presence or absence of $F_i$ indicates a slight decrease in mobility when $b_2$ was in complex with $F_i$ (compare the back to front panel in Fig. 3B). Two-dimensional gel electrophoresis of all the spin-labeled mutants at positions 101–
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FIG. 3. Rotational correlation times of the radicals bound to b₂ in the presence and absence of ATPase. The rotational correlation times were determined directly from the ESR spectra as described under "Materials and Methods." A, rotational correlation times (ns) calculated from the spectra in Fig. 1, A and B. Back panel, spin-labeled b₁ in the presence of F₁. Middle panel, spin-labeled b₂ in the presence of δ-depleted F₁. Front panel, spin-labeled b₁ alone. The appropriate amino acid positions are given in the figure. B, rotational correlation times (ns) calculated from the spectra in Fig. 5. Back panel, spin-labeled b₁ in the presence of F₁. Front panel, spin-labeled b₁ alone. Again the amino acid positions for spin-labeling are given in the figure.

FIG. 4. Binding of subunit δ to a complex consistent with δ-depleted F₁ and b₂. A, room temperature ESR spectrum of a complex of δ-depleted F₁ with IAAT-spin-labeled subunit b₁AlaS30C. B, ESR spectrum A upon the addition of isolated subunit δ. C, ESR spectrum of IAAT-labeled b₁AlaS30C in complex with F₁. The protein concentrations were between 30 and 40 μM. The b₂ to F₁ ratio was 1:2 in all experiments.

114 also confirmed that binding of the spin-labeled b-mutants to F₁ had occurred under the experimental conditions (data not shown). The data suggest that the packing interactions between amino acid positions 101 and 114 and F₁ are significantly less tight than those observed for the more C-terminal region of the b₂.

DISCUSSION

Tremendous progress in our understanding of the mechanism of ATP hydrolysis and synthesis by the ATP synthase has been made in the last decade, gaining even more momentum when a high resolution structural model of the beef heart mitochondrial ATPase was presented 10 years ago by Walker and co-workers (15). An interesting problem of energy transduction within the F₅₆,F₅₇-ATP synthase is raised by the subunit composition of the enzyme (for review, see Ref. 64). During a 360° rotation of γ, each of the catalytic β subunits presumably catalyzes the synthesis of 1 ATP, whereas the subunits c ring makes a full rotation, and 10 or 12 protons are translocated. The question then arises how the movement of 10–12 proteins in the c ring is tied to the rotation of 3 β-subunits in F₁ and how those differently geared reactions are mechanically coupled. Junge and co-workers (13, 14) have proposed a mechanism where the energy derived from a 30° rotation of c₁₂ relative to subunit a is accumulated in the b₂ subunits of the stator (and possibly through interactions with a) until the fourth proton is translocated. Only then, according to this hypothesis, does subunit γ rotate by 120° to initiate the binding change and release of ATP. In other words γ does not rotate until the elastic stress stored in b₂ reaches a threshold limit after passage of the fourth proton and after the fourth 30° rotation of the c ring occurs. To fully understand this mechanical phenomenon, it is imperative to comprehend the structure of the stator subunits and their protein-protein interactions. In this paper we have investigated the packing interactions between the dimer of subunit b and the F₁-ATPase in the presence or absence of subunit δ. Fig. 1, A and B, shows the ESR spectra of single cysteine mutations of a water-soluble, truncated form of subunit b (33, 34). The rather sharp signals with significantly reduced high field amplitude are typical for radicals bound to macromolecules that retain a significant degree of mobility. Such spectra are consistent with the elongated, mostly α-helical structure that has been proposed for the b dimer. Comparison of the left to the right hand panels of Fig. 1, A and B, indicates that at certain amino acid positions (position 62, 84, and from 120 toward the C terminus), binding of F₁ strongly effects the mobility of the radical, suggestive of rather tight packing interaction between the proteins (compare the signal amplitudes of the respective high field signals in Fig. 1, A and B, right to left panel). The packing interaction seems to be most pronounced in the upper half of b toward the C terminus. The effect is also obvious when the rotational correlation times of the resulting spectra are compared as shown in Fig. 3A. Although binding of F₁ (Fig. 3A, back panel) to b₂ (Fig. 3A, front panel) has almost no effect on the rotational correlation time of spin labels attached to amino acids below about position 80, the effects are quite significant from about amino acid 80 to the C terminus of b. These results support earlier work (65) where cross-links between b-positions 92, 109, and 110, with F₁ and both the full-length and truncated b were observed.

Interestingly, almost no effect both on the spectral shape and on the calculated rotational correlation times can be observed when b₂ is incubated with a δ-depleted form of F₁. In previous work it had been suggested that subunit δ directly conferred binding of b₂ to F₁ (4) and binding of F₁ to F₁ (63). Very recent work from Senior and coworkers (66), however, showed that binding of b₂ does not seem to depend on the presence of...
subunit δ in F₁ if Mg²⁺ ions are present in the medium (66). This group suggests that a structural Mg²⁺ may be involved in the binding process. To further investigate this discrepancy and to determine whether subunit b binding to the δ-depleted F₁ took place in our experiments, we performed two-dimensional gel electrophoresis, where the first dimension was a non-denaturing PAGE. If a b₂ complex with δ-depleted F₁ had formed, b would migrate with the F₁-ATPase and should be visible in the second dimension, which was a denaturing SDS gel. The experiments (Fig. 2) showed that the b mutants indeed bound to the δ-depleted F₁, supporting Senior’s findings. It should be noted that our experimental set-up contained Mg²⁺-ions (as discussed in Ref. 66) as well as the high concentrations of protein that are needed for ESR spectroscopy, which are well above the Kᵦ for b₂ dissociation (2 μM (62)).

The fact that binding of the labeled b to δ-depleted F₁ did not result in changes in the shape and the rotational correlation times of the corresponding ESR spectra indicates that the packing interactions of F₁ with b₂ differed significantly when δ was not present. This may result in “nonproductive” interaction that may render the enzyme less active than normal F₁. The experiments shown in Fig. 4 indicated that δ was able to bind to a complex consistent of b₂ and δ-depleted F₁. The resulting spectra were identical to the spectra observed when spin-labeled b₂ was added to the complete, 5-subunit F₁.

In separate experiments we mutated 14 consecutive amino acid residues in b₂, almost 4 helical turns, between amino acid positions 101 and 114. The reason we did this stemmed from the rather unusual multicomponent ESR spectrum that we obtained for the spin-labeled position 109 and which suggested that in the presence or absence of F₁ at least two different spin-label populations coexisted with significantly different mobilities. A similar multicomponent spectrum was only observed for amino acid position 130, but there it was only in the presence of F₁ and could result from asymmetric interaction of the spin-labeled b₂ with F₁. Position 109, when spin-labeled,
showed the different components both in the absence and presence of F₁ and β-depleted F₁. The amino acid sequence of subunit b shows an extremely high number of polar and charged amino acids between about position 100 and 120 that do not seem to be suitable to form hydrophobic b-b and/or β₁F₁ protein-protein interactions surfaces. The ESR spectra resulting from spin-labeled mutations 101–114 (Fig. 5) and the corresponding rotational correlation times (Fig. 3B) indicate that in contrast to most of the other mutants we investigated (Fig. 1, A and B), binding of F₁ did not alter the shape of the spectra or the rotational correlation times of the radicals significantly. We eliminated the possibility that the modified b subunits did not bind to F₁ by performing two-dimensional gel electrophoresis as described before for all spin-labeled mutations. The gels clearly show that the modified b subunits did bind to F₁. We also confirmed b binding by Western blot analysis (data not shown). The data suggest that the 101–114 region of b₂ does not pack tightly with F₁, which would eliminate at least one protein-protein interaction surface (b₂ interaction to F₁) and may at least partially explain the highly hydrophilic character of this part of b. These presented data do not contradict the earlier discussed findings that residues 109 and 110 were found to cross-link with subunits α and β of F₁. In these described experiments cross-linkers were used that should span about 12–15 Å and that should, therefore, be able to chemically link amino acids that are somewhat spatially removed from each other.

A further interesting result of these investigations concerns amino acid positions 102, 109, 112, and 113 within this highly hydrophilic stretch of b that, when spin-labeled, exhibit multi-component ESR spectra (Fig. 6). The shape of the spectra again did not change significantly in the presence or absence of F₁ as discussed above (as can be seen from the overlay of the spectra in Fig. 6). The 2A_{NN} values of the less mobile components of the different mutants are between 50 and 60 G and are significantly different from the hyperfine splitting of the mobile components that range between 37 and 39 G. These differences in mobility at identical positions of a homodimeric component that range between 37 and 39 G. These differences in mobility at identical positions of a homodimeric component that range between 37 and 39 G. These differences in mobility at identical positions of a homodimeric component that range between 37 and 39 G. These differences in mobility at identical positions of a homodimeric component that range between 37 and 39 G.

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The Subunit $b$ Dimer of the $F_\text{0}F_\text{1}$-ATP Synthase: INTERACTION WITH F1-ATPase AS DEDUCED BY SITE-SPECIFIC SPIN-LABELING

Christian Motz, Tassilo Hornung, Michael Kersten, Derek T. McLachlin, Stanley D. Dunn, John G. Wise and Pia D. Vogel

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